Hemochromatosis

Genetics, pathophysiology, diagnosis, and treatment

Since its discovery in the nineteenth century, hemochromatosis has fascinated and challenged biomedical scientists and clinicians. Once thought rare, it is now acknowledged as one of the commonest inherited disorders, affecting one in two hundred people of western Caucasian descent. It is estimated to affect over one million people in the USA alone. Moreover, it is now possible to diagnose affected people before they develop multisystem iron overload disease, and offer simple, effective and inexpensive treatment, although there remains a shortage of adequate screening programs.

This is the most comprehensive clinical reference yet on hemochromatosis. The international author team includes specialists in internal medicine, hematology, hepatology, genetics, biochemistry and molecular biology, and the contents cover all aspects of pathophysiology, epidemiology, diagnosis and treatment. The latest developments in the genetics of the disorder are well explained, and there are sections on screening, diagnostic techniques, and clinical complications. Social and ethical issues are also considered.

Highly illustrated, up to date and authoritative, this is the definitive resource for all clinicians involved in the management of hemochromatosis, and will also be invaluable to scientists interested in iron metabolism and iron overload.

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Edited by

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Foreword

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When Dr John Sheldon published his classic monograph on hemochromatosis in 1935, he documented the evidence, both from the literature and from his own practice, that such patients were suffering from an inherited disorder of iron metabolism, but he could hardly have anticipated the remarkable developments of the next 60 years. These developments were the outcome of continued, detailed basic and clinical observations by investigators who were able to exploit the major technological advances in laboratory research to probe the cytopathologic, biochemical and, ultimately, the molecular basis of one of the most common human genetic disorders.

It was the landmark discovery in the 1970s by Dr Marcel Simon and his colleagues in Brittany, France that clearly established the autosomal recessive inheritance of hemochromatosis and its close linkage with the major histocompatibility class I complex A3 on the short arm of chromosome 6. Subsequent confirmation of this finding by investigators scattered throughout the world documented a prevalence of homozygotes with hemochromatosis of between 1 in 250 and 1 in 300 of the Caucasian population, and a phenotypic expression that was virtually invariable in individuals possessing the abnormal allele on both chromosomes 6. With all the evidence pointing to the relentless accumulation of body iron in such individuals during the formative evolution of their disease, the search for the abnormal gene became an imperative of the highest priority. In 1996, Feder and his colleagues, after a highly productive collaboration between industry and academia, were able to clone the candidate gene (initially termed 'HLA-H,' and subsequently correctly named *HFE*) and demonstrated the presence in homozygous form of the missense mutation C282Y in 83% of 178 patients with phenotypic evidence of hemochromatosis. From the nucleotide sequence of the *HFE* gene, a protein product could be predicted that

denoted structural homology with the major histocompatibility class I molecules. The expression of the *HFE* gene in the crypt cells of the intestinal villus and in other transport epithelia, and the binding characteristics of the protein product with β_2 microglobulin and the transferrin receptor offer intriguing glimpses into potential roles in the regulation of iron transport and absorption. It is therefore timely for Drs Barton and Edwards to have assembled experts from around the world to provide a state-of-the-art review on iron metabolism and iron overload diseases. They have invited specialists in internal medicine, hematologists, hepatologists, geneticists, biochemists, and molecular biologists to share their experience of hemochromatosis in this highly accessible series of chapters. The multiorgan manifestations of iron overload with its attendant toxic consequences offer insights into the many roles for iron in cellular metabolism. No single group of investigators can hope to encompass all the important areas of research needed to answer the many remaining unresolved questions relevant to the pathogenesis, pathology, diagnosis, and treatment of hemochromatosis. How does a gene expressed in a progenitor cell, but not in the absorptive cell of the intestine, regulate iron absorption? Why do some homozygotes for the C282Y mutation not show phenotypic expression of iron overload? Are there other mutant genes, not yet identified, that account for a small but definite subgroup of phenotypic hemochromatosis patients who do not carry the C282Y mutation? Only by providing a forum for sharing the latest research information will there be synergy to address these challenges.

Jim Barton and Corwin Edwards are to be congratulated for providing this forum in such a well-organized series of manuscripts. The rewards of these collaborative efforts in terms of patient survival and relief of suffering are already being realized. Future returns of these initiatives will be found not only in terms of scientific insights, but also in practical value to an increasingly cost-conscious health delivery system.

Part I

Introduction to hemochromatosis

1

A brief history of hemochromatosis

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Introduction

In 1847, Virchow reported the occurrence of golden brown granular pigment in sites of hemorrhage and congestion; this pigment was soluble in sulfuric acid, yielded a red ash on ignition, and then produced a Prussian blue reaction[1]. Eighteen years later, Trousseau first described the syndrome of hepatic cirrhosis, pancreatic fibrosis, and cutaneous hyperpigmentation[2], but he did not recognize the involvement of iron in its pathogenesis. In 1867, Perls formulated the first practical acid ferrocyanide reaction for histologic analysis of iron, and applied the staining reaction to a variety of tissues[3]. Trousseau's report was followed by Troisier's account of 'diabète bronzé et cirrhose pigmentaire' in 1871[4]. In 1889, von Recklinghausen reported the use of the methods of Virchow and Perls to identify excess iron in tissues obtained at autopsy of persons who had 'hämochromatose'[5]. Following the theories of Virchow, von Recklinghausen erroneously believed that the iron-containing pigment was derived from blood (due to hemorrhage or hemolysis), rather than from primary iron deposition, but he also described another finely granular yellow pigment that occurs with hemosiderin in the liver and other tissues in hemochromatosis that does not react to the iron methods (hemofuscin = lipofuscin)[5]. During the next several decades, additional cases were reported using variations of the terminology of the French and German physicians. In 1935, the English gerontologist Sheldon summarized 311 carefully selected 'haemochromatosis' cases from the literature, establishing this as the name for the disorder and for his detailed monograph[6].

Etiology

Sheldon concluded that the absorption of iron and possibly that of other metals is increased in hemochromatosis, suggested that the disorder is an inborn error of metabolism that primarily affects men, and rejected hypotheses that diabetes, infections, intoxication, alcoholism, and other conditions cause hemochromatosis[6]. Clinical case series published in the interval 1935–1955 hinted that the disorder was more common than had been appreciated previously[7]. In the 1960s, MacDonald diverted attention from the true etiology of organ and tissue injury in hemochromatosis by concluding incorrectly that hemochromatosis and iron overload are consequences of alcoholism and other nutritional factors[8]. However, many other investigators reported additional evidence for central roles of a heritable factor and absorption of excess iron in hemochromatosis, and recognized that the clinical phenotype of hemochromatosis was variable and depended on sex, age, and coincidental occurrence of other disorders. Nonetheless, alcohol can increase the absorption of iron and lower the threshold for hepatic injury in hemochromatosis, alcoholism is common among case series of hemochromatosis patients, and excess alcohol use frequently mimics the primary abnormalities of iron metabolism typical of hemochromatosis. In most target organs of iron overload in hemochromatosis, a sequence of subcellular injury, cell death, and fibrosis occurs due to the excessive production of injurious free radicals in the presence of ever-increasing iron burdens. A murine genetic knockout model of hemochromatosis now provides an additional important means to assess the pathogenesis of iron overload[9, 9a].

Metal absorption and transport

The absorption of both inorganic and organic forms of iron is inappropriately increased, mechanisms to eliminate excess body iron are extremely limited, and transfer of iron

from enterocytes to the blood is the rate-controlling step in abnormal iron absorption in hemochromatosis. By demonstrating that the absorption of inorganic cobalt is also increased in hemochromatosis, Valberg and colleagues confirmed Sheldon's postulates regarding increased absorption of non-ferrous metals[10]. Other metals are also absorbed or retained in increased amounts in hemochromatosis, although their possible role in the pathogenesis of clinical abnormalities remains obscure. Enterocyte iron and non-ferrous metal uptake from the intestinal lumen probably depends on divalent cation transporters such as *Nramp2*/DCT1 and calreticulin[11–13], and on the interaction of wild-type and mutant *HFE* gene products with transferrin receptor at the basolateral membranes of enterocytes[14]. This provides a unifying concept of abnormal metal absorption in hemochromatosis. Further, the persistent deficiency of unsaturated transferrin and behavior of non-transferrin-bound plasma iron in hemochromatosis largely explain the histologic patterns of excess iron and non-ferrous metal deposition, especially in the liver[15, 16].

Diagnosis

In the first few decades after its original descriptions, hemochromatosis was often diagnosed at autopsy, primarily by recognition of the 'classic' triad of end-stage iron overload: cutaneous hyperpigmentation, diabetes mellitus, and hepatic cirrhosis. In 1962, Scheuer and colleagues reported a method of histologic grading of hepatic biopsy specimens prepared using Perls' technique. This study emphasized the characteristic gradient of iron distribution in hepatocytes in hemochromatosis, made it possible to semi-quantify hepatic iron content with minimal apparatus and thus diagnose the disorder ante-mortem, and demonstrated that some relatives of index cases had similar abnormalities of hepatic iron deposition[17]. Hepatic histology also provided prognostic information. The availability of reliable clinical measurements of serum iron, total serum iron-binding capacity, and serum ferritin in the 1960s and 1970s provided a basis for ascertaining the clinical phenotype of hemochromatosis. Consequently, variability of the phenotype among probands and affected family members became more apparent[18]. The hepatic iron index distinguished presumed homozygotes from heterozygotes and persons with alcoholism and other forms of iron overload and hepatic disease[19], and became the standard of diagnosis for more than a decade.

In 1975, Simon and colleagues reported that the genetic factor associated with hemochromatosis was closely linked to the human leukocyte antigen (HLA)-A locus[20]. By the late 1970s, HLA immunophenotyping was used to identify relatives of probands who also inherited two HLA-linked hemochromatosis alleles, sometimes before iron overload occurred. In 1996, Feder and colleagues discovered two missense mutations in an unusual major histocompatibility (MHC) class I gene on 6p known as *HFE*. The C282Y mutation is present in homozygous configuration in approximately 80% of persons with heavy iron overload attributable to hemochromatosis[21]. The H63D mutation, although more frequent in the general population, is much less frequently associated with iron overload. The ability to detect C282Y and H63D mutations now makes possible the detection and diagnosis of many asymptomatic, healthy persons from the general population likely to develop iron overload.

Complications of iron overload

Sheldon's compilation of histologic observations explained or presaged identification of virtually all complications of iron overload recognized today in untreated patients. However, data accumulated on patients and their families in the 20 years after the publication of Sheldon's *Haemochromatosis* indicate that many affected persons had symptoms and signs other than those of the classic diagnostic triad, and that many of these preceded the development of the classic features. Bassett and coworkers demonstrated that the hepatic iron concentrations had a direct relationship with the occurrence of hepatic cirrhosis[19]. Further, the incidence of primary hepatic cancer is markedly increased among persons with hemochromatosis with hepatic cirrhosis[22], but cancer of the liver almost never develops in persons without cirrhosis. Now, the most common causes of death among persons with hemochromatosis are cirrhosis and its complications[23]. Before insulin therapy was available, diabetes mellitus was a major cause of acute illness and death. Today, the clinical picture of diabetes mellitus in hemochromatosis is more complex. Its clinical expression depends on pancreatic iron overload, the degree of hepatic disease, coincidental inheritance of other diabetogenic genes, and other factors. Vasculopathy and neuropathy, rarely observed by early investigators, are now as common in hemochromatosis patients as in others with diabetes mellitus. Accordingly, diabetes is now the second most common cause of death in modern hemochromatosis case series[23]. Once a common form of acute illness and death in hemochromatosis, cardiac siderosis with cardiomyopathy and arrhythmias is increasingly rare[24], but remains a common presentation and potentially avoidable cause of

death in young persons with severe iron overload, especially those with 'juvenile' hemochromatosis. Schumacher described the distinctive forms of arthropathy that affect approximately one-half of persons with hemochromatosis[25]. However, some cases are diagnosed before iron overload has developed, and most fail to improve after iron depletion, suggesting that factors other than inheritance of hemochromatosis alleles is responsible for this common and often disabling sequel. Even the cutaneous abnormalities of hemochromatosis are more diverse than the hyperpigmentation described in the nineteenth century[26].

Abnormal immunity and susceptibility to infection

Over many years, reports of unusual fulminant bacterial infections that occurred in persons with chronic hepatic disease or increased iron stores have included some hemochromatosis patients. Although these incidents are clinically important, the commonness of hemochromatosis and the rarity of the reports suggest that the occurrence of some of these infections in persons with hemochromatosis is coincidental. In 1978, however, de Sousa and co-workers began to discover more fundamental relationships of hemochromatosis, iron overload, and immunity[27]. Their subsequent investigations and those of others demonstrate the direct effects of excess iron on cells of the immune system, the decreased expression of CD8+ lymphocytes in hemochromatosis, and the role MHC class I-like proteins in intestinal iron absorption[28]. Further, discovery of the *Nramp* family of divalent cation transporters reveals additional relationships in the absorption and metabolism of iron with cellular immunity in experimental animals and humans[11, 12, 29].

Treatment

In 1952, Davis and Arrowsmith reported treating three persons with hemochromatosis with repeated phlebotomy[30]; long-term studies demonstrated thereafter that treated patients lived longer[31, 32]. In a prospective trial, Niederau and colleagues demonstrated that persons without hepatic cirrhosis or diabetes mellitus who undergo iron depletion have normal actuarial survival[23]. Early diagnosis and treatment prevent hepatic cirrhosis and diabetes mellitus in those who do not have other risk factors for the development of these disorders, although therapeutic phlebotomy does not reverse established hepatic or pancreatic injury. Cardiac disease and hypogonadism are sometimes reversed by aggressive phlebotomy therapy, but arthropathy is usually unaffected. Lingering questions about the role of iron overload in the pathogenesis of certain hemochromatosis-associated complications or the effectiveness and cost-effectiveness of therapy in subgroups of patients are only answerable with randomized control therapeutic trials. However, the overall success of phlebotomy trials indicates that treatment must now be recommended for most patients[33].

Population genetics

The original descriptions of hemochromatosis from countries of western Europe suggested its prevalence in this geographic area. Extension of Simon's original work revealed that HLA-A and -B and other MHC types mark haplotypic variants among hemochromatosis patients in different populations, and provided indirect evidence that hemochromatosis alleles occur frequently in many European population groups. In 1988, a report of the evaluation of 11 065 healthy blood donors in Utah using phenotypic criteria revealed convincingly that hemochromatosis genes are common[34]. After's Feder's 1996 publication[21], other investigators confirmed that approximately 12% and 25% of western Caucasians in many geographic locations are heterozygotes for the C282Y and H63D mutations, respectively[35], confirming the conclusions of the Utah survey[34]. *HFE* mutations, especially the C282Y allele, are associated primarily with western Caucasians. The origins, dissemination, and population frequencies of *HFE* alleles are associated with old northern European peoples, especially the Vikings or Celts, and have been reconciled with the hemochromatosis-associated HLA haplotypes discovered by Simon and others. However, the cause(s) for the prevalence of hemochromatosis alleles remains obscure.

Social issues

At a meeting at the Centers for Disease Control and Prevention in Atlanta in early 1996, international experts in hemochromatosis, geneticists, epidemiologists, and patient advocates collectively recommended initiation of population-based screening and treatment for hemochromatosis in western European and derivative countries based on its frequency, its susceptibility to early diagnosis, the availability of a meaningful treatment intervention, and evidence of cost–benefit. Soon thereafter, discovery of the *HFE* gene caused these recommendations to be reconsidered, and raised new questions about the optimal

diagnosis strategies[36]. These included the social and ethical consequences of hemochromatosis diagnosis (particularly on a large scale), the disparities of phenotyping and genotyping, and the availability of adequate numbers of diagnosticians and genetic counselors familiar with hemochromatosis to mount population-based screening programs.

Conclusions

Since its discovery in the nineteenth century, hemochromatosis has fascinated and challenged basic and clinical scientists. Long-held notions of its rarity have yielded to increasing recognition of its prevalence among Caucasians of western European descent, and of its place among several heritable iron overload disorders that occur frequently among large population groups. It has become possible to diagnose affected persons before they develop multisystem iron overload disease, and provide simple, effective, and inexpensive treatment. However, there are unrecognized mechanisms of iron absorption and homeostasis, additional ill-effects of iron overload, unforeseen consequences of altered immunity and limited expression of HLA types, and adverse (or beneficial) effects of increased non-ferrous metal absorption in hemochromatosis that remain undefined. In aggregate, the many persons who are heterozygous for hemochromatosis-associated alleles could be at increased risk to develop heart disease, cancer, or other common disorders; answering these questions is possible now that *HFE* genotyping can identify most hemochromatosis gene carriers. Genetic maneuvers to restore normal iron absorption in affected persons await discovery. Further, hemochromatosis is likely to be the disorder by which new social and ethical standards surrounding revelation of personal genetic information are formulated in western populations. Truly, hemochromatosis is 'the genetic disease of the twenty-first century'[37].

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2

Hemochromatosis: a genetic definition

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Early definitions of hemochromatosis

The definition of hemochromatosis, a disorder that causes iron overload, has undergone a great metamorphosis from the initial description of bronze diabetes in 1865 to the identification of a hemochromatosis gene in 1996. The original definition was a simple description of the two abnormalities that were noted at the time: skin pigmentation and diabetes mellitus[1]. Six years later, the definition was modified to include the observation of iron deposition in a cirrhotic liver[2]. In 1889, von Recklinghausen advanced the definition to emphasize the presumed pathophysiologic abnormality: iron from circulating blood accumulated in and caused pigmentation of the liver[3].

Definitions in the twentieth century

During most of the twentieth century, hemochromatosis was considered to be an idiopathic disorder. Some authors included in the definition a statement about possible causes of iron overload, such as an unidentified environmental effect, disordered copper metabolism[4], or alcohol abuse[5]. Sheldon considered the possibility that hemochromatosis is an inborn error of metabolism[6]. Prior to 1949, hemochromatosis was regarded as untreatable, because there was no known reliable therapy for iron overload or its complications. The first reports of phlebotomy therapy for hemochromatosis appeared at mid-century[7–9]. By 1955, hemochromatosis was believed to be a rare idiopathic disorder of iron metabolism, primarily of middle-aged men, that caused heavy iron overload. Signs and symptoms included grey-green skin pigmentation, hepatomegaly, splenomegaly, ascites, diabetes mellitus, hypogonadism, heart failure, hepatic cirrhosis, and hepatocellular carcinoma[10].

Most reports described only patients who had end-stage hemochromatosis with heavy iron overload and organ damage. However, hemochromatosis was not acknowledged to be a heritable disorder, and family members of probands were not routinely examined. Thus, there were only a few descriptions of young individuals who had no organ damage. Most patients described were men approximately 50 years of age; few women were diagnosed[6, 10, 11]. The apparent gender bias added to confusion about the nature of the disorder. It is now understood that affected women are granted some reprieve from iron accumulation due to their losses of iron during menstruation, pregnancy, and childbirth.

Changing definition based on segregation analysis

In the 1970s, a major advancement occurred: the suggestion that hemochromatosis is an inherited disorder[12–21]. The mode of genetic transmission, however, was obscured because penetrance of hemochromatosis is incomplete, and it is more penetrant in males than in females. The inherited nature of hemochromatosis was accepted due to three observations. First, hemochromatosis occurs with greater frequency than expected among siblings of affected individuals[15–21] and among the offspring of consanguineous marriages[15]. Second, segregation analysis of informative pedigrees revealed that hemochromatosis is transmitted as an autosomal recessive trait[15]. Third, the results of segregation analysis also demonstrated that diabetes mellitus and hemochromatosis segregate independently as genetic traits[17]. These findings resulted in a change of the definition of hemochromatosis to indicate that it is an autosomal recessive disorder that causes iron overload.

Definition based on linkage with HLA-A3

In the mid-1970s, the observation that hemochromatosis is associated with the HLA serotype A3 caused thinking about the disorder to change[18] for two main reasons. First, confirmation of the tight genetic linkage of hemochromatosis to the major histocompatibility complex, and physical mapping of the HLA class I region to 6p21.3 raised the possibility of identifying the hemochromatosis gene[19–22]. Second, the hypothesis of a founder mutation for hemochromatosis provided the single most important piece of information that later contributed to cloning of the hemochromatosis gene[23–27].

Definition based on results of populations screening: phenotypic expression and HLA typing

Further understanding of the phenotypic expression of hemochromatosis was achieved in the 1980s. Incremental understanding of iron balance in affected individuals and postulates about the natural history of the disorder contributed to an evolving definition[28, 29]. The phenotypic expression of hemochromatosis, including the iron phenotype among homozygotes and heterozygotes, was characterized[14, 20, 21, 28–38]. Among heterozygotes, 10–25% have an elevated transferrin saturation value or serum ferritin concentration, but most do not develop heavy iron overload or its complications unless they have an additional condition that contributes to increased iron absorption[38].

A large screening study for hemochromatosis among healthy volunteer blood donors was performed in Utah in 1988[39]. The results demonstrated that it is possible to identify young, asymptomatic homozygotes prior to the onset of illness. In fact, most of the homozygotes who were identified by a transferrin saturation value $>60\%$ were not iron loaded. Evaluation of the first-degree relatives of the probands (by HLA typing and measurement of transferrin saturation and serum ferritin concentration) resulted in the identification of additional, asymptomatic homozygotes. The Utah blood bank screening study confirmed previous estimates of the frequency of homozygosity for hemochromatosis among Caucasians of European ancestry (5 per 1000). These results and those of many other population and family screening studies added evidence to support a revised definition of hemochromatosis: an autosomal recessive trait that can be identified prior to the development of iron overload and organ damage[39–42].

Definition after identification of the hemochromatosis gene

A hemochromatosis-associated gene (*HFE*) was isolated in 1996[43]. The existence of a founder mutation enabled researchers to localize the candidate region by analysis of conserved haplotypes on chromosomes bearing a hemochromatosis gene. Initial genetic analysis suggested that the hemochromatosis locus was within one centimorgan of HLA-A. Subsequent studies revealed that this estimate was low due to reduced recombination in this region[27]. Positional cloning based on borders defined by conserved regions identified a sequence having a mutation common in hemochromatosis chromosomes[43]. Homozygosity for a mutation resulting in an 845G→A change (cysteine to tyrosine at residue 282; C282Y) is present in a majority, but not all, patients with hemochromatosis[43]. In four reports, the frequency of homozygosity for the C282Y mutation among hemochromatosis homozygotes varied from 82–90%[43–46]. In a study restricted to pedigrees having two or more affected individuals, the C282Y mutation was detected in 100% of cases[47].

The etiology of iron overload in the 11–18% of individuals with the hemochromatosis phenotype who are not homozygous for the C282Y mutation remains unknown, but two possibilities exist: there are other undescribed mutations that occur in the *HFE* gene, or other genes are involved in the regulation of iron absorption. A second mutation (187C→G; H63D) in *HFE* has been identified[43]. This may represent a common polymorphism, although there is evidence that the 187C→G mutation also contributes to iron loading[48, 49]. Therefore, hemochromatosis is now defined as a disorder of iron metabolism that is inherited as an autosomal recessive trait due to two mutant *HFE* alleles. The current definition does not require the presence of symptoms or signs of illness, or the presence of iron overload.

Relationship of definition of hemochromatosis to identification of homozygotes

Since the discovery of a specific mutation associated with hemochromatosis, there has been increased interest in performing large-scale molecular screening programs. There are approximately 1 200 000 hemochromatosis homozygotes in the United States (5 homozygotes per 1000 Caucasians). Screening of populations at risk using DNA-based genetic analysis will identify large numbers of homozygotes who can be treated before iron overload or its complications develop. Healthy homozygotes identified

during screening, who undergo therapeutic phlebotomy, could serve as a resource of blood that is usable for transfusion or production of blood components.

Problems with the genetic definition

Large-scale screening programs using genetic testing will have the advantage of identifying a large number of asymptomatic individuals who are homozygotes for the 845G→A (C282Y) mutation in the *HFE* gene. Not all iron-overloaded patients, however, will be identified by this screen. Based on current indications, screening for the 187C→G (H63D) mutation will also be needed to identify persons at risk for iron overload. Most importantly, there are iron-loaded individuals who lack detectable mutations in *HFE*. Until the basis of iron overload in these people is determined, screening based on iron status will continue to be necessary.

In this book, chapters are devoted to a discussion of screening for hemochromatosis, including the most reliable tests that are currently available, and the populations that are at greatest risk for the disorder. The cost of screening through detection of the *HFE* gene mutation has not yet been determined, but it is likely that some combination of transferrin saturation, serum ferritin concentration, and an *HFE* mutation test can be used to identify homozygotes. After experience is gained in screening populations with mutational analysis of the *HFE* gene, and after additional discoveries of other mutations or genes that increase iron absorption, it should be possible to refine the definition of hemochromatosis further. The discovery of the *HFE* gene has provided the most direct way to diagnose most cases of hemochromatosis. The ability to screen large populations based on a simple genetic test will undoubtedly reduce the morbidity associated with iron overload. Some chapters in this text address the potential role that mutations of the *HFE* gene play in the development of hemochromatosis and iron overload, although it is ironic that the way in which the gene was identified revealed little regarding its function.

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Part II

Genetics of hemochromatosis

3

Inheritance of hemochromatosis: family studies

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Introduction

Hemochromatosis is an inherited disorder of iron metabolism characterized by excessive iron absorption that leads to parenchymal iron overload and eventually to organ damage[1–5]. For nearly half a century, hemochromatosis was recognized as a separate disease entity before it was suggested by Sheldon that it could be an inborn error of metabolism[1, 2]. For many years the disease was named idiopathic or primary hemochromatosis, but over time evidence for the hereditability of hemochromatosis became compelling. The discovery by Simon[6, 7] in the 1970s that hemochromatosis was closely associated to certain human leukocyte antigens (HLA), i.e., HLA-A and HLA-B, is persuasive evidence of its hereditability. Subsequent epidemiologic surveys showed that hemochromatosis is one of the most common heritable disorders, with a prevalence of homozygosity of approximately 0.5% and a prevalence of heterozygosity of 10–15%. The high prevalence makes homozygote–heterozygote matings common, and thus a pseudodominant mode of inheritance. The discovery of specific molecular genetic markers for hemochromatosis has had a profound influence on understanding of this disorder. It will enable us to perform valid screening of the populations and to identify reliably persons at risk in affected families, and to obtain data on the expression and penetrance of the gene(s) in homozygous and heterozygous individuals.

HLA-linked hemochromatosis

HLA alleles

The modern epoch of genetic investigation into hemochromatosis began when Simon et al.[6, 7] discovered the positive association between the HLA-A3 and hemochromatosis, confirmed in subsequent studies on Caucasian subjects of European origin[4, 8–34]. This led to the clarification of the mode of inheritance[24]. The frequencies of different HLA alloantigens in hemochromatosis are displayed in Table 3.1 and those of the HLA-haplotypes associated with the disorder in Table 3.2. The frequency of HLA-A3 in unrelated persons with hemochromatosis ranges from 55–83%. In contrast, HLA-A3 is found with a significantly lower frequency (20–32%) in Caucasian subjects without evidence of abnormal iron metabolism. Due to the marked increase in the prevalence of HLA-A3 in hemochromatosis patients, there is a decrease in the frequency of the other HLA-A antigens such as HLA-A2, HLA-A9 and HLA-A10[29]. HLA-B7 and HLA-B14 were also found more frequently among patients with hemochromatosis, but usually only when accompanied by HLA-A3[24, 25, 29, 35]. HLA-B7 was present in 34–60% of patients and in 19–28% of control subjects. HLA-B14 occurred in 0–31% of patients with hemochromatosis and in 2–9% of normal subjects. In the Caucasian Danish population, HLA-B7, HLA-B14, HLA-B35, and HLA-B47 are associated with HLA-A3[36]. However, the allelic association of HLA-B7, HLA-B14, and HLA-B47 with HLA-A3 was significantly more frequent in hemochromatosis patients than in control subjects[29]. In contrast, the frequencies of HLA-B35 and HLA-A3, B35 were significantly lower in Danish hemochromatosis patients than in controls[29]. Even though HLA-A3 and HLA-B35 are associated in the normal population, the frequency of HLA-B35 has not been increased in persons with hemochromatosis in most studies on HLA alloantigens and hemochromatosis. An exception to these observations is the association between the hemochromatosis allele and the HLA-B35 allele in the regions of northeastern Italy[31, 37, 38].

The relative risk (RR) value indicates how many more times hemochromatosis is likely to occur in individuals

Table 3.1. Frequency of HLA alleles in persons with hemochromatosis (H) and in normal subjects (N)

				Frequency, %					
				HLA-A3		HLA-B7		HLA-B14	
Country	Reference	Unrelated patients, *n*	Normal controls, *n*	H	N	H	N	H	N
Australia	12	73	522	70	26	60	27	10	8
Australia	13	77		62	24[a]	49	21[a]		
Australia	15	10	700	60	20	50	21	20	7
Belgium	17	12	40	83	25	75	15	0	8
Canada	20	18	253	61	25	50	22	17	8
Denmark	29	70	1967	80	27	42	27	10	5
France	16	48	591	75	26	38	19	29	9
France	21	154	439	73	30	39	27	31	8
France	25	66	444	79	26	53	20	9	9
Great Britain	14	35	95	69	31	34	20	20	6
Germany	8		5046		30		27		5
Germany	18	22		73		45		0	
Germany	19	35	100	74	21	60	22	3	6
Germany	28	18	892	67	30	39	27	6	4
Germany	34	59	892	66	30	61	27	5	4
Hungary	33	13	1910	69	22	38	15	8	6
Ireland	9	7		100		86		0	
Ireland	22	20	143	60	24	60	27	20	13
Italy	30	67	700	63	23	27	9	5	5
Italy	31	16	1348	81	22	25	11	0	5
Italy	38	32	128	81	22	28	9	3	4
Scotland	10	6		83		83		0	
South Africa	32	17	1059	65	29	47	28	6	8
Spain	27	9	450	67	19	56	16	0	10
Sweden	26	50	500	66	32	40	28	22	2
USA	4	23	201	56	22	35	34	4	4
USA	11	23	201	56	22	35	34	4	4

Note:
[a] Controls from[23].

carrying a specific HLA antigen. In 70 unrelated Danish subjects with clinically overt hemochromatosis[29], the frequency of HLA-A3 was 80%; in 1967 normal control subjects, the frequency was 27% (RR = 10.9). The frequency of HLA-B7 was 60% in patients and 27% in controls (RR = 4.1), and the frequency of HLA-B14 was 10% in patients and 5% in controls (RR = 2.4). HLA-B47 occurred with a frequency of 4.3% in patients and 0.5% in controls (RR = 9.7). The frequency of HLA-A3, B7 in patients was 51% and 12% in controls (RR = 7.6). The frequency of HLA-A3, B14 was 10% in patients and 1% in controls, with an RR of 7.7. The frequency of HLA-A3, B47 was 4.3% vs. 0.5% in controls (RR = 9.7). Only 6 of the 70 patients did not carry any of the three typical HLA alloantigens (HLA-A3, HLA-B7, HLA-B14)[29].

Table 3.3 shows that the RR value for having hemochromatosis when carrying HLA-A3 is similar in Denmark[29], Germany[19], and Paris[25], somewhat lower in Brittany[7,21] and Australia[12], and lowest in Sweden[26]. The RR value for HLA-B7 is similar in Denmark, Germany, Paris, and Australia, and low in Brittany and Sweden. The RR value for HLA-B14 is low in Denmark, Germany, and Paris, higher in Brittany, and highest in Sweden. The antigen combination HLA-A3, B7 produced a similar RR value in patients in Denmark and Sweden, whereas the combination HLA-A3, B14 yielded an intermediate RR in Denmark, a higher RR in Brittany, and the highest RR value in Sweden. Lin et al.[39] proposed a method in which Bayes' rule was used to calculate the probability that designated HLA marker haplotypes,

Table 3.2. Frequency of HLA haplotypes in persons with hemochromatosis (H) and in normal control subjects (N)

				Frequency, %									
				HLA-A3, B7		A3, B14		A3, B5		A3, B35		A3, B47	
Country	Reference	Unrelated patients, *n*	Normal controls, *n*	H	N	H	N	H	N	H	N	H	N
Australia	13	77		22	8	4	3	2	0	0			
Belgium	17	12	40	67	8	0	8						
Denmark	40	74	497	26	7	3	0.6	10	0.3			7	0.2
France	16	48	591	21	5	12	1						
France	35	342 (609[a])	291 (475[a])	25	6	18	2	0				0	
Germany	8		5046		6		0.1						
Germany	19	35	100	54	8								
Germany	34	59	892	71	6	3	0.3	3	0.4	8	3		
Ireland	9	7	86	86	21	14							
Ireland	22	20	143	40	12	4	0						
Italy	30	38		14	4	5	5	7	1			0	
Italy	31	16	1348							44	8		
Italy	38	32	2819	10	0.7	0		10	1	22	2	0	
Scotland	10	6	186	83	16	17							
Sweden	26	50	190	19	7	11	0.5	0				0	
USA	11	23	201	26	11	4	0						
USA	82	202	321	24	5	4	1	<1	<1			<1	<1

Note:
[a] HLA haplotypes.

Table 3.3. Relative risk (RR) values for specific HLA alleles in hemochromatosis

Country ref	HLA-A3	HLA-B7	HLA-B14	HLA-B47	HLA-A3, B7	HLA-A3, B14	HLA-A3, B47
Australia[12]	6.7	4.1					
Denmark[29]	10.9	4.1	2.4	9.7	7.6	7.7	9.7
France–Brittany[7]	9.9						
France–Brittany[21]	6.6	<1.0	5.2			13.8	
France–Paris[25]	10.6	4.3					
Germany[19]	10.8	5.3	<1.0				
South Africa[32]	4.4						
Sweden[26]	4.2	1.7	11.5		7.0	23.4	

brought into the family by spouses, had attendant hemochromatosis genes. The haplotypes HLA-A3, B14 and HLA-A3, B7 were high-risk markers, associated with a 24–100% risk of coinheritance of a hemochromatosis allele. When HLA-A3 and HLA-B14 were absent from the unshared haplotype, the risk of having another hemochromatosis allele was 2–3%, i.e., less than that of the general population.

Many studies from different countries have shown that HLA-A3 is the major and only independent marker for hemochromatosis, whereas HLA-B7, HLA-B14, HLA-B35 (and HLA-B47 in Denmark) are minor or secondary markers occurring in increased frequencies only when linked to the critical HLA-A allele[29, 31, 35, 38]. Among Danish hemochromatosis patients carrying HLA-B7, 86% also possessed HLA-A3, although all patients with HLA-B14 and HLA-B47 carried HLA-A3[29]. The presence of HLA-A3 is associated with the highest RR of having hemochromatosis; the RR is not increased even if HLA-A3 is accompanied by HLA-B7, HLA-B14 or HLA-B47[29, 35]. No association was found between HLA-C antigens and hemochromatosis[21, 29].

One French study[21] reported an increased prevalence of HLA-DRw6 in patients with hemochromatosis, whereas a Danish study[29] found no association between HLA-DR antigens and hemochromatosis.

The high frequency of HLA-A3 was initially described in the population of Brittany[6, 7, 24], and is a common feature of hemochromatosis in Caucasians of European origin. In contrast, the frequencies of HLA-B7 and HLA-B14 are dependent on the geographic region. For example, the prevalence of HLA-B7 is high in Germany and Denmark, whereas the prevalence of HLA-B14 is low[19, 28, 29]. In Brittany[24], England[14], and Central Sweden[26], the prevalence of HLA-B14 is significantly higher than the prevalence of HLA-B7. Due to the strong association between the HLA alloantigens and the hemochromatosis gene (ignoring the small probability of recombination), HLA-typing has until now been an indispensable tool in the identification of the genotype in an affected pedigree, and an adjunct to the biochemical screening of iron status in relatives of probands with hemochromatosis.

HLA haplotypes

Pedigree analyses have led to the identification of the HLA-A and HLA-B haplotypes associated with hemochromatosis[13, 26, 30, 31, 34, 35, 40] (Table 3.2). The majority contain the HLA-A3 allele, whereas the occurrence of HLA-B alleles varies among populations from different geographic areas. Probably the ancestral hemochromatosis gene originated by rare mutation(s) on a chromosome carrying the HLA haplotype A3, B7[24]. Over time, genetic recombinations between HLA-A and HLA-B loci[41] and the migration and mingling of different populations have remodelled the HLA markers[35]. This explains why the major marker HLA-A3 is now associated with various HLA-B alleles in different populations, and why the diversity of haplotypes will increase in proportion with the period of time elapsed since the initial mutation occurred.

Population studies have demonstrated a strong linkage disequilibrium between the hemochromatosis allele and HLA-A3, a less pronounced disequilibrium with HLA-B7 and, in some populations, with HLA-B14[4, 13, 24, 35]. Linkage refers to the tendency of genes at two loci on the same chromosome to segregate together, which means that they are inherited together[42]. The associations of the hemochromatosis allele with the HLA-B alleles (B5, B7, B14, and B47) appear to be due to linkage disequilibria between the HLA-A3 allele and the HLA-B alleles[36]. The HLA-B alleles display an increased frequency merely because they are linked to HLA-A3[30, 31, 35, 40]. In Danes[40], 19 different haplotypes have been identified in 39 chromosomes of patients with hemochromatosis. The HLA-A3 allele was present in 56% of the haplotypes, and 26% carried the HLA-A3, B7 haplotype. Among 31 different HLA haplotypes in 51 non-hemochromatosis chromosomes, only 1.9% carried HLA-A3, B7.

A founder effect denotes that a particular gene is introduced into a population by a single carrier, from which it spreads by multiplication from generation to generation. It is believed that the hemochromatosis mutation arose in a gene that was spread subsequently by the founder effect, and that the majority of hemochromatosis chromosomes appear to be derived from a founder individual. For example, the candidate hemochromatosis gene could be traced through eight generations to the beginning of the eighteenth century in a Swedish pedigree[26]. In a geographically isolated region of northeastern Quebec, genealogical reconstruction showed that 15 of 57 obligate carriers of the hemochromatosis allele could be traced to a unique ancestor in the eighteenth century[43]. The founder concept was supported by the presence of high coefficients of consanguinity and kinship compared to control populations[43], and probably also explains the close association between the hemochromatosis allele and the haplotype HLA-A3, B35 in northeastern Italy[31, 37, 38] (Table 3.2). An apparent exception occurred in South Africans of Afrikaner origin[44]. This population, dating back to the settlement in 1652, started out with few founders, yet the expected founder effect in the form of a single haplotype was not detected[35], and no association was observed between the hemochromatosis allele and the HLA-A3 allele[44]. However, these results were revised in a subsequent more comprehensive study[32] which confirmed that the well-recognized association between hemochromatosis and the HLA-A3 and HLA-B7 alleles also existed in the Afrikaner population (Tables 3.1, 3.2).

The geographic distribution pattern of HLA alloantigens associated with hemochromatosis suggests a common ancestral source of the HLA-A and HLA-B markers and thus the hemochromatosis gene. An early chromosomal recombination between HLA-A and HLA-B loci might have replaced HLA-B7 by HLA-B14, and the two ancestral haplotypes thus created would each have followed a different migratory profile[24]. The HLA marker was subsequently modified by genetic recombinations and geographical scattering due to migration. The hemochromatosis gene might be of Celtic origin, with spread of the gene by migration[24], or of Scandinavian (Danish) origin[29], from which it subsequently might have been spread by the Vikings who extended their settlements towards the north and east to Norway and Sweden, towards the west to England, and towards the south and southwest to Germany, Normandy, and Brittany.

Inheritance of hemochromatosis

Abnormalities in iron metabolism can be demonstrated consistently in relatives of persons affected with hemochromatosis[24]. In the 1950s and 1960s, the findings of abnormalities in iron status among parents or offspring of hemochromatosis patients motivated the majority of researchers to suggest that the disorder was inherited in an autosomal dominant manner[45–47], although some authors favored an intermediate inheritance (i.e., recessive with partial expression in heterozygotes)[48]. Some proposed an autosomal recessive mechanism[49]. In the 1970s and 1980s, genetic transmission of hemochromatosis was proposed to occur via polygenic and oligogenic mechanisms, although intermediate and recessive mechanisms were also suggested[24, 42, 50, 51].

Overt hemochromatosis is far less common than minor abnormalities in the iron status of parents and children of the proband. Clinically overt disease was most frequently seen in adult siblings, especially brothers[24]. Genetic analysis of such data led to the hypothesis that hemochromatosis is an autosomal recessive condition with full phenotypic expression in adult males. Saddi and Feingold[49, 52] proposed that minor biochemical abnormalities of iron metabolism in other family members could indicate heterozygosity for a hemochromatosis allele. Based on their genetic analysis of 96 pedigrees, they presented evidence that the mode of genetic transmission was autosomal recessive and that homozygosity was required for clinical expression of the disorder. They found an increased rate of consanguineous matings among the parents of the probands compared to that of the reference population. Three distinct levels of iron stores were identified: normal, slightly increased, and heavily increased. None of the subjects among the parents or offspring of the proband was affected by the disorder. To test the recessive hypothesis, they performed a segregation analysis in which the observed proportions of affected individuals were compared with the expected values, taking into account that the penetrance varies with gender. Penetrance concerns the relationship between genotype and phenotype. Assuming a penetrance of 1.0 and 0.2 in males and females, respectively, the results of the segregation analysis were highly compatible with a disorder inherited as an autosomal recessive trait[52]. The consanguinity reported in other studies[45, 50, 52, 53] also supported a hypothesis of recessive inheritance. Coefficients of consanguinity of 174×10^{-5}, 271×10^{-5}, and 440×10^{-5} have been determined[45, 50, 52]; the mean coefficient of apparent consanguinity in France during the period 1926–1930 was much lower (86×10^{-5})[54]. Subsequent pedigree studies employing HLA analysis arrived at a similar conclusion: that hemochromatosis is inherited as an autosomal recessive trait[7, 24, 35, 50, 51, 55] (Fig. 3.1).

The degree of dominance is estimated by the ratio of the heterozygote effect to the homozygote effect. This ratio, symbolized by the letter d, ranges from 0 (indicating strictly recessive inheritance) to 1 (indicating strictly dominant inheritance); the probability that an offspring has inherited a particular allele from a parent is 0.5. Genetic recombination (chromosomal crossing over) occurring during meiosis is the process by which DNA is exchanged between paternal and maternal chromosomes. In the case of genetically linked alleles, the probability that an offspring will inherit a pair of alleles that occur on the same chromosome is 0.5 multiplied by the probability that the two alleles do not undergo genetic recombination. The probability of genetic recombination is symbolized by θ. When $\theta = 0.0$, no genetic recombination is ever observed, and when $\theta = 0.5$, recombination occurs half of the time. A value for θ of 0.5 is equivalent to no linkage, i.e., the probability of joint inheritance for the two alleles is similar to that for random assortment of unlinked alleles. Likelihood analysis is an intuitive way of assessing data[42, 56]. A likelihood is the probability of a set of observations occurring, given a specific model and a set of parameter values. The logarithm of the likelihood ratio is expressed in the LOD score, i.e., the logarithm of the odds (the odds being the likelihood ratio). The likelihood of a particular genetic model, given a set of observations in a number of pedigrees, is the product of the likelihoods of that model on each pedigree separately[42, 56]. LOD scores calculated on different pedigrees can therefore be added to determine the support for a given genetic model in the group as a whole[42, 56]. Linkage is estimated by the LOD score that indicates the relative certainty with which linkage can be accepted or rejected. A LOD score of 0.0 gives no information on linkage, whereas a LOD score of +5, for example, indicates that the odds are 10^5 to 1 that the hemochromatosis locus and the HLA locus are genetically linked.

Kravitz et al.[55] assessed the linkage between the HLA loci and the hemochromatosis locus using likelihood analysis in a large Mormon pedigree of a proband with hemochromatosis using transferrin saturation as the quantitative phenotypic trait. At one maximum of $d = 0.17$, the highest likelihood was obtained for a recombination fraction of $\theta = 0.0$, indicating close linkage. At the other maximum at $d = 0.61$, there was no evidence for linkage. A LOD score of +6.9 indicated strong linkage between the hemochromatosis locus and the HLA-locus. The analysis indicated that the inheritance of hemochromatosis was recessive, with partial expression in heterozygotes. Because two

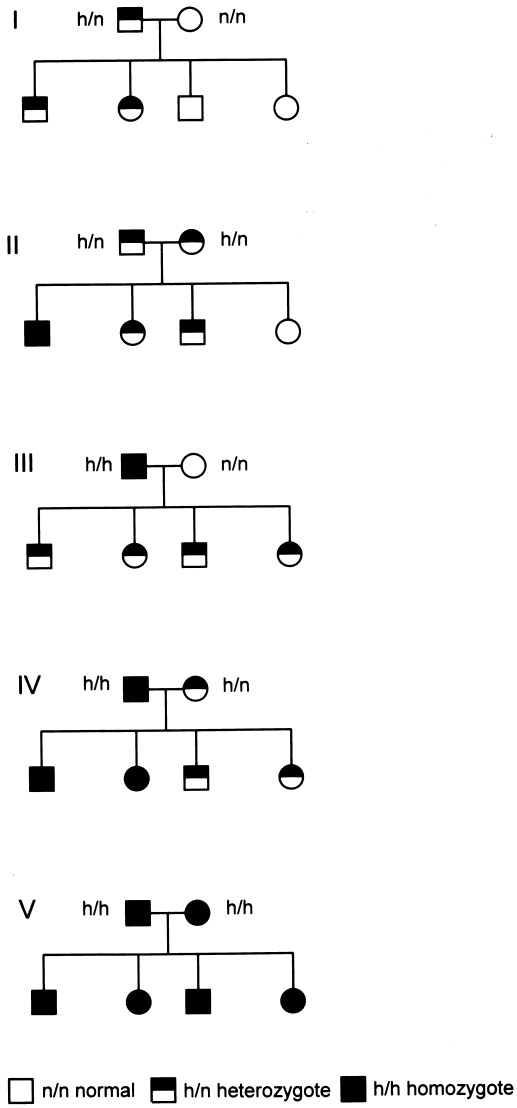


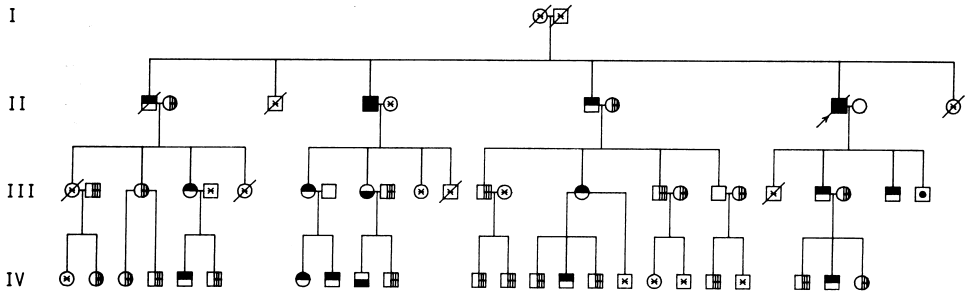
Fig. 3.1. Pattern of inheritance of hemochromatosis (Mendelian autosomal recessive). I. One heterozygous and one normal parent; none of their offspring will be homozygous for the hemochromatosis allele, 50% will be heterozygous, and 50% will not have the hemochromatosis allele. II. Two heterozygous parents: 25% of their offspring will be homozygous, and 50% will be heterozygous for the hemochromatosis allele; 25% will not inherit the hemochromatosis allele. III. One homozygous and one normal parent: all offspring will be heterozygous for the hemochromatosis allele. IV. One homozygous and one heterozygous parent: 50% of their offspring will be homozygous and 50% heterozygous for the hemochromatosis allele. This combination shows apparent dominant (pseudodominant) mode of inheritance. V. Two homozygous parents; all their offspring will be homozygous for the hemochromatosis allele. This combination also demonstrates a pseudodominant mode of inheritance.

hemochromatosis alleles are required for phenotypic expression of hemochromatosis, expression in a family member occurs only when the HLA haplotypes are identical to those of the proband. The specific HLA haplotypes linked to a hemochromatosis allele vary from pedigree to pedigree, but are consistent within each family. However, genetic recombination explains occasional exceptions to this rule[13, 39, 51, 55, 57–67].

The HLA class I complex is located on the short arm of chromosome 6 (6p)[68, 69]. Simon et al.[7] suggested that the hemochromatosis allele and the HLA loci were closely linked, and the linkage was verified by likelihood analysis of hemochromatosis pedigrees in which HLA data and biochemical measures of iron stores had been obtained[50, 57, 61]. Simon et al.[50, 51] obtained a LOD score of + 2.2 for linkage of the hemochromatosis allele and the HLA region provided a recombination fraction (θ) of 0.005. Kravitz et al.[55] found a LOD score of +6.9 in a recessive model with partial biochemical expression in heterozygotes. Cartwright et al.[57] obtained a LOD score for linkage of +9.8 for a recombination fraction (θ) of 0.0 and a gene frequency of 0.056. Dadone et al.[61] obtained a LOD score of +16.4 for an estimated recombination fraction (θ) of 0.015 and a gene frequency of 0.069. Lalouel et al.[62] obtained powerful evidence for linkage: a LOD score of +38.0 for an estimated maximum likelihood recombination fraction (θ) of 0.011 and an estimated gene frequency of 0.06. The cumulative LOD score reaches more than +60 when other published results are also considered[12, 16, 61], signifying that the likelihood of the existence of linkage is 10^{60}-fold greater than not. Thus it became possible, within a given pedigree, to assign the hemochromatosis allele to a certain HLA-haplotype (Fig. 3.2). Based on an observed recombination, Edwards et al.[60] suggested that the hemochromatosis allele on chromosome 6p could be located distal to the HLA-A locus, between the HLA-A and HLA-C loci, or between the HLA-C and HLA-B loci. The candidate hemochromatosis gene *HFE* is 4 megabases telomeric to the HLA-A locus on chromosome 6p[70], and genotyping for *HFE* mutations will probably replace HLA analysis in family screening.

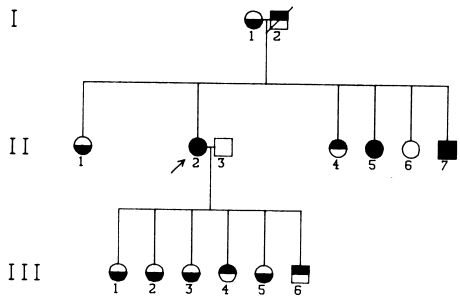
Iron status markers in hemochromatosis

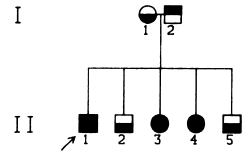
Clinically overt hemochromatosis with organ damage due to iron overload is characterized by a marked increase in mobilizable body iron stores. Typical laboratory biochemical findings are an elevated serum iron concentration, a decreased serum transferrin value, an increased serum transferrin saturation, and an elevated serum ferritin concentration[4, 5, 61, 66]. The best and most widely applied discriminators for screening of populations and families are



Family A

homozygote, HLA-A3,B7,DRw6/A3,B7,DR-
homozygote, HLA-haplotypes deduced
heterozygote, HLA-A3,B7,DRw6
heterozygote, HLA-A3,B7,DR-
heterozygote, HLA-A3,B7,DRw6?DR-?
normal or heterozygote
normal
not HLA-typed





Family D

Family E

homozygote, HLA-A3,B15,Cw3/A1,B8
heterozygote, HLA-A1,B8
heterozygote, HLA-A3,B15,Cw3
heterozygote, HLA-haplotypes deduced
normal
homozygote, HLA-A3,B7/A3,B15,Cw3
heterozygote, HLA-A3,B7
heterozygote, HLA-A3,B15,Cw3

Fig. 3.2. Hemochromatosis allele tracking by HLA linkage in a Faroese (family A) and two Danish pedigrees (family D and E). The probands are indicated by arrows. The siblings with both haplotypes in common with the proband are considered to be homozygous (h/h) for the hemochromatosis allele and are at risk for developing iron overload. The siblings who share one HLA-haplotype with the proband are considered to be heterozygous (h/n) for the hemochromatosis allele and are putative carriers. The siblings with no HLA-haplotype in common with the proband are considered not to have the hemochromatosis allele, and to be 'normal' (n/n). (Reproduced with permission[65].)

the biochemical iron status markers, serum transferrin saturation and serum ferritin concentration[4, 5, 57–59, 61, 62, 66, 71–73]. There is a large intraindividual variability of the serum iron concentration and thus transferrin saturation[74]. Therefore, when using the transferrin saturation as a discriminator, it is necessary that the measurements be obtained under standardized conditions and that the high values are reproducible, i.e., at least two measurements are necessary to classify an individual in the diagnostic phase. The serum transferrin saturation value is considered to be phenotypic indicator of the presence of the hemochromatosis allele[57, 61, 62, 66, 71]. The serum ferritin concentration reflects the size of the mobilizable body iron stores, i.e., the phenotypic expression of iron overload. It seems likely that *HFE* mutation analysis will become used commonly in the screening for hemochromatosis[70]. Genotyping assesses the predisposition to disease, but cannot replace the conventional iron status markers that monitor the phenotypic expression and thus the impact of the hemochromatosis gene(s). Furthermore, the indication for prophylactic or therapeutic iron depletion (phlebotomy) relies on the serum ferritin concentration.

Iron status markers in homozygotes

The diagnostic value of iron status markers has been assessed in single patients with clinically overt hemochromatosis and in pedigree studies. Until genetic analysis becomes routinely available, relatives of hemochromatosis probands are characterized according to their HLA-A, B haplotypes. Relatives that share both haplotypes with the proband are classified as 'homozygotes' (h/h), those with one haplotype in common with the proband are classified as 'heterozygotes' (h/n), and those having no haplotype in common with the proband are considered to be 'normal' (n/n)[57–59, 61, 62, 65–67, 71, 75, 76]. Persons with clinically recognizable disease have a high serum iron concentration, low serum transferrin concentration, a high serum transferrin saturation value, and a high serum ferritin concentration[5, 57–59, 66, 75, 77]. The transferrin saturation has the highest diagnostic efficiency[61, 62, 66, 71]. In HLA-homozygous relatives without clinical disease, serum transferrin saturation values are likewise elevated, but with a greater scatter of the values[66].

Dadone et al.[61] evaluated iron status markers in hemochromatosis pedigrees using discriminant analysis. Family members were assigned a genotype according to their HLA-A, B haplotypes. Transferrin saturation values were superior to serum ferritin concentrations in predicting the genotype. A critical transferrin saturation value of 62% accurately predicted 'homozygosity' for hemochromatosis alleles in 92% of the cases, and incorrectly classified 8% of them as 'heterozygotes.' Homozygous males had significantly higher transferrin saturation than females. Serum ferritin concentration accurately predicted homozygosity for hemochromatosis in 53% of the subjects, a figure that increased to 71% after logarithmic transformation. Consequently, serum ferritin concentration appeared unsuitable as a discriminator in this series. These investigators also tested the mode of inheritance (dominant vs. recessive) of the iron status markers, and found that serum ferritin concentration and hepatic iron content were inherited as recessive traits, whereas transferrin saturation was inherited as an intermediate trait with a value of $d = 0.16$[61]. Bassett et al.[62] studied iron status markers in kinships with hemochromatosis; genotypic assignment was made by HLA-typing. The predictive value of a positive test (PV_{pos}), and predictive value of a negative test (PV_{neg}) were calculated. The accuracy for positive prediction indicates the probability that a person with a positive test, in fact, has the disorder, and the accuracy for a negative prediction indicates that a person with a normal test does, in fact, not have the disorder. A threshold value for transferrin saturation of $>50\%$ yielded a PV_{pos} of 0.74, and a PV_{neg} of 0.93. A threshold value for serum ferritin concentration (>200 μg/l in men and >150 μg/l in women) yielded slightly higher predictive values (PV_{pos} of 0.88 and PV_{neg} of 0.94, respectively). The diagnostic efficacy was not improved when transferrin saturation values and serum ferritin concentrations were combined. Serum ferritin concentration correlated well with mobilizable body iron stores measured by quantitative phlebotomy, and was thus an accurate indicator of the degree of iron overload. Each μg/l of ferritin corresponded to 7.5 mg of body iron.

Borwein et al.[71] examined the diagnostic efficacy of transferrin saturation values and serum ferritin concentrations in hemochromatosis pedigree studies using likelihood analysis; genotypic assignment was made by HLA-typing. A threshold value for transferrin saturation of 55% provided the best statistical combination of true-positive and true-negative results. The maximum true-positive and true-negative rates for distinguishing between homozygous and non-homozygous individuals (both 92%) were obtained with a transferrin saturation cut-off value of $\geq 55\%$. A transferrin saturation of $\geq 55\%$ or a cut-off value of $>$90th percentile for serum ferritin concentration were adequate for the detection of homozygosity. A transferrin saturation of $>74\%$ and a serum ferritin $>$90th percentile ruled in homozygosity, whereas a transferrin saturation of $< 55\%$ and a serum ferritin concentration $\leq$90th percentile excluded homozygosity with confidence. Milman[5] performed studies of iron status

markers in 162 patients with hemochromatosis and pedigree analysis with HLA typing in 21 families comprising 149 first- , second- and third-degree relatives; the genotype was derived from the HLA type. Twelve homozygous siblings with preclinical disease, 65 heterozygotes, and 9 normal relatives were identified. In patients with clinical disease, transferrin saturation and serum ferritin concentration had similar maximum efficacies of 0.97 and 0.99, respectively. Patients with clinical disease had transferrin saturation values ≥ 52%, and 97.5% had values >60%; all patients with clinical disease had serum ferritin ≥800 μg/l. Homozygous subjects with preclinical hemochromatosis had transferrin saturation values ≥47%; 91.7% had values >50%, and 58.3% had values >60%. All subjects with preclinical hemochromatosis had serum ferritin ≤ 600 μg/l. Transferrin saturation was the best single marker; transferrin saturation values >50% identified all individuals with clinical and preclinical hemochromatosis. This threshold value corresponds to the 90th percentile for healthy Danish men and the 95th percentile for healthy Danish women. In a screening survey for hemochromatosis among healthy blood donors, Edwards et al.[72] used a critical transferrin saturation value of >61%, and found that 60% of men and 100% of women fulfilling this criterion had the disorder. This discriminatory value, however, gave a higher prevalence of hemochromatosis in men than in women, suggesting that in women, a critical transferrin saturation value of >49% should be employed.

Iron status markers in heterozygotes

The autosomal recessive inheritance of hemochromatosis implies that partial expression of the disorder is expected in subjects who are heterozygous for the hemochromatosis allele. So far, 'heterozygosity' has been identified by HLA-typing, i.e., those individuals having one haplotype in common with the proband. The frequency of heterozygosity for the hemochromatosis allele among populations of northern European origin is 9–17%[32, 57, 61, 72, 78–81]. Therefore, by chance it occurs often in pedigrees with hemochromatosis that some relatives carry a hemochromatosis allele other than those in the proband. Thus some of the individuals initially designated as heterozygotes are, in fact, homozygotes for hemochromatosis allele(s). The risk of misclassification has been addressed in several studies[5, 58, 61, 67, 75, 76, 82]. The concept of how heterozygosity may influence iron status awaits reevaluation using genotyping[70].

Studies of HLA typing have shown that approximately one-third of heterozygotes have minor abnormalities of iron status parameters, and some develop minor iron loading[5, 57–59, 62, 71, 75, 76, 78, 82–84]. Compared to normal subjects (n/n), heterozygotes have a slightly, (but significantly) higher transferrin saturation[57, 59, 62, 66, 71, 76, 78, 82–84]. Beaumont et al.[58] found that 18% of 141 heterozygous family members of hemochromatosis probands had at least one positive test (serum iron concentration, serum unsaturated iron-binding capacity, or serum ferritin concentration) for iron loading. Serum ferritin concentrations were not significantly different in heterozygous women than in controls, whereas heterozygous men had significantly higher concentrations than control subjects. Of the men, 26% had serum ferritin values >200 μg/l, and 32% of the women had ferritin values >150 μg/l. Cartwright et al.[57] reported that 31% of 145 heterozygotes had minor abnormalities in iron status markers, and displayed significantly higher transferrin saturation and hepatic iron content than normal subjects. Increased transferrin saturation values of 51–77% were observed in 31% of the male heterozygotes and in 17% of female heterozygotes. However, there were no significant differences between heterozygotes and normal subjects with respect to serum ferritin concentration. Valberg et al.[59] found that 25% of 69 heterozygotes had minor abnormalities in iron status markers. Among heterozygous men, 28% had a transferrin saturation of >50%, and 11% a saturation of >60% (range 52–75%); 18% of heterozygous women had a transferrin saturation of >50% (range 51–85%). Heterozygous women had significantly higher serum ferritin concentrations than controls; 9% had values greater than the 90th percentile for age and sex obtained in a population survey. Heterozygous men had serum ferritin concentration values similar to those of control subjects; only 6% had values greater than the 90th percentile for age and sex obtained in a Canadian population survey. Borwein et al.[71, 78] demonstrated that 6% of 82 heterozygotes had transferrin saturation >55%. Heterozygotes had serum ferritin concentrations similar to those of controls; 9% had values above the 90% confidence interval. Bassett et al.[75] reported that 25% of 98 heterozygotes had at least one abnormality in iron status markers, suggesting increased body iron stores. However, there was no significant difference in serum ferritin concentrations in heterozygotes and normal subjects. These investigators also found that 12% of 86 heterozygous and homozygous normal subjects had transferrin saturation values >50%, and 5% had elevated serum ferritin concentrations (>200 μg/l in men, >150 μg/l in women)[62].

Powell et al.[76] studied a series of 98 putative heterozygous relatives of hemochromatosis probands; 38% had some phenotypic expression of a hemochromatosis allele. Six percent had an elevated serum transferrin saturation,

6% had elevated transferrin saturation and elevated serum ferritin concentration, and 26% had increased serum ferritin concentration alone. The putative heterozygous subjects were followed for several years and displayed no evidence of an increase in iron load, except for one subject. This individual was probably homozygous for the hemochromatosis allele, but was misclassified as a heterozygote due to chromosomal recombination between the hemochromatosis and the HLA-A loci[76]. Milman[5] examined 84 heterozygous individuals; 12% had transferrin saturation values of >50%, 4% had values >62%. Serum ferritin concentration was not significantly different in heterozygotes and controls. In a pedigree study of 29 unrelated probands and 172 relatives, 11 of 105 (10.5%) individuals initially classified as heterozygotes were reclassified as homozygotes due to abnormal iron status explained by either: homozygous–heterozygous matings ($n=7$), heterozygous–heterozygous matings ($n=2$), chromosomal recombination of the HLA-A locus ($n=1$), or increased body iron stores ($n=1$)[67]. Adams[83] analyzed iron status in 255 heterozygotes classified by HLA typing. The mean transferrin saturation value and serum ferritin concentration was significantly higher in heterozygotes than in normal subjects; 9% had a transferrin saturation >55%, and 11% had a serum ferritin concentration greater than the 90th percentile for gender and age. Mean hepatic iron concentration was higher in heterozygotes than in normal controls, but all heterozygotes had a hepatic iron index <2.0, i.e., they had no clinically significant iron load.

Crawford et al.[84] studied iron status in 256 individuals designated as heterozygotes by HLA haplotyping. The mean transferrin saturation was significantly higher in heterozygotes than in control subjects, whereas no difference was observed in serum ferritin concentrations. The relationship between increasing age and serum ferritin concentration was similar in heterozygotes and controls. However, heterozygous women carrying the ancestral hemochromatosis haplotype had significantly higher serum ferritin concentration than those carrying another hemochromatosis haplotype; this difference was not present in men. Among heterozygous women and normal controls, 0.8% and 5% were iron depleted (serum ferritin concentration <10 μg/l), suggesting that heterozygosity offers partial protection against iron deficiency. Bulaj et al.[82] examined the iron status of 505 male and 553 female heterozygotes, and of 321 normal (n/n) persons defined by HLA typing of 202 pedigrees. The effects of age, gender, gender of the parent transmitting the affected chromosome, and of specific HLA haplotypes on iron stores were analyzed. The mean transferrin saturation value was higher in heterozygotes than in normal subjects, and did not increase with age. Four percent of the male heterozygotes and 8% of the female heterozygotes had initial transferrin saturation values above the critical levels used in the diagnosis of hemochromatosis (>62% in men, >50% in women); 18% of male heterozygotes and 11% of female heterozygotes had transferrin saturation values that were more than two standard deviations above the mean values for normal subjects. The serum ferritin concentration was higher in heterozygous men than in normal subjects at all ages. Serum ferritin concentration in male heterozygotes rose gradually until the sixth decade and declined slightly hereafter; 20% had serum ferritin concentrations above the 95th percentile value for the age-matched male controls. Serum ferritin concentrations in female heterozygotes >30 years of age were higher than in normal subjects. In heterozygous women, the serum ferritin concentration rose until the seventh decade; 8% had a serum ferritin concentration greater than the 95th percentile value for the age-matched male controls. The prevalence of iron deficiency (serum ferritin concentration < 12 μg/l) in women of the fertile age was significantly lower in heterozygous women than in normal women; the incidence of iron deficiency was not different in heterozygous and normal men. There were no significant differences in serum ferritin concentrations in heterozygotes carrying an HLA-A3 allele and those with any other HLA-A allele. The clinical and biochemical expression of the hemochromatosis allele was more marked in heterozygotes with paternally transmitted mutations than in those with maternally transmitted mutations.

In general, most studies conclude that median or geometric mean serum ferritin concentrations in heterozygotes are not significantly different from those in normal subjects[57, 75, 78], although one large study has reported moderately increased levels in heterozygotes of both sexes[82]. Heterozygous subjects with high serum ferritin concentration should therefore be suspected of being misclassified homozygotes. However, some heterozygous men and women have a slightly higher hepatic iron content than normal subjects[57, 75], although the serum ferritin concentrations apparently are not significantly different than those of normal subjects[57, 75]. It appears that major iron overload associated with serum ferritin concentrations greater than 700–800 μg/l and organ damage does not occur in heterozygotes. Any such cases are probably homozygotes misclassified as heterozygotes[67].

In normal subjects, the intestinal iron absorption is inversely correlated to the serum ferritin concentration, i.e., body iron stores[85, 86]. A feedback mechanism downregulates iron absorption when body iron stores increase and ensures that iron stores remain at a relatively constant

level, protecting against iron overload. In patients with hemochromatosis, the relation between iron absorption and body iron stores reaches a plateau at a much higher level of iron stores than in normal subjects, resulting in an excessively high iron absorption in persons with hemochromatosis[85, 87–89]. Walters et al.[85] demonstrated a significant negative correlation between serum ferritin concentration and iron absorption in 52 normal subjects. There was a similar significant inverse relationship between serum ferritin concentration and iron absorption in 15 patients with hemochromatosis, but absorption was higher in relation to iron stores at all levels. Adams et al.[88] reported a significant negative correlation between hepatic iron content and iron absorption in 21 subjects who were homozygous for hemochromatosis allele(s). Valberg et al.[59] measured intestinal iron absorption in subjects who were homozygous and heterozygous for the hemochromatosis allele. Homozygotes had excess iron absorption in proportion to the serum ferritin concentration, whereas iron absorption in relation to respective ferritin concentrations was within the normal control range in most heterozygotes. Borwein et al.[71] measured iron absorption in subjects who were homozygous, heterozygous, and normal for the hemochromatosis allele. In homozygotes, there was a deregulation of the absorptive mechanism, whereas heterozygotes had a preserved absorptive feedback mechanism similar to that of normal subjects. McLaren et al.[89] assessed iron absorption in six patients with hemochromatosis and in five normal subjects. Iron absorption was inversely correlated to the serum ferritin concentration in both normal subjects and hemochromatosis patients, but was higher in relation to serum ferritin concentration among the latter. Analysis of mucosal iron kinetics showed that the rate constant for mucosal iron uptake was inversely correlated to the serum ferritin concentration, and that there was no significant difference between hemochromatosis patients and normal subjects. However, the rate constant for transfer of mucosal iron to the plasma was higher in hemochromatosis patients than in normal subjects[89]. Dadone et al.[61] found that serum ferritin concentration and hepatic iron content were inherited as recessive traits, whereas transferrin saturation was inherited as an intermediate trait (indicating that the expression in heterozygotes was similar to that in normal individuals). Other studies[5, 57, 61, 75, 78, 82] corroborate that, in those heterozygotes with partial expression of the hemochromatosis trait, iron loading is not sufficiently pronounced to cause clinical manifestations.

Family studies of HLA-linked hemochromatosis

Since Sheldon[1, 2] suggested that hemochromatosis is an inborn error of metabolism, many family studies have been performed, a moderate number of which were published in the 1950s and 1960s [43, 44, 46, 85, 88–92]. After the fortuitous observation that HLA-A3 was closely linked to the hemochromatosis allele, the research on family studies of the disorder was intensified in Western populations. HLA-typing was the prerequisite for these pedigree analyses and was used to designate the genotype, whereas the phenotypic expression of the genotype was assessed by the biochemical iron status markers, transferrin saturation and serum ferritin concentration. Pedigree studies have revealed the pitfalls that may occur when determining the genotype from the HLA type, such as the matings between two heterozygous subjects or between homozygous and heterozygous subjects, and the effect of consanguineous matings. Furthermore, family analyses have demonstrated that genetic recombination occurs between the HLA-A locus and the hemochromatosis locus, and have made it possible to estimate the expression or penetrance of the hemochromatosis gene in both homozygous and heterozygous subjects.

Heterozygote-heterozygote matings

In most families with hemochromatosis, the disorder is expressed in a horizontal pattern: siblings of the proband are affected far more frequently than offspring[50, 51, 65, 67]. There is no doubt that the majority of cases of hemochromatosis is due to heterozygote–heterozygote matings, in which an affected individual inherits a hemochromatosis allele from each parent. Examples of such matings are displayed in Fig. 3.1–II, Fig. 3.2 and Fig. 3.3. Due to the high gene frequencies in Caucasian populations of northern European origin, matings between two heterozygous individuals are not uncommon. The estimates of gene frequency vary from 0.045 to 0.081, corresponding to homozygote frequencies of 0.002 to 0.0071, and heterozygote frequencies of 0.086 to 0.149, respectively[12, 42, 57, 61, 72, 78, 79, 93–97]. The chance of a heterozygote mating another heterozygote has been calculated according to different gene frequencies (Table 3.4). With a gene frequency of 0.06, random heterozygote–heterozygote matings occur in approximately 1272 of 100 000 matings, i.e., in 1.3% of all random matings. When the hemochromatosis gene has been introduced into a family, the risk of heterozygote–heterozygote marriages increases in proportion to the coefficient of consanguinity within the family. The risk of heterozygote–heterozygote matings is an important

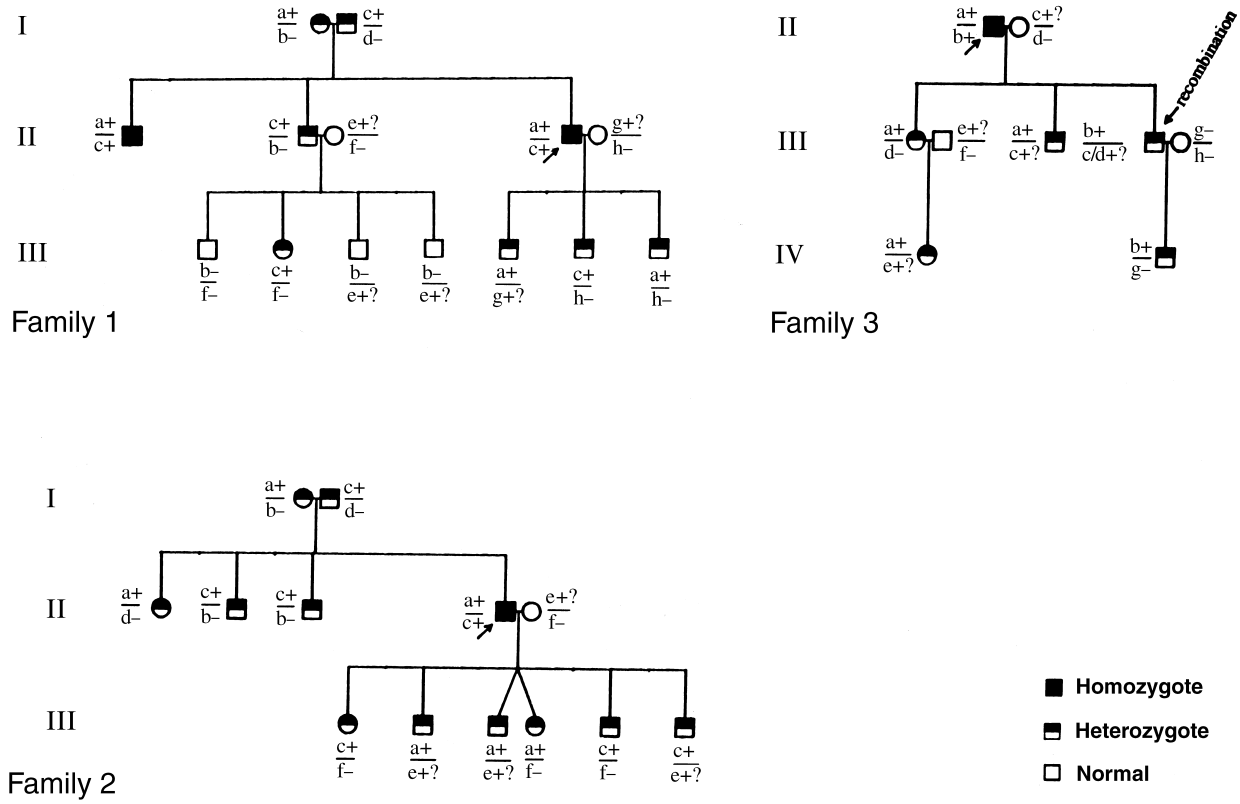


Fig. 3.3. Part of the pedigree trees in three Danish families with hemochromatosis. Probands are indicated by arrows. Person symbols indicate putative genotype according to HLA-typing. Letters a to j symbolize HLA haplotypes and postfix the presumed genotype, +/− indicate the presence/absence of the hemochromatosis allele. In subjects with ?, the presumed hemochromatosis genotype has been reclassified according to the phenotypic expression of the disorder, i.e., iron status markers. A genetic recombination is indicated in family 3. (Reproduced with permission[67].)

argument in the discussion of whether the population should be offered general prophylactic screening of the *HFE* genotype.

Homozygote–heterozygote matings

In a proportion of families, a vertical pattern of disease transmission is observed, one in which full expression of hemochromatosis is observed in the offspring of the proband[50, 51, 65, 67, 75, 98]. A vertical pattern of inheritance has been interpreted as evidence for autosomal dominant transmission[87]. However, simulation of dominant (i.e., pseudodominant) transmission is expected with a common recessive disorder such as hemochromatosis, in which 9–15% of the individuals in a population are heterozygous for the mutant allele(s)[57, 61, 72, 78–81]. Examples of such matings are shown in Fig. 3.1–IV, Fig. 3.3 and Fig. 3.4. The chance of a homozygous subject's mating a heterozygote is shown in Table 3.4. With a gene frequency of 0.06 (yielding a calculated frequency of homozygosity of 0.36% and a frequency of heterozygosity of 11.3%), random homozygote–heterozygote matings occur in 81 out of 100 000 matings, i.e., in 0.0812%. The risk for such matings increases in proportion with the coefficient of consanguinity in the population or family. In the screening of affected families, the risk of homozygote–heterozygote matings is an argument that the offspring (and siblings) of a proband should be evaluated.

Bassett et al.[98] observed a vertical pattern of inheritance in four families in a study of 22 hemochromatosis families, of which three are shown in Fig. 3.4. In the four families, all offspring with iron overload inherited a common HLA-haplotype from the unaffected parent, suggesting that this haplotype was carrying the hemochromatosis allele. Likewise, all unaffected offspring had inherited the other HLA haplotype from the unaffected parent, suggesting that his haplotype was not carrying the hemochromatosis allele.

Table 3.4. Frequencies of random matings at different gene frequencies in hemochromatosis[a]

Mating type	Probability	Distribution of 1000 matings according to gene frequency			
		$p=0.05$	$p=0.06$	$p=0.07$	$p=0.08$
h/h×h/h	$p^2\times p^2$	0.006	0.013	0.024	0.041
h/h×h/n	$2\times p^2\times 2pq$	0.475	0.812	1.276	1.884
h/h×n/n	$2\times p^2\times q^2$	4.513	6.362	8.476	10.834
h/n×h/n	$2pq\times 2pq$	9.025	12.724	16.952	21.668
h/n×n/n	$2\times 2pq\times q^2$	171.475	199.340	225.220	249.180
n/n×n/n	$q^2\times q^2$	814.506	780.749	748.052	716.393
Number of matings		1000	1000	1000	1000

Note:

[a] The hemochromatosis allele is denoted h, and its frequency p. The normal allele is denoted n, and its frequency q; $p+q=1$.

Milman et al.[67] classified first-, second-, and third-degree relatives according to HLA typing as putative homozygotes (h/h), putative heterozygotes (h/n), and putative normals (n/n) of 29 hemochromatosis probands. Spouses were HLA typed and a priori presumed to be normal (n/n). The phenotype was assessed by iron status markers (transferrin saturation, serum ferritin concentration, mobilizable body iron stores). In six families, there were divergences between the HLA-derived genotypes and the phenotypic expression, suggesting the occurrence of homozygous–heterozygous marriages. The pedigrees of three of these families are shown in Fig. 3.3. The possibility of homozygote–heterozygote matings should always be considered by the clinician performing the screening of families with hemochromatosis. Testing for *HFE* mutations will strengthen the genetic classification of relatives in future family screenings.

Homozygote–homozygote matings

A mating between two homozygous individuals will produce a vertical pseudodominant disease pattern, in which all the offspring may be affected (Fig. 3.1–V). This situation is extremely rare, and most likely to occur in families with a high coefficient of consanguinity. With a gene frequency of 0.06, random homozygote–homozygote matings will occur in approximately 1.3 out of 100 000 matings, i.e., 0.0013% (Table 3.4). Simon et al.[51] described a family in which five of seven sibs were affected by hemochromatosis. Both parents were bronze-colored; the mother died of hepatic disease, and the father by accident. Therefore, parents of the proband might have been homozygous for the hemochromatosis allele. Accordingly, the French hemochromatosis pedigree series[45, 50, 52] had a higher coefficient of consanguinity compared with that of the general French population[54].

Genetic recombination

Genetic recombination, or chromosomal crossover, is the process in which DNA is exchanged during the meiosis between paternal and maternal chromosomes being inherited from the father and from the mother. Genes are located on a linear fashion on chromosomes, and this has the logical consequence that genes located on the same chromosome may be transmitted together, i.e., that their segregation will not be independent. However, during the first meiotic division, certain chromosome segments are exchanged between homologous chromosomes from the parents[99]. Hence, even genes located on the same chromosome will not always be transmitted together. The probability of transmission of two linked genes will depend on the distance between them, and how often they are separated by crossing over. If the distance between the genes is large enough, or if there exist recombination 'hot-spots' between the genes, multiple recombinations may occur on the chromosomal segment between them. This implies that genes located on the same chromosome may segregate independently.

Reliable figures concerning the frequency of recombination of the hemochromatosis allele will probably be available soon. Until then, the recombination frequency can only be estimated due to the close linkage of the hemochromatosis locus to the HLA-A locus. When both the hemochromatosis allele and its marker allele (the HLA-A locus) cross over together, there is usually no difficulty in

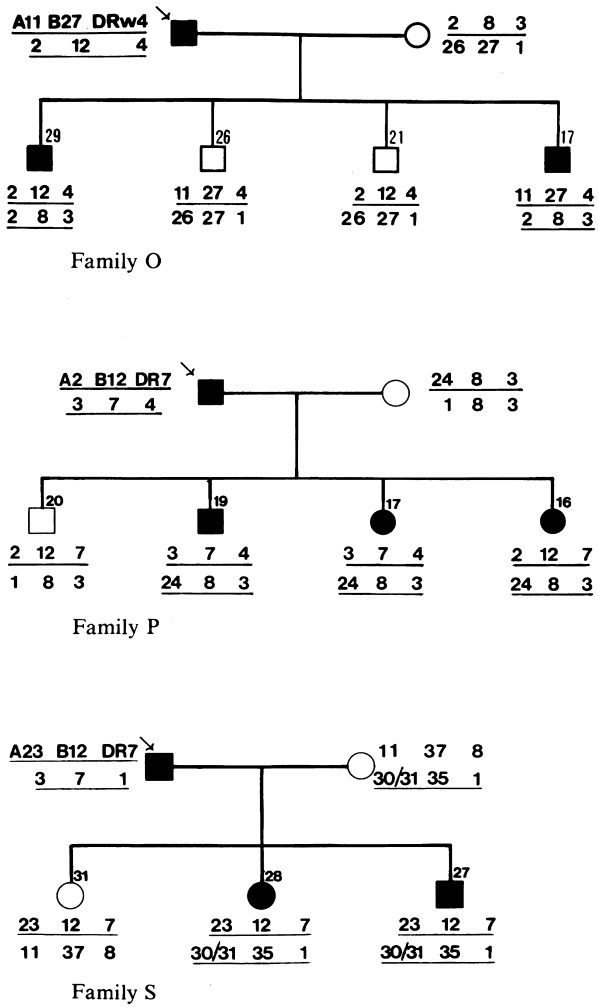


Fig. 3.4. Three hemochromatosis families illustrating vertical inheritance, probably due to homozygous-heterozygous marriage. HLA haplotypes who putatively carry the hemochromatosis allele are underlined. Arrow indicates probands and small numerals indicate ages of offspring. (Reproduced with permission[98].)

recognizing the presence of a recombination, except when there is homozygosity for the particular HLA-A allele. However, when the hemochromatosis allele recombines without its marker HLA-A allele, or the marker allele recombines without the corresponding hemochromatosis allele, interpretation of genetic transmission becomes difficult. Assuming that there is a common ancestral hemochromatosis gene linked to one particular ancestral HLA-haplotype, subsequent genetic recombination between the hemochromatosis locus and the HLA locus or between the HLA-A and HLA-B loci will result in association of the hemochromatosis allele with many HLA-haplotypes, and thus introduce new HLA marker haplotypes linked to the hemochromatosis gene. Chromosomal recombination may also result in the misclassification of hemochromatosis homozygotes as heterozygotes, or vice versa.

Recombination between the HLA-A and HLA-B loci occurs with a probability of 0.0087, i.e., less than 1%[41]. Due to the tight linkage between the hemochromatosis and the HLA-A loci, chromosomal recombination between these two is likewise rare, and in most studies is estimated to occur with a frequency of $<1\%$[24, 51, 55, 57, 59, 63]. However, Dadone et al.[61] reported a maximum likelihood estimate of the recombination fraction (θ) of 0.015, and Lalouel et al.[63] and Simon et al.[35] a value of 0.011. Consequently, recombination is considered to have a low appreciable general effect on pedigree studies. Valberg et al.[59] found one recombination among 99 relatives of 12 probands with hemochromatosis. Edwards et al.[60] reported a pedigree in which a chromosomal recombination had occurred between the HLA-A and HLA-B loci. It appeared that the hemochromatosis locus segregated with the HLA-A locus, indicating tight linkage and close proximity of the two loci. Dadone et al.[61] found two possible recombinations among 421 relatives of 18 probands with hemochromatosis. David et al.[64] described a family in which the proband had two HLA-identical, and thus presumed homozygous, siblings who had no abnormalities of iron status markers, probably due to genetic recombination of the hemochromatosis locus. Milman et al.[65] found three possible recombinations between the hemochromatosis and the HLA-A loci among 95 relatives of four probands. In another series, Milman et al.[67] examined 172 relatives of 29 unrelated probands and observed that two of 14 HLA homozygotes had normal iron status, and were therefore probably recombinant heterozygotes. One HLA heterozygote, who had highly abnormal iron status, was probably a recombinant homozygote (Fig. 3.3).

Expression of the hemochromatosis gene according to HLA-studies

Genotyping for the hemochromatosis gene(s) has been performed indirectly by HLA typing. The penetrance of the gene(s) has been assessed according to the phenotypic expression of iron status markers (transferrin saturation, serum ferritin concentration, hepatic iron content). Four grades of genetic expression may be adapted: (i) full expression with a transferrin saturation $>60\%$, a serum ferritin >700 μg/l indicating clinically significant iron overload, and increased risk of damage to target organs; (ii)

intermediate expression with a high transferrin saturation and serum ferritin concentration between the 90th percentile and 700 μg/l, respectively, indicating moderate iron overload without immediate risk of target organ damage; (iii) partial expression with a high transferrin saturation and a serum ferritin concentration below the 90th percentile; and (iv) no expression, i.e., a normal transferrin saturation (< 50% in women, < 60% in men) and a serum ferritin below the 90th percentile. Variable expression may be due to true variations in genotype expression. Studies of *HFE* will clarify this[70]. Differences in expression could also be caused by external modulating factors such as iron intake, menstruation, pregnancy, donation of blood, and pathological bleeding.

Rate of expression in homozygotes

The rate of clinical expression of the hemochromatosis allele in HLA-typed homozygous subjects is 85–100% in men and 14–20% in women[52, 57]. The expression in premenopausal women is low. Among 179 Danish patients with clinically overt hemochromatosis, the male/female ratio was 4[5], a lower value than in other series in which the ratios were 8–11[2–4, 100, 101]. Thirty-nine of the 179 patients were women, and only nine were premenopausal at the time of the diagnosis[5]. A majority of clinically overt cases of hemochromatosis in women are not diagnosed until after the menopause, probably due to their physiological iron losses related to reproduction. The few reports of homozygous subjects who have normal iron status and are HLA-identical with the proband[61, 64, 67, 76] should probably not be interpreted as lack of expression of the hemochromatosis allele, but rather as a misclassification of true heterozygotes due to chromosomal recombination between the hemochromatosis and the HLA-A loci[61, 64, 67, 76].

Rate of expression in heterozygotes

The expression of the hemochromatosis allele is far more variable in HLA-classified heterozygotes than in homozygotes. Among heterozygotes, 12–38% display minor abnormalities in the iron status parameters, and may develop a minor iron load[57–59, 62, 66, 71, 75, 76, 78, 82]. As a general rule, heterozygotes may have a slight to moderate iron load, but do not accumulate iron to an extent that causes damage to the target organs. Heterozygotes who develop clinically significant iron overload are probably misclassified homozygotes due to chromosomal recombination between the hemochromatosis locus and the HLA-A locus[67, 76].

Hemochromatosis gene

In 1996, Feder et al.[70] discovered a candidate hemochromatosis gene *(HFE)*, which is present in homozygous form in 82–100% of patients with typical hemochromatosis[70, 102–105]. Persons with hemochromatosis who do not have mutations of *HFE* alternative may have yet undiscovered genetic mechanisms. *HFE* mutations occur with a high frequency in western Caucasian populations for unknown reasons. This might be due to a selective advantage in heterozygotes who were relatively protected against the deleterious effects of iron deficiency due to an increased iron absorption from the diet[35, 42, 82, 84, 106, 107]. The accumulation of larger body iron reserves may improve survival during infancy, childhood, adolescence, and pregnancy in comparison with persons without hemochromatosis alleles. Negative selection against homozygous subjects is minimal, because the disorder in the majority of patients exerts its harmful effects after the reproductive period is over[5]. An alternative hypothesis is that *HFE* mutations became frequent due to positive selection resulting from tight linkage to some unknown important gene or genes in the HLA region[106].

Location of the hemochromatosis genes on chromosome 6 according to HLA studies

The tight linkage of the hemochromatosis and HLA-A loci and the segregation of the hemochromatosis locus with the HLA-A locus and not with the HLA-B locus in a recombinant family[60] indicates that the putative hemochromatosis gene should be located on the short arm of chromosome 6, in close proximity to the HLA-A locus[4, 24, 42]. However, it could not be substantiated whether the hemochromatosis locus was situated telomeric to the HLA-A locus, between the HLA-A and HLA-C loci, or between the HLA-C and HLA-B loci[4]. An examination of many haplotypes showed that the hemochromatosis gene was closer to the HLA-A locus than HLA-B locus, possibly telomeric to the HLA-A locus[35, 76].

Positional cloning of the HFE-gene on chromosome 6

By 1993, linkage analysis had placed the hemochromatosis gene within 1 centiMorgan of the HLA-A locus[108, 109], strongly associated with the microsatellite marker D6S105–8 located at least 2 centiMorgan telomeric to the HLA-A locus[109–111]. The identification of new markers telomeric to D6S105 enabled Feder et al.[70] to identify a new candidate gene with two different mutations in patients with hemochromatosis, four megabases away from the

initial candidate region close to the HLA-A locus[105]. The identification of the *HFE* gene was performed by positional cloning; this is the isolation of disease genes on the basis of their chromosomal position, and does not require knowledge of the function or sequence of the defective protein[70]. Feder et al.[70] defined a hemochromatosis gene candidate region of 250 kilobases between the markers D6S2238 and D6S2241 common to all ancestral hemochromatosis chromosomes. Using cDNA selection, exon trapping, and genomic DNA sequencing, 15 genes were identified. These consisted of 12 histone genes and three novel genes, of which only one had a nucleotide change consistent with the ancestral hemochromatosis mutation. This MHC class I-like gene, which had a nucleotide sequence homology to HLA-A2, was initially termed 'HLA-H'[70]; the WHO nomenclature committee for factors of the HLA system have assigned the name *HFE*, instead of 'HLA-H'[112]. Two missense mutations that can occur in *HFE* are known. The mutation consistent with the ancestral hemochromatosis haplotype resulted in the substitution of the amino acid cysteine with tyrosine at the amino acid position 282 (Cys282Tyr; C282Y); 83% of the hemochromatosis patients examined by Feder et al.[70] were homozygous for C282Y. The second missense variant resulted in the substitution of the amino acid histidine for aspartic acid at amino acid position 63 (His63Asp; H63D). It does not seem to play any obligatory role in causing hemochromatosis, although compound heterozygotes may develop clinically significant iron overload[70]. The high frequency of homozygosity for the C282Y mutation in patients with hemochromatosis has been confirmed in studies from USA[70, 102], France[103], Australia[104], and England[105].

Hemochromatosis gene function

It is not clear how a mutation in an aberrant MHC class I-like molecule could impair the normal regulation of intestinal iron absorption and contribute to the progressive iron overload in hemochromatosis. By analogy with other MHC class I molecules, the C282Y mutation is predicted to disrupt the formation of a disulfide bridge required for the non-covalent association of ß$_2$-microglobulin and for correct cell-surface presentation. This means that the function of the aberrant MHC class I molecule most likely is severely impaired[70]. The only evidence for the involvement of a defective MHC class I molecule in hemochromatosis is the marked, progressive hepatic iron overload observed in β_2-microglobulin deficient mice[113]. RNA analysis reveals that *HFE* is widely expressed in human tissues, especially in the small intestine and the liver. However, analysis of protein expression, identification of potential ligands, and ultimately transgenic mouse experiments will be required to formally resolve the status of *HFE* as a candidate for the hemochromatosis gene[114].

Association of hemochromatosis to other inherited disorders not located on chromosome 6

There is good evidence to suggest that the C282Y mutation of *HFE* occurred in an individual with the ancestral hemochromatosis haplotype[24, 35, 84]. Considering the high prevalence of the *HFE* mutations, it appears unlikely that the ancestral haplotype was associated with any phenotypically characteristic or serious genetic disorders located on other chromosomes. The association of hemochromatosis with other inherited diseases can be dealt with according to whether the other concurrent disease is located to chromosome 6. *HFE* mutations may be introduced into families with a pre-existing inherited disorder located on a chromosome other than no. 6. New mutations on chromosomes other than no. 6 may occur in some individuals who are homozygous or heterozygous for *HFE* mutations. This may lead to a coincidental association of hemochromatosis and other diseases. Sporadic instances of persons with hemochromatosis and other concurrent inherited diseases have been reported, such as brachydactyly type D, an autosomal dominant trait not yet assigned to any specific chromosome[115].

There is an association between sporadic porphyria cutanea tarda (PCT) and the C282Y mutation[116]. The gene encoding PCT is on 1p34[117] and positional cloning of the gene has been performed. PCT is inherited as an autosomal dominant trait, and patients with PCT have greater frequency of the C282Y mutation than healthy control subjects (RR 6.2; $p < 0.0001$). The presence of the C282Y mutation greatly facilitates the penetrance of the PCT gene. This means that subjects with the PCT gene may not develop clinical disease unless they also possess the C282Y mutation, suggesting that occurrence of the two mutant genes in the same genome is probably coincidental.

Because hemochromatosis is an autosomal recessive trait, both the parental haplotypes on chromosome 6 must have the specific mutation to cause clinical disease in the offspring. If a concurrent inherited disorder was also an autosomal recessive trait, both the parental haplotypes on the particular chromosome must likewise have the particular mutation to induce disease. Such combinations are probably less frequent than if the concurrent inherited disorder is inherited in an autosomal dominant manner, as mentioned above for brachydactyly type D and PC. Unless there is a high coefficient of consanguinity in the family, such coincidental occurrences are seen in one or a

few individuals and are seldom transmitted to the offspring.

The concomitant presence of hemochromatosis and type III hyperlipoproteinemia, an autosomal recessive disorder mapped to chromosome 19, has been reported in one individual[118]. Approximately 1% of the general population is homozygous for the disease-producing mutation of apolipoprotein E2. The fortuitous occurrence of hemochromatosis and type III hyperlipoproteinemia is therefore estimated to be 3 in 100 000 in the general population, and there is no evidence of a direct genetic linkage between the two disorders[118]. Although there exists no genetic association between X-linked pyridoxine-responsive sideroblastic anemia and hemochromatosis, it appears that the fortuitous presence of the C282Y mutation, even in its heterozygous form, increases the magnitude of iron overload in these patients[119]. The occurrence of hemochromatosis has been reported in one patient with mixed gonadal dysgenesis (karyotype 46 XY, 45 XO)[120], in a patient with Turner's syndrome (karyotype 45 XO)[121], and in a patient with Klinefelter's syndrome (karyotype 47 XXY)[122]. All these cases occurred by coincidence and do not imply genetic linkage.

There may be an association between hereditary α_1-antitrypsin deficiency and hemochromatosis[123–125]. Hereditary α_1-antitrypsin is encoded by a gene mapped to chromosome 14. Of the three most important α_1-antitrypsin phenotypes PiM, PiS, and PiZ, only the homozygous type PiZZ is associated with clinically symptomatic α_1-antitrypsin deficiency. Rabinovitz et al.[123] reported a significantly increased prevalence of the PiMZ phenotype among 15 hemochromatosis patients. Kaserbacher et al.[124] concluded that patients with hemochromatosis had no significantly increased risk of inheriting abnormal α_1-antitrypsin phenotypes, whereas patients carrying the α_1-antitrypsin Z gene had a six-fold increase in risk of having clinically significant hemochromatosis. In 67 consecutive Swedish patients with hemochromatosis, Elzouki et al.[125] found a significantly increased prevalence of the homozygous PiZZ phenotype, whereas the prevalence of the heterozygous PiZ phenotype was similar to that of the general population. In contrast, Fargion et al.[126] found no association between the PiZZ phenotype and hemochromatosis in a series of 115 Italian hemochromatosis patients. Thus, to prove (or disprove) that there is an association between these two disorders, prospective screening of a general population with genetic tests for α_1-antitrypsin and *HFE*-genotypes is needed.

Gene mutations on chromosome 6 causing disease

Many mutations have been mapped to chromosome 6[117] (Table 3.5). Most are located on 6p, and at least 16 are located within, or in close proximity to, the band where *HFE* is localized. It is presumed that only those genes located on 6p may be in linkage disequilibrium with *HFE*. The most interesting from the point of view of hemochromatosis is the gene causing insulin-dependent diabetes mellitus (IDDM), also called type 1 diabetes mellitus; this gene is termed *IDDM1*.

Association of hemochromatosis and other inherited disorders on chromosome 6

It is logical to pose the question whether the *HFE* gene is associated with or linked to other disease-related genes on chromosome 6. However, a conclusive answer cannot be given until positional cloning of the possible associated candidate genes has been done, e.g., the gene located on chromosome 6p that causes type 1 diabetes mellitus. At present, conclusions must therefore be based on probabilities of associations based on clinical and genetic estimates. A considerable number of inherited disorders are caused by mutations in genes located on chromosome 6. However, there has been no systematic genetic study of other diseases in patients with hemochromatosis. Provided the hypothesis concerning an ancestral hemochromatosis haplotype is correct, and considering the high prevalence of *HFE* mutations, it seems unlikely that the initial C282Y mutation was accompanied by another mutation that causes clinically significant disease.

However, during centuries of genetic admixture of the ancestral hemochromatosis haplotype with other haplotypes, it is possible that chromosomal recombinations or new mutations may have occurred in a few individuals, so that the ancestral haplotype has been 'supplemented' with other inherited disorders located on chromosome 6. The clinical manifestation of a concurrent inherited disease also depends on whether the disease is transmitted in a recessive or dominant manner. In particular families, this may lead to the association or linkage of hemochromatosis to other inherited disorders. Very likely, the combination of two genetic diseases may reduce the reproductive potential of an individual, whereby the particular haplotype will tend to be eliminated from the population. Although patients with hemochromatosis have not been examined prospectively for other genetic disorders on chromosome 6, in none of the published patient series has there been reported any suggestion for an association or linkage of the hemochromatosis allele to other genetic

Table 3.5. Genes on Ch6p that may be the site of disease-producing mutations, and genes on Ch6q that may confer susceptibility to diabetes mellitus[a]

Location[b]	Symbol	Status[c]	Title	Disorder
6p25-p24	OFC	C	Orofacial cleft	Orofacial cleft
6p25	FKHL7	C	Iridogoniodysgenesis	Iridogoniodysgenetic anomaly
6p25-p24	F13A1	C	Coagulation factor XIII, A-polypeptide	Coagulation factor XIIIA deficiency
6p23	ATX1	C	Spinocerebellar ataxia-1	Spinocerebellar ataxia-1
6p23	DEK	P	DEK gene	Acute non-lymphocytic leukemia
6p23	SCZD3	P	Schizophrenia disorder 3	Schizophrenia-3
6p22.3	TPMT	P	Thiopurine S-methyltransferase	6-mercaptopurine sensitivity
6p22-p21	BCKDHB, E1B	C	Branched-chain keto acid dehydrogenase E1	Maple syrup urine disease, type Ib
6p21.3	AS	P	Ankylosing spondylitis	Ankylosing spondylitis
6p21.3	ASD2	P	Atrial septum defect	Atrial septum defect, secundum type
6p21.3	C2	C	Complement component-2	Complement component-2 deficiency
6p21.3	C4A, C4S	C	Complement component-4A	Complement component-4A deficiency
6p21.3	C4B, C4F	C	Complement component-4B	Complement component-4B deficiency
6p21.3	COL11A2	C	Collagen XI, α_2-polypeptide	Stickler syndrome, type II
6p21.3	CYP21	C	Cytochrome P450, subfamily XXI; steroid 21-hydroxylase; adrenal hyperplasia, congenital	Congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency
6p21.3	DFNA13	P	Deafness, autosomal dominant-13	Deafness, autosomal dominant-13
6p21.3	DYX2	P	Dyslexia-2	Dyslexia, specific-2
6p21.3	GLYS1	P	Renal glucosuria	Renal glucosuria
6p21.3	**HFE**	**C**	**Hemochromatosis**	**Hemochromatosis**
6p21.3	**IDDM1**	**L**	**Insulin dependent diabetes mellitus-1**	**?Type 1 diabetes**
6p21.3	NEU	C	Neuraminidase	Sialidosis, type I
6p21.3	PDB	L	Paget disease of bone	?Paget disease of bone
6p21.3	RP14	P	Retinitis pigmentosa-14	Retinitis pigmentosa-14
6p21.3	RWS	L	Ragweed sensitivity	?Ragweed sensitivity
6p21.3	TAP2	C	Transporter-2	Bare lymphocyte syndrome
6p21.3	TNXA	P	Tenascin XA	Ehlers–Danlos syndrome
6p21.3-p21.2	LAP	L	Laryngeal adductor paralysis	?Laryngeal adductor paralysis
6p21.1	PXAAA1	C	Peroxisomal AAA-type ATPase 1	Peroxisomal biogenesis disorder
6p21.1-p12	PKHD1	C	Polycystic kidney and hepatic disease-1	Polycystic kidney disease, autosomal recessive
6p21.2-p12	PAFAHT	C	Platelet-activating factor acetylhydrolase	Platelet-activating factor acetylhydrolase deficiency
6p21.1-p11	RHAG	C	Rhesus blood group-associated glycoprotein	Hemolytic anemia, Rh-null, suppressor type
6p21.1	RDS	C	Retinal degeneration, slow	Retinitis pigmentosa, peripherin-related
6p21	CBFA1	C	Cleidocranial dysplasia	Cleidocranial dysplasia
6p21	MUT	C	Methylmalonyl coenzyme A mutase	Methylmalonic aciduria, mutase deficiency type
6p12	GLCLC	C	Glutamate–cysteine ligase, catalytic	Hemolytic anemia due to γ-glutamylcysteine synthetase deficiency
6p	ICS1	L	Immotile cilia syndrome-1	?Immotile cilia syndrome
6p	NYS2	P	Nystagmus-2	Nystagmus-2, autosomal dominant
6p	PUJO	C	Pelviureteric junction obstruction	Pelviureteric junction obstruction
6q21	IDDM15	P	Insulin-dependent diabetes mellitus-15	Insulin-dependent diabetes mellitus
6q24-q27	IDDM5	P	Insulin-dependent diabetes mellitus-5	Insulin-dependent diabetes mellitus
6q25-q27	IDDM8	P	Insulin-dependent diabetes mellitus-8	Insulin-dependent diabetes mellitus

Notes:
[a] Adapted from ref.[117], 1997.
[b] Chromosomal site: p = short arm; q = long arm; number = band.
[c] Status: C = confirmed; P = provisional; L = tentative or inconsistent.

disorders on chromosome 6, with the exception of diabetes mellitus[2–4, 24, 25, 35, 42, 50, 55, 57–59, 61–63, 67, 71, 72, 75, 78, 82].

The combination of hemochromatosis and celiac disease has been described in one patient[127]. Coeliac disease is significantly positively associated with the HLA-B8, HLA-DR3 and HLA-DR7 alloantigens on 6p, and is negatively associated with HLA-B7[128]. The combination of hemochromatosis and sarcoidosis has been reported in one Danish patient[5] and in two Spanish monozygotic (genetically identical) twins[129]. Sarcoidosis is significantly associated with HLA-DR3 and HLA-DR6 alloantigens located on 6p[130]. However, the genetics of celiac disease and sarcoidosis have not been clarified. No relation has been found between schizophrenia and the HLA alloantigens[131]. However, a provisional gene (SCZD3) located on chromosome 6p23 seems to be a susceptibility allele for this disorder (Table 3.5). Milman[5] reported two patients, a mother and her son, who presented with hereditary hemochromatosis and schizophrenia. Multiple sclerosis is significantly associated with the same alloantigens as hemochromatosis, i.e., HLA-A3, HLA-B7, and HLA-DR2 on chromosome 6p[132]. Milman[5] reported one patient who had hemochromatosis and multiple sclerosis. Myasthenia gravis is significantly associated with HLA-A1, HLA-B8 and HLA-DR3 on chromosome 6p[133]. Milman[5] also reported one patient with hemochromatosis and myasthenia gravis. Although the concomitant presence of these various disorders with hemochromatosis is probably fortuitous, their possible genetic association deserves evaluation in larger family and case series.

Genetic factors in insulin-dependent diabetes mellitus

Insulin-dependent diabetes mellitus (type 1 diabetes mellitus) is a multifactorial autoimmune disease caused by the interaction of environmental factors in persons with an inherited predisposition. In Caucasians, the overall lifetime risk of type 1 diabetes mellitus is 0.4%[134]. The risk is 6% in siblings of patients with type 1 diabetes mellitus, and the concordance rate in monozygotic twins is 36%[135]. The high discordance rate in monozygotic twins indicates that the susceptibility genes have a low penetrance, i.e., most individuals who are genetically predisposed do not develop type 1 diabetes mellitus.

The genetics of type 1 diabetes mellitus is the best delineated of all polygenic conditions. Genetic factors account for about one-third of susceptibility to type 1 diabetes[136]. Over 20 different regions of the human genome have some linkage with type 1 diabetes mellitus[134]. The strongest linkage is with the HLA-locus in the MHC region of 6p designated *IDDM1*. In the early 1970s, associations were reported between type 1 diabetes and HLA-B15 and HLA-B8[137], allowing the genetic subdivision of diabetes mellitus into type 1 (insulin-dependent) and type 2 (non-insulin-dependent). After combining the results of ten studies on HLA-alloantigens and type 1 diabetes, Ludvigsson et al.[138] calculated RRs of developing diabetes mellitus when having HLA-B8, HLA-B15, and HLA-B7 of 2.4, 2.0, and 0.46, respectively. HLA-A3, HLA-B14, and HLA-B35 occurred with (insignificantly) lower frequencies in diabetes patients than in controls[138]. After combining the results of four studies on HLA-B7 and type 1 diabetes, Ludwig et al.[139] surmised that patients with diabetes had a significantly lower frequency of HLA-B7 than controls (13% vs. 27%; $p < 0.0005$). A positive association has also been described between type 1 diabetes mellitus and HLA-Cw3 (RR = 2.2)[139].

It has been reported subsequently that the association between type 1 diabetes is much stronger with the MHC class II antigens HLA-DR3 and HLA-DR4 found in 95% of Caucasians with type 1 diabetes and in 50% of controls[140]. These associations are due to linkage disequilibria of HLA-DR3 and HLA-DR4 with diabetes susceptibility loci. The RR of developing diabetes in persons who inherit HLA-DR3 and HLA-DR4 is approximately 8.0 and 9.7, respectively[137]. However, the HLA-DRB1 alleles encoding HLA-DR3 and HLA-DR4 are too common in the general population to be true susceptibility determinants[141]. There are linkage disequilibria between HLA-B7 and HLA-DR2[137], and a negative association between type 1 diabetes and HLA-B7 and HLA-DR2[142]. The frequency of HLA-A3 is significantly decreased in persons with type 1 diabetes mellitus due to the strong linkage disequilibrium between HLA-A3 and HLA-B7[36, 142].

Linkage disequilibrium is strong between the HLA-DR and the HLA-DQ subregions with a low frequency of chromosomal recombination. The discovery of a polymorphism of the DQB1 gene allowed subdivision of HLA-DR4 haplotypes into high- and low-risk categories[143]. Nepom et al.[144] showed that this restriction fragment length polymorphism corresponded to a serologically defined antigen (TA10), and that the two alleles of the DQB1 locus were associated with HLA-DR4. One allele, HLA-DQ8 (or TA10–), was observed in 90% of Caucasian diabetes patients, whereas HLA-DQ7 (or TA10+) was seen in only 10%. In contrast, the two alleles were equally represented in non-diabetes control subjects carrying HLA-DR4[145].

There is ample evidence to suggest that the HLA-DQ subregion encodes susceptibility determinants for type 1 diabetes[134]. Variants of the DQß-chain encoded in the DQB1*0201 and DQB1*0302 alleles which are found in

HLA-DR3 and HLA-DR4, respectively, are apparently diabetogenic in Caucasians. In contrast, DQβ-chain variants encoded by the alleles DQB1*0602 and DQB1*0603, found in HLA-DQ6 and HLA-DQ18, respectively, apparently have a protective effect against IDDM[134]. The region of the insulin gene on chromosome 11p, designated *IDDM*2, is also associated with and linked to type 1 diabetes mellitus. Other weaker diabetes susceptibility loci are *IDDM*3, *IDDM*4, and *IDDM*5, that map on chromosome 15q, 11q, and 6q, respectively[134]; their gene products and mode of action are unknown.

The association of hemochromatosis and genetic insulin-dependent diabetes mellitus

Although diabetes mellitus is not uncommon in persons with hemochromatosis, it is essential to recognize that diabetes may not necessarily be an obligate feature in hemochromatosis patients[4, 5]. However, diabetes mellitus is the most commonly observed endocrine disorder in hemochromatosis[2–5]. In end-stage hemochromatosis, pancreatic iron deposition is often visible, and pancreatic fibrosis is almost invariably present[4]. The heaviest iron deposits occur in acinar cells of the exocrine glands, although no effects on exocrine pancreatic function have been documented. Within the pancreatic islets, iron is found only in the ß-cells and associated with progressive loss of their endocrine granules[146]. The islets are of normal size and shape, in contrast to those found in type 1 diabetes mellitus, and do not exhibit the amyloid of type 2 diabetes[146]. Impaired insulin secretion and insulin resistance have been recognized among hemochromatosis patients who have diabetes mellitus[147–149]. Insulin resistance may be due to the excess iron in the hepatocytes and to hepatic cirrhosis[147, 148]. Impaired insulin removal by the liver may also contribute to increased plasma insulin levels[147, 150].

The frequency and severity of pancreatic dysfunction depends on the size of the body iron overload[5], and of the fraction of surplus iron deposited in the pancreatic islet cells. In large series of patients with advanced iron overload due to hemochromatosis[2, 3], glucose intolerance was found in approximately 80% of the subjects; in series of patients with minor or moderate iron overload, the frequency of diabetes mellitus is significantly lower. When treatment dependence is used as the diagnostic criterion, the incidence of diabetes mellitus in 237 patients from ten different studies was 51%[4, 25]. In 179 Danish patients with clinically overt hemochromatosis, the prevalence of treatment-dependent diabetes mellitus was 54% (non-insulin-dependent 18%, insulin-dependent 36%). Patients with treatment-dependent diabetes had higher serum transaminase concentrations, higher serum ferritin concentrations, and greater mobilizable iron stores than patients without diabetes[5]. Accordingly, women had a significantly lower prevalence of treatment-dependent diabetes than men[5]. It appears that, in hemochromatosis patients in whom the diagnosis was made in the preclinical or early iron loading phase, a lower prevalence of diabetes mellitus and less severe diabetes was observed than in series of patients who had symptomatic iron overload[151]. In patients with clinically overt hemochromatosis, the prevalence of diabetes was 40–55%[5, 152, 153], whereas in subjects with biochemical evidence of hemochromatosis without clinical signs, the prevalence of diabetes was 13–17%[153, 154].

Homozygosity for the C282Y mutation occurs with a presumed prevalence of 0.4–0.5%, and type 1 diabetes mellitus occurs with a similar lifetime cumulative prevalence of approximately 0.4%[134]. Due to the high prevalence of diabetes mellitus in patient series with hemochromatosis, it was reasonable to postulate that there is a genetic linkage between the hemochromatosis gene(s) and the diabetes gene(s)[147, 155–157]. However, Simon et al.[158] found a significantly higher prevalence of diabetes mellitus among the first-degree relatives of diabetes patients than among first-degree relatives of hemochromatosis patients, and thus rejected the hypothesis. There is no documentation of a genetic linkage between hemochromatosis and genetic type 1 diabetes. For a number of reasons, present evidence highly favors the view that diabetes mellitus and hemochromatosis segregate independently as genetic traits. First, it is unlikely that a gene encoding for type 1 diabetes mellitus is situated on the same 6p where the initial mutation encoding hemochromatosis on the ancestral haplotype occurred. The C282Y mutation probably occurred centuries ago, when no knowledge of or treatment for diabetes mellitus existed. It seems likely that frequent death before reproduction would have occurred in individuals who had a *HFE* mutation and an *IDDM*1 gene encoding for insulin-dependent diabetes mellitus. This would keep the prevalence of the hemochromatosis trait low. However, the hemochromatosis trait is one of the most prevalent inherited disorders, arguing against an association with type 1 diabetes mellitus. Second, there is no documentation of an increased frequency of type 1 diabetes in subjects who, according to HLA-haplotyping, are homozygous (h/h) for the hemochromatosis allele, but have preclinical disease with no or minor iron overload[5]. Furthermore, there are no reports of an increased prevalence of type 1 diabetes mellitus in subjects who are heterozygous (h/n) for a hemochromatosis allele[57–59, 62, 66, 71, 75, 78, 82]. Third, most studies do not suggest that there is an increased prevalence of

hemochromatosis in patients with diabetes mellitus[159–161]. In 572 diabetes mellitus patients >30 years of age, O'Brien et al.[159] found the prevalence of hemochromatosis was 0.52%. Singh et al.[160] screened 406 patients with diabetes mellitus; the prevalence of hemochromatosis in this group was 0.49%. George et al.[161] found a prevalence of hemochromatosis of 0.50% in 1149 patients with diabetes mellitus. These prevalences of ~0.5% are similar to those reported in the general population, and do not support a genetic relationship between the two disorders. Phelps et al.[162] reported a prevalence of hemochromatosis of 0.96% in a series of 418 Australian diabetes patients. Manachino et al.[163] found a prevalence of hemochromatosis of 1.3% in 530 Italians with diabetes. Even slightly increased prevalences of this magnitude are surprisingly low considering the natural history of the two disorders, and are not in favor of a genetic association.

Fourth, there is no evidence that patients with non-insulin-dependent diabetes have increased body iron stores[164]. Dineen et al.[164] examined hepatic iron stores in 15 patients with non-insulin-dependent diabetes and 17 age-matched controls. No significant difference was found in either the cellular hepatic iron distribution or the amount of hepatic iron in the diabetes and the control group. Fifth, the clinical spectrum of diabetes mellitus in hemochromatosis is variable, and has no consistent pattern of presentation. The spectrum ranges from latent diabetes, in which the only finding is abnormal glucose tolerance, to non-insulin-dependent diabetes treatable with dietary restrictions or oral antidiabetes agents, to insulin-dependent disease[3–5, 25]. Such a wide clinical variety of manifestations argues against one particular trait associated with hemochromatosis, but favors the view that the severity of pancreatic dysfunction and diabetes are closely related to the severity of iron deposition in the pancreatic islet cells and thus to body iron overload[5]. This is substantiated by the observation that 40–50% of hemochromatosis patients with diabetes show improved carbohydrate tolerance after phlebotomy therapy[147, 148]. Insulin resistance is also markedly improved after depletion of body iron stores by phlebotomy treatment, resulting in lower insulin requirements in patients with insulin-dependent diabetes mellitus[147, 148]. Hramiak et al.[149] examined hemochromatosis patients with and without diabetes mellitus and hepatic cirrhosis; all had impaired glucose tolerance. Patients without diabetes and cirrhosis had normal insulin sensitivity but decreased insulin secretion. In these patients, phlebotomy treatment increased insulin secretion and normalized glucose tolerance. Patients without diabetes and with cirrhosis had reduced insulin sensitivity, but maintained normal insulin secretion; these parameters were not changed by phlebotomy. Patients with diabetes had reduced insulin sensitivity and insulin secretion, and these parameters were also unaffected by phlebotomy treatment.

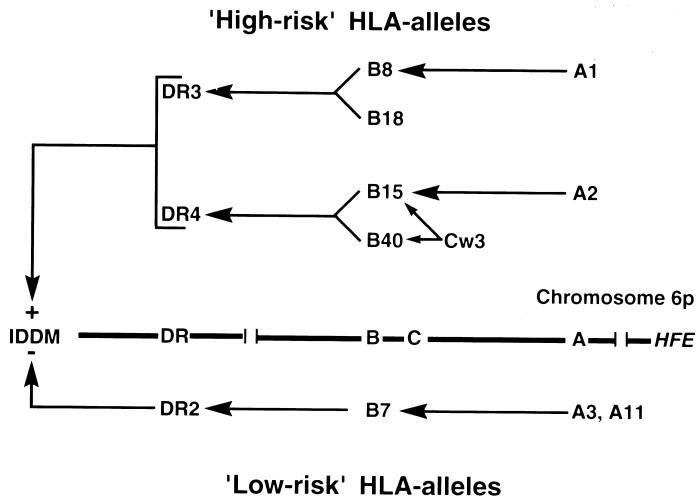


Fig. 3.5. Model of HLA-linked susceptibility and resistance alleles in type 1 (insulin-dependent) diabetes mellitus being in linkage disequilibria with particular determinants of the major HLA class I and class II loci. (Adapted from ref.[142].)

Sixth, a morphologic study of the pancreas in hemochromatosis by Rahier et al.[146] revealed that the pathologic features of diabetes mellitus in hemochromatosis are different from those in genetic type 1 diabetes and in non-insulin-dependent diabetes mellitus. The pancreatic damage in hemochromatosis is caused by intracellular iron toxicity, whereas in type 1 diabetes it is due to the formation of autoantibodies against the β-cells. Finally, the 'high-risk' genes for type 1 diabetes mellitus are associated with HLA-A1, HLA-A2, HLA-B8, HLA-B15, HLA-B18, HLA-B40, HLA-Cw3, HLA-DR3 and HLA-DR4, whereas the 'low-risk' genes are associated with HLA-A3, HLA-A11, HLA-B7, and HLA-DR2[138, 139, 142] (Fig. 3.5). Hemochromatosis patients have a low frequency of the 'IDDM-high-risk' HLA-A and HLA-B alloantigens (HLA-A1, HLA-A2, HLA-B8, HLA-B15, HLA-B18, HLA-B40) and a high frequency of the 'IDDM-low-risk' HLA-A and HLA-B alloantigens (HLA-A3, HLA-A11, HLA-B7)[29]. Furthermore, hemochromatosis patients have a significantly lower frequency of the 'IDDM-high-risk' HLA-Cw3 than healthy controls[29], although in another study there was no apparent association between hemochromatosis and HLA-Cw antigens[21].

Two studies have examined the association between hemochromatosis and HLA-DR types[21, 29]. Fauchet et al.[21] observed a significant, positive association of HLA-DRw6 in 100 hemochromatosis patients in comparison with that

in 100 control subjects. The frequency of HLA-DRw3 was (insignificantly) lower in patients (11%) than in controls (20%), whereas the frequency of HLA-DRw4 was similar in patients (20%) and controls (17%) Milman et al.[29] found that the frequency of HLA-DR2 was (insignificantly) higher in 44 hemochromatosis patients (43%) than in 119 control subjects (32%). The frequencies of both HLA-DR3 and HLA-DR4 were (insignificantly) lower in hemochromatosis patients than in controls (HLA-DR3 11% vs. 22% and HLA-DR4 34% vs. 43%, respectively). The results of the HLA-alloantigen studies thus argue against the existence of an association between hemochromatosis and genetic type 1 diabetes[21, 29].

Hemochromatosis and a possible protective effect against genetic insulin-dependent diabetes mellitus

Apparently there is no linkage between the hemochromatosis locus and the *IDDM*1–gene, consistent with the observation that there is no association between the HLA-A and HLA-DR3 and HLA-DR4 loci[21, 29]. However, hemochromatosis is strongly associated with HLA-haplotypes known as 'low-risk' markers for type 1 diabetes mellitus. The haplotype HLA-A3, B7 is significantly associated with HLA-Cw7, HLA-DR2 and HLA-DQw6 (DQA1*0102, DQB1*0602) which have a 'protective' effect against type 1 diabetes. The haplotype HLA-A3, B14 is associated with HLA-DR1, which does not predispose to the development of type 1 diabetes. The haplotype HLA-A3, B35 is associated with HLA-Cw4, HLA-DR1 (DRB1*0101) and HLA-DQw5 (DQA1*0101, DQB1*0501), none of which is associated with type 1 diabetes. In 1976, Ludwig et al.[165] hypothesized that HLA-B7 was associated with a protective gene in type 1 diabetes. In 1981, Saddi et al.[25], in contrast to their report in 1974[157], suggested that the ancestral haplotype had a protective effect against the development of insulin-dependent diabetes mellitus due to the strong linkage between HLA-A3 and HLA-B7. When 66 patients with hemochromatosis were divided into two groups according to whether they had insulin-dependent diabetes mellitus, the HLA-B7 antigen was markedly more frequent in the non-diabetes patients than in those with diabetes (71% vs. 42%, respectively)[25]. All HLA-B7 positive patients were also positive for HLA-A3. Accordingly, HLA-B7 has been found associated with a protective haplotype against type 1 diabetes mellitus[142]. These studies provide ample evidence that hemochromatosis is associated with a 'protective gene' against type 1 diabetes. As suggested by Kravitz et al.[55], patients with hemochromatosis may have obtained a selective advantage in maintaining HLA-A3 and HLA-B7 together during evolution. This advantage could be due to increased intestinal iron absorption and 'protection' against the development of type 1 diabetes mellitus.

Conclusions

The gene associated with hemochromatosis is in tight linkage with the MHC class I HLA-A3 antigen, the locus of which is located on 6p. Genetic recombination between the HLA-A loci, HLA-B loci, and the hemochromatosis locus occurs with a frequency of $<1\%$. Two HLA haplotypes are characteristic of hemochromatosis, i.e., HLA-A3, B7 and HLA-A3, B14, suggesting the occurrence of a single mutation on the ancestral haplotype followed by a chromosomal recombination between the HLA-A and HLA-B loci. Many family studies have confirmed that hemochromatosis is transmitted as an autosomal recessive (intermediate) disorder with partial expression in heterozygotes. Phenotypic expression is most reliably assessed by the serum transferrin saturation; values $>60\%$ are highly indicative of homozygosity for hemochromatosis allele(s). The serum ferritin concentration indicates the quantity of body iron stores, which in homozygous subjects increases with age, and may vary from normal to massive. Approximately one-third of hemochromatosis heterozygotes have increased transferrin saturation values or serum ferritin concentration, indicating slight to moderate iron load; rarely do these persons develop iron overload-associated disease or organ damage. According to the prevailing frequency of the *HFE*-gene, random heterozygote–heterozygote matings occur with frequencies ranging from 903 to 2167 per 100 000 matings, homozygote–heterozygote matings occur with frequencies from 48 to 188 per 100 000 matings, and homozygote–homozygote matings with frequencies from 0.6 to 4 per 100 000 matings. Positional cloning has been performed of the hemochromatosis gene (*HFE*), which has been mapped four megabases telomeric to the HLA-A locus on 6p. The significance and implications of these findings awaits further evaluation. The hemochromatosis locus does not seem to be associated with or linked to other genetic disorders located on chromosome 6. In particular, there appears to be no association with the *IDDM*1–gene encoding type 1 diabetes mellitus (insulin-dependent diabetes). There is some evidence that the HLA-A3, B7 hemochromatosis haplotype may have a 'protective' effect against type 1 diabetes mellitus.

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4

Hemochromatosis: population genetics

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Introduction

Hemochromatosis has a distinctive distribution that reflects the movements of people of northwestern Europe during historic times. The 'major' hemochromatosis gene in northern Europeans is linked to the HLA gene locus, and occurs with greatest frequency among the populations of the countries that surround the North Sea. These countries include Norway, Denmark, Iceland, western and southern Germany, the United Kingdom, Ireland, and France (particularly in the Brittany peninsula)[1] (Fig. 4.1; Table 4.1). Within Sweden, there is considerable variation in prevalence of hemochromatosis from one area to another[2–5]. The gene frequency appears to be lower in southern Italy[6] and in western Russia[7]. Hemochromatosis occurs in Portugal[7, 8], Spain, Estonia[9], Hungary[10], and northern Italy, but more data are needed to clarify the prevalence and the gene frequency in these countries. Hemochromatosis is rare in Finland, Greece, Turkey, North Africa and Asia[11]. The prevalence of hemochromatosis has not been ascertained in most of eastern Europe. Frequency of the hemochromatosis gene is low in Ashkenazic Jews[7, 11]. In countries that have large populations derived principally from the North Sea littoral, including Canada, the United States, South Africa, Australia and New Zealand, the prevalence of hemochromatosis mirrors that of the parent populations of Europe.

Source of the hemochromatosis mutations

The striking geographic distribution of hemochromatosis has led to the conjecture that hemochromatosis is the result of a relatively recent mutation (one that has occurred in hundreds of generations) that occurred in a Celtic population. However, it could be argued as reasonably that the mutation occurred originally in a Viking or a Germanic population. In addition, the distinctions among these groups should be viewed as cultural rather than genetic. More than a thousand years ago, northwestern Europeans invaded what had been the Roman Empire by land and by longboat, colonizing areas now included in southern Germany, Austria, France, Spain, Portugal, northern Italy, Sicily, and the British Isles. They also ascended the rivers that enter the Baltic Sea and colonized present-day Ukraine, Belarus, and western Russia. In the past few hundred years, their descendants have colonized much of North America, Australia, and New Zealand. With this diaspora of northwestern European people, the hemochromatosis gene mutations have also been dispersed worldwide, except in those regions wherein indigenous people were able to resist encroachment. Hemochromatosis therefore appears to be rare or nonexistent in Asia, in the countries of the old Ottoman Empire, and in much of Latin America. The low gene frequency of hemochromatosis in Ashkenazic Jews, consistent with studies of the frequency of other gene markers in this population, attest to a remarkable ability of this group to maintain its genetic identity by resisting assimilation by the larger populations within which it has coexisted for nearly 2000 years.

For a gene to become so prevalent in a population, it may also confer a selective advantage. There is little doubt, for example, that *falciparum* malaria is responsible for the high prevalence of the hemoglobin S mutation and of glucose-6–phosphate dehydrogenase deficiency in Africa, the Hb E mutation in southern Asia, and of thalassemias in both Asia and the Mediterranean littoral. Similarly, the hemochromatosis mutation(s) may have conferred some survival benefit for the populations in which they became so prevalent. Because hemochromatosis is rarely associated with disability and death during reproductive years,

Fig. 4.1. Estimated hemochromatosis gene frequency in Europe and the Mediterranean littoral. The highest gene frequencies occur in the nations that surround the North Sea and that are in the northeastern Atlantic. Similarly high gene frequencies occur in countries whose populations are derived from those of this region of northwestern Europe, including Canada, the United States of America, Australia and New Zealand, and the Caucasian population of South Africa. This map is based on estimates shown in Table 4.1, and principally reflects the distribution and allele frequency of the 845A C282Y mutation of the *HFE* gene.

it seems unlikely that hemochromatosis would confer a selective disadvantage. In northwestern Europe where the hemochromatosis mutations became highly prevalent, human dietary iron intake may have been limited. Fish have been a major source of dietary protein among many populations in which hemochromatosis is common. In historical times, western Europe was a region in which milk and cheese were major sources of protein, but these foods are poor sources of iron. Historically, this was also an area in which chlorosis (iron-deficiency anemia) became a serious epidemic disease. It was also a region in which the men were repeatedly subjected to bloodletting by broad-

Table 4.1. Estimated prevalence of hemochromatosis and *HFE* allele frequencies world-wide

	HH prevalence (%)		Allele frequencies			
Country	Observed	Predicted[a]	(C282Y)	(H63D)	Basis[b]	References
Portugal	1.9		—	—	p	8
South Africa (Boers)	1.3	—	—	—	p	12
Australia	1.0		0.1	—	f, p, d	13–17
Ireland	—	1.0	0.1	0.10	d	7
Iceland	—	0.05	0.07	0.10		7
Germany	1.0	0.16	0.04	0.15	p, d	7, 18
Denmark	—	1.0	0.1	0.12	d	7
US/Canada	0.5	0.4	0.062	0.162	p, d	19–28
Norway	—	0.4	0.06	0.11	d	7
Estonia	0.4	—	—	—	a	9
France	0.4	0.08	0.029	0.12	p, d	1, 29–31
United Kingdom	0.3	0.36	0.06	0.12	p, d	7
Italy (North)	0.20	0.25	0.045	0.12	p, d	6, 32
Scotland	0.14	—	—	—	a	33
Netherlands	—	0.09	0.10	0.29	d	7
Spain	—	0.09	0.03	0.26	d	7
Sweden	0–0.08	—	—	—	p	2–5
Russia	0.01	0.02	0.014	0.08	p, d	7, 34, 35
Greece	—	0.01	0.01	0.12	d	7
Ashkenazic Jews	—	0.01	0.01	0.09	d	7
Turkey	—	0	0	0.14	d	7
North Africa/Arabia India/Pakistan	—	0	0	0.10	d	7
Sri Lanka	—	0	0	0.08	d	7
Africa (Sub-Saharan)	—	0	0	0.10	d	7
China	—	0	0	0.03	d	7, 36
Pacific Islands	—	0	0	0	d	7
Native Americans	—	0	0	0	d	7

Note:

[a] Prevalence of hemochromatosis predicted from C282Y allele frequency.

[b] Basis of estimates: a = autopsy studies; p = population surveys; d = DNA analysis for *HFE* alleles.

axe, sword, and spear. When blood transfusions were not available, those who had abundant iron stores may have been more likely to survive or to recover more quickly and transmit their DNA to new generations. Thus, the ability to absorb iron at an accelerated rate from a relatively iron-poor diet may have conferred a survival advantage.

Frequencies of hemochromatosis-associated mutations

Numerous studies have demonstrated a high prevalence of hemochromatosis in northwestern Europeans and in the Caucasian populations of Canada, the United States, Australia, New Zealand, and South Africa. The prevalence rates of hemochromatosis in these countries are consistent with a gene frequency of 0.06–0.1 in these Caucasian populations. Approximately 10–15% of the Caucasians of these countries are heterozygotes who carry a single copy of an hemochromatosis mutation. The great prevalence of hemochromatosis in these populations indicates that this is the most common single-gene hereditary disorder in Caucasians of northern European ancestry. Hemochromatosis is ten times more common than is cystic fibrosis (once thought to be the most common single-gene disease in Caucasians), five times more common than Hodgkin disease or acute leukemia, and one hundred-fold more common than plasma cell myeloma. It

is, of course, not as prevalent as diabetes mellitus or essential hypertension, but these disorders appear to be inherited in a polygenic manner.

Hemochromatosis-associated *HFE* mutations and Hardy–Weinberg equilibrium

If only one gene and one mutant allele were responsible for hemochromatosis, then the genotype frequencies could be estimated, assuming the conditions of Hardy–Weinberg equilibrium:

$$(w+h)^2 = w^2 + 2\,wh + h^2,$$

where w represents the frequency of the normal or wild-type allele in a population, and h represents the frequency of the mutant or hemochromatosis allele.

The w^2 represents the prevalence of homozygous normal persons in the population, $2wh$ represents the prevalence of heterozygotes, and h^2 represents the prevalence of homozygotes for the mutant hemochromatosis allele. Instead, extensive studies of the *HFE* gene have disclosed the existence of two common mutant alleles in addition to the wild-type allele. One of these is at nucleotide position 845, which is G in the normal or wild-type allele, and A in most patients with hemochromatosis. The other is at nucleotide position 187, which is C in the wild-type allele, and G in many patients with hemochromatosis. The 845G→A mutation encodes for a change in amino acid sequence from cysteine to tyrosine at amino acid 282 (C and Y, respectively). Thus, this mutation is called the C282Y mutation. The 187C→G mutation encodes for a change in amino acid sequence from histidine to aspartic acid (H and D, respectively), and this mutation has been called H63D. The observed frequencies of genotypes comprising homozygotes and heterozygotes for these three alleles reported in four recent studies in North America and observed genotype frequencies for patients with iron overload and for normal control subjects are displayed in Table 4.3[20, 23, 27, 37]. The results of other studies performed in Europe, the United States, or Australia have not been included in this tabulation because sufficient numbers of patients and control subjects are shown in Table 4.2 to permit inferences to be made. However, the data that have been reported from Europe are consistent with inferences made from data in Tables 4.2 and 4.3.

For a population in which there are three alleles of a gene, the genotype frequencies are determined by Hardy–Weinberg equilibrium. For simplicity, let w = frequency of the wild-type allele, y = the frequency of the allele due to the 845G→A mutation, and let d = the frequency of the allele due to the 187C→G mutation. Then,

Table 4.2. *HFE* genotypes in persons with iron overload and in normal subjects[a]

	Marked iron overload	Mild iron overload	Normal
Genotypes	(*n*, %)	(*n*, %)	(*n*, %)
C282Y/C282Y	295 (83.3)	21 (61.8)	2 (0.4)
C282Y/wt	4 (1.1)	6 (17.6)	40 (9.7)
C282Y/H63D	18 (5.1)	6 (17.6)	11 (2.5)
H63D/H63D	4 (1.1)	0 (0)	4 (0.2)
H63D/wt	7 (2.0)	1 (2.9)	124 (28.3)
wt/wt	26 (7.3)	0 (0)	260 (59.4)
Total	$n=354$	$n=34$	$n=438$

Note:
[a] These data were compiled from four North American studies[20, 23, 27, 28].

Table 4.3. Analysis of null hypothesis for association of H63D *HFE* allele with hemochromatosis[a]

	Normal control subjects, *n* (%)	Hemochromatosis patients, *n* (%)
H63D	410 (19.2)	117 (42.9)
Wild-type	1721 (80.8)	156 (57.1)

Note:
[a] These data were combined and analyzed for five North American studies[20, 23, 27, 28, 38] and six European studies [1, 6, 29, 31, 36]. Only H63D (mutant) and wild-type (normal) alleles were enumerated in this analysis. Total of all subjects = 2404; $\chi^2 = 78.9$ ($p < 0.000\,001$).

$$(w+y+d)^2 = w^2 + y^2 + d^2 + 2wy + 2wd + 2yd,$$

where homozygotes are represented by w^2, y^2 and d^2, and heterozygotes by $2wy$, $2wd$ and $2yd$.

Each of these genotypes is shown in Table 4.3. From the observed allele frequencies in the 438 normal control subjects, the frequency of the wild-type allele = 0.776, that of the 845G→A mutation = 0.062, and that of the 187C→G mutation = 0.162. The frequency of the C282Y allele was lowest in the series by Feder et al.[27], and highest in the series of Beutler et al.[20]. To the extent that these 438 normal subjects were representative of their parent populations, the following genotype frequencies may be predicted for this population:

homozygous wild-type: 0.602 predicted, 0.594 observed;
homozygous C282Y: 0.004 predicted, 0.004 observed;

homozygous H63D:	0.026 predicted, 0.002 observed;
heterozygous C282Y:	0.096 predicted, 0.091 observed;
compound heterozygous C282Y/H63D:	0.021 predicted, 0.025 observed; and
heterozygous H63D:	0.251 predicted, 0.283 observed.

Except for the paucity of H63D homozygotes, the observed genotype frequencies are not very different from those predicted for Hardy–Weinberg equilibrium. Furthermore, the predicted genotype frequencies agree quite well with those observed by the UK Haemochromatosis Consortium in persons of northwestern European origin[20].

The role of the C282Y mutation in hemochromatosis

There appears to be a consistent discrepancy between the estimates of hemochromatosis prevalence based on population studies using indices of iron overload for ascertainment, and the prevalence that can be predicted from the observed C282Y allele frequencies in various countries, as shown in Table 4.1. For example, the predicted prevalence for C282Y homozygosity in Canada and the United States is approximately 4 per 1000, but many studies have estimated prevalence of as high as 5 per 1000. Thus, the prediction of disease prevalence on the basis of C282Y allele frequency is only about 80% of that estimated from population studies that are based on chemical tests for iron overload. This discrepancy is not easily explained. It may indicate that more rigorous criteria are needed for diagnosis of hemochromatosis. Barton et al. found that, when they selected probands based on criteria of severe iron overload, DNA studies demonstrating C282Y homozygosity could account for approximately 80% of probands, compared with only 59% when they used criteria that identified persons with milder iron overload or persistently increased values of transferrin saturation alone[38]. On the other hand, Jazwinska et al. found that 100% of their clinically diagnosed cases in Australia were homozygous for the C282Y allele[14]. However, they selected only persons with relatively severe iron overload, and included multiple members from families with hemochromatosis probands, thereby causing a bias in their results toward a much higher C282Y allele frequency. At present, it is not clear whether the discrepancies between the prevalences of hemochromatosis observed and those predicted from allele frequencies are principally due to over-diagnosis by chemical criteria, or whether they may reflect presently unrecognized genetic causes of hemochromatosis.

The role of the H63D mutation in hemochromatosis

There is an emerging consensus that the C282Y allele, homozygosity for which occurs in approximately 80% of patients with marked iron overload, is, in fact, the 'major' hemochromatosis allele. The significance of the H63D allele has been the subject of dispute. However, in each of the four studies combined in Table 4.3, the H63D allele was present in substantially greater frequency among iron-overload cases than in normal controls. When more extensive data are combined for chromosomes that can carry the H63D allele, because they do not carry the H63D allele, the association of the H63D allele with iron overload is highly significant. This can be demonstrated in a four-fold contingency table (Table 4.3) that compares the number of observed non-C282Y (non-845A) chromosomes carrying H63D (187G) with the number of observed chromosomes that are wild-type for five North American[20, 23, 27, 28, 38] and six European[1, 6, 29, 31, 35, 39] studies. For this comparison that includes a total of 2404 chromosomes, $c^2 = 78.9$, and $p < 0.000\,001$.

Feder et al.[27] first identified the *HFE* gene and its C282Y and H63D alleles, and recognized the 'enrichment' of their iron overload group of patients with the H63D allele, that was present in 89% of patients (exclusive of those who were homozygous C282Y), whereas only 17% of controls had the H63D allele. They found that the association of the H63D allele with iron overload is highly significant ($p < 0.0001$). Paradoxically, however, they discounted the relevance of the H63D allele to hemochromatosis. Beutler et al. similarly evaluated the association of the H63D allele with iron overload in all reported studies prior to the summer of 1997 (thus, omitting four of the sets of data analyzed in Table 4.3)[20, 40, 41]. They compared the observed frequency of C282Y/H63D compound heterozygotes with C282Y heterozygotes, and found an extraordinarily low probability that the H63D allele is associated with iron overload merely due to chance ($p < 10^{-10}$). Thus, these three parallel analyses of probability provide overwhelming evidence that both the C282Y and the H63D alleles are related to hemochromatosis. One may arrive at the same conclusion if one similarly examines only the data reported from Europe. It seems erroneous to assert that the H63D allele is merely a polymorphism unrelated to hemochromatosis. The H63D allele is less frequently associated with marked iron overload than is the C282Y allele (apparent from inspection of the data in Table 4.2). Of the reported C282Y/C282Y homozygotes, more than 90% have had

marked iron overload, whereas H63D/H63D homozygotes are rare; of the 39 reported, approximately one-third have had marked iron overload. Although the H63D allele frequency in normal control subjects was nearly three times that of the C282Y allele, the C282Y allele was associated with iron overload ten times more often than was the H63D allele. C282Y/H63D compound heterozygotes usually had mild iron overload, whereas C282Y/C282Y homozygotes usually had marked iron overload (Table 4.2). It is reasonable to postulate, therefore, that the C282Y allele is associated with a considerable increase in the rate of iron absorption from the intestinal tract, and that the H63D allele is also associated with increased iron absorption, but less than that caused by the C282Y allele. This would explain the much lesser penetrance of the H63D allele. Other studies suggest that C282Y/C282Y homozygotes have at least 50% penetrance (i.e., approximately 50% of these homozygotes have clinical features of hemochromatosis)[1, 41, 42], whereas C282Y/H63D compound heterozygotes appear to have $<1\%$ penetrance (fewer than 1% of these compound heterozygotes exhibit clinical disease).

Ascertainment bias (the selection of patients with marked iron overload for DNA studies) is the best explanation for the observed higher frequency of the C282Y allele in hemochromatosis in all reported series. The H63D allele is not expected (and has not been observed) in C282Y/C282Y homozygotes, nor has the C282Y allele been observed in 37 reported H63D/H63D homozygotes. Intragenic recombination is quite rare. If such recombination had occurred, a few instances might be encountered in which both mutations 845A and 187G are present in one *HFE* gene. To date, however no such recombinants have been described.

As with the C282Y allele, the H63D allele occurs in its highest known frequency in people of Europe or of European ancestry, in whom the average H63D allele frequency is approximately 0.14. Outside of these populations, the H63D allele frequency appears to decline as a function of distance from Europe. It is approximately 0.09 in Ashkenazic Jews and in Saudi Arabs, 0.09 in Pakistanis, Indians, and Sri Lankans, and approximately 0.019 in persons in eastern and southeastern Asia. It is rare in the aborigines of the Pacific and the Americas. Indians, Sri Lankans, Pakistanis, Arabs, Nigerians, and Kenyans appear to have higher H63D allele frequencies than do populations that have been more isolated. These differences could be due to the greater age of the H63D allele than the C282Y allele, thereby permitting a wider diffusion of the gene into non-European populations.

Iron overload in African Americans

It has been asserted that hemochromatosis is rare, or even non-existent, in African Americans[19, 43]. Most studies of hemochromatosis prevalence in this country have been conducted in predominantly Caucasian populations, so it is not possible to confirm empirically the alleged rarity of hemochromatosis in African Americans. However, the prevalence of hemochromatosis can be predicted for persons of this ethnic group on the basis of other studies that have demonstrated that the gene pool of African Americans includes a large component that is of northern European origin. These studies have demonstrated that, on average, 25–45% of the genes of African Americans are of Caucasian derivation. African Americans are of British and African heritage[44–47]. If it is assumed that 'British' genes comprise a third of the African American gene pool, then the frequency of the C282Y allele in this population should be approximately one-third of that in the north European Caucasian population ($(0.33 \times 0.06)^2 = 0.0004$). Thus, homozygous hemochromatosis should be expected to occur with a prevalence of 0.4 per thousand in the African American population, or about one-ninth as frequently as in those of northwestern European ancestry. However, because African Americans comprise only about one-tenth of the United States population, a completely random survey of the population of the United States should reveal approximately 90 times as many Caucasian cases of hemochromatosis as African American cases. However, some African Americans are less likely than many Caucasian Americans to receive expert medical care, and they have a higher mortality rate at younger ages than do Caucasian Americans. These factors could partly explain why African American cases of hemochromatosis have been reported rarely. However, large-scale analyses of the prevalence of the C282Y and H63D mutations in African Americans have not been reported. Estimates of the prevalence of hemochromatosis in African Americans is complicated by the fact that another form of iron overload disorder is prevalent in sub-Saharan Africa[19, 48]. African iron overload (known in the past as 'Bantu siderosis') also appears to be determined genetically. It is not HLA-linked, and its features are somewhat different from hemochromatosis of northern Europeans. In particular, iron deposition in African iron overload is more marked in Kupffer cells and less striking in hepatocytes than in 'classical' hemochromatosis in Caucasians. The prevalence of African iron overload may be as high as 30% in central and southern Africa. However, its contribution to iron overload in African Americans is unclear.

Other mutations possibly associated with the hemochromatosis phenotype

Iron overload develops in mice that have a 'knockout' deletion of the β_2-microglobulin gene[37, 49]. The β_2-microglobulin gene is not on chromosome 6 (as is the *HFE* gene) and thus it is not HLA-linked. An analogous mutation of this gene has not been found in human hemochromatosis. However, other genes on other chromosomes may be implicated in the etiology of hemochromatosis. *Nramp2* is a transmembrane protein that is involved in intracellular iron transport, both in the apical cells of the duodenal mucosa, and in the erythroid precursor cells in hematopoietic bone marrow[50]. A constitutive mutation in Nramp2 might lead to increased iron absorption from the intestinal tract. Homozygosity for *Nramp2* deficiency causes microcytic anemias in mice and rats. At this time, it is not known whether mutations of *Nramp2* occur in human populations. Juvenile hemochromatosis, a severe form of hemochromatosis that is manifested in children or teenagers, is not linked to Ch6p[51]. It is not yet known whether compound heterozygotes for a juvenile hemochromatosis mutation and for the C282Y or H63D mutation have less severe clinical manifestations. There appears to be an additive effect on iron absorption and iron overload when either the C282Y allele or the H63D allele occurs together with an otherwise unrelated gene in heterozygotes for β-thalassemia or hereditary spherocytosis, and possibly for other hereditary anemias[26]. Thus, persons who are heterozygous both for one of the two known hemochromatosis alleles and for either hereditary spherocytosis or thalassemia trait exhibit features of severe iron overload. Similarly, the author has observed that persons who have hemoglobin Lepore trait and who are also heterozygous for the C282Y allele may have marked iron overload. Severe iron overload also occurs in persons with chronic severe anemias, e.g., pyruvate kinase deficiency, congenital dyserythropoietic anemias, and in congenital atransferrinemia. Iron overload may occur even in those patients who have not received erythrocyte transfusion or oral or parenteral iron. It is not clear whether these cases are conditioned, in part, by the presence of a mutant allele for the *HFE* gene. For two genes that are either on different chromosomes or are widely separated on the same chromosome, the Hardy–Weinberg principle is not applicable. Instead, the probability of concurrence is simply the product of the two gene frequencies. Thus, in a population in which the gene frequency of the C282Y allele is 0.06 and the gene frequency of a β-thalassemia allele is 0.02 (as is common in Italian Americans), the likelihood of compound heterozygosity is approximately 1.2 per 1000, or about one-third the prevalence of homozygosity for C282Y. We are only beginning to elucidate the genetic basis for hemochromatosis. Although it seems remarkable that only two mutant alleles of the *HFE* gene have been identified to date, it seems likely that other mutations of the *HFE* gene will be found. Although it appears that other mutations of *HFE* are quite rare, nearly 10% of persons with hemochromatosis are homozygous for the wild-type *HFE* allele.

Conclusions

Hemochromatosis is highly prevalent in populations of those countries surrounding the North Sea or northeastern Atlantic littoral, and in their derivative populations of North America, Australia, New Zealand and South Africa. This disorder is quite uncommon in other populations. An HLA-linked gene, now designated *HFE*, has three recognized alleles: the normal or wild-type allele, and two mutant alleles that are both highly associated with hemochromatosis: C282Y and H63D. Approximately 90% of cases of hemochromatosis can be attributed to the presence of these two mutant alleles, either in the homozygous configuration (C282Y/C282Y or H63D/H63D), or in compound heterozygosity (C282Y/H63D). The means by which these alleles increase iron absorption is unclear, although penetrance of the C282Y allele is greater than that of the H63D allele. Undiscovered genetic mechanisms must occur to explain the iron overload that occurs in nearly 10% of patients with a hemochromatosis clinical phenotype who lack both of these alleles.

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5

Variation of hemochromatosis prevalence and genotype in national groups

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Introduction

Once considered to be a rare disorder, hemochromatosis is now recognized as the most common known autosomal recessive disorder in Caucasians, which occurs commonly in many European or European-derived populations. The major factor leading to this recognition was the clarification of its genetic mode of transmission. The finding by Simon and co-workers of the association of hemochromatosis with the MHC class I antigen HLA-A3 and the subsequent demonstration of the haplotypic nature of that association marked the beginning of a new era in the clarification of the true frequency of hemochromatosis[1]. Assignment of a genotype on the basis of HLA typing has permitted an estimate of frequencies of the disorder in several areas of the world that are greater than the calculations previously based on the identification of clinical symptoms or on the findings of autopsy studies[2]. The recent finding by Feder and co-workers of the *HFE* gene[3], one mutation which accounts for the majority of hemochromatosis patients, permits a new approach to the study of the epidemiology of the disorder. In spite of the frequency of hemochromatosis in most Caucasian populations, there is considerable variation in its prevalence among different countries and even within national boundaries. This chapter provides an overview of the geographical variations of the HLA phenotypes marking the *HFE* gene and of the *HFE* genotypes found in hemochromatosis patients and normal populations throughout the world, and offers some explanations for the variations observed.

Ethnic origin and world-wide spread of the hemochromatosis trait

The frequency of hemochromatosis is relatively great in many European populations and in populations that are mainly of European descent, such as those in the United States, Canada, Australia, and South Africa. In general, the frequency of homozygotes for the disorder in Caucasians may be as high as 3–10 persons per 1000. The world-wide distribution of hemochromatosis in Caucasian populations and its strong linkage to particular HLA phenotypes are classical arguments that favor a common European origin of the hemochromatosis trait. The hypothesis that the original hemochromatosis mutation was a rare, if not unique, event that produced an ancestral HLA marking subsequently modified by recombinations was first evinced more than 10 years ago by Simon and co-workers based on a study of 609 HLA haplotypes marking the hemochromatosis gene[4]. Under that mutation–recombination hypothesis, it was assumed that patterns of population migrations would account for the geographic distribution of the disorder and the antigens and haplotypes marking the hemochromatosis trait. The similarity between the geographical distribution of hemochromatosis and the supposed settlement and migration pattern of the Celtic peoples led to the general belief that the hemochromatosis gene is of Celtic origin[1]. However, detailed comparisons of ethnic and racial backgrounds of hemochromatosis populations have not been mentioned until recently, when Smith and co-workers reported a study of screening for hemochromatosis among employees of a Massachusetts corporation[5]. They found that 100% of identified cases were of British–Irish ancestry, suggesting that the association of hemochromatosis with a Celtic ancestry is statistically significant. Whatever the origin of the hemochromatosis gene, the mutation–recombination hypothesis is now strongly supported by the finding of the *HFE* gene mutation (C282Y, nucleotide 845), homozygosity for which accounts for the vast majority of hemochromatosis patients in the world. In contrast, *HFE* mutations are not associated with the common syndrome of African iron overload[6], wherein a still non-identified genetic factor is involved[7].

Merryweather-Clarke and co-workers analyzed 2978 samples from persons distributed world-wide, and showed that the C282Y mutation of the *HFE* was most prevalent in northern European populations and absent from samples of non-European subjects (Africans, Asians, Australasians, and Americans)[8]. These results and others are illustrated in Table 5.1, wherein the frequencies of *HFE* mutations are compared to the frequencies of HLA-A and -B alleles in the different racial and ethnic groups. The major histocompatibility complex (MHC) is the most polymorphic genetic system in humans, and the tremendous genetic diversity found in HLA loci makes these genes valuable tools for studying the evolution of human populations[15]. The degree of diversity of the various MHC loci is also an indication of their evolutionary age, the more ancient loci being those with the most diverse profiles. At the level of particular HLA alleles, the more ancient ones in human evolution are found in all races and ethnic groups, reflecting their common ancestry.

Under these assumptions, it can be concluded from Table 5.1 that the HLA-B locus is older because it is more polymorphic (more alleles are represented) and the variations in the frequencies of the different alleles do not show any striking racial or ethnic pattern. With the exception of HLA-A2, the frequencies of HLA-A alleles show a diverging pattern in the different racial groups. Indeed, the more frequent antigens consistently show a racial or ethnic predominance: A3 and A1 occur predominantly in Caucasians; A11 and A24 occur predominantly in Asians (and less markedly in southern or eastern Europeans); and A23, A28, and A30 occur predominantly in Africans. HLA-A29 is an allele with a homogeneous representation in all populations, perhaps reflecting common ancestry. Within this immunogenetic background, the C282Y mutation appears to be a recent mutation of Caucasian origin, in accordance with its estimated age of 60–70 generations[16]. However, the H63D mutation is more widespread, although peak frequencies are also observed in Caucasian populations. In these, the H63D mutation is generally found in polymorphic frequencies, i.e., higher than 10%. This observation and the linkage disequilibrium between this mutation and the HLA antigens A29 and B44 in normal individuals and in patients with 'non-classical' forms of hemochromatosis[13] led us to speculate that this particular association could have been positively selected in human evolution, possibly in relation to iron overload.

This question is further analyzed in this chapter by searching for possible correlations between the frequencies of the *HFE* mutations and the various HLA-A and -B antigens. The strong correlation between the frequencies of the H63D mutation and those of the HLA antigens A29 and B44 is confirmed. This is not the result of any predominant racial or ethnic effect, because the HLA alleles in question are common to all populations and the significance of the correlation is highest when analyzed in Caucasian populations only ($r = 0.84$; $p = 0.0001$ and $r = 0.87$; $p = 0.00001$, respectively, for A29 and B44). This is illustrated in Fig. 5.1, in which the frequencies of the alleles HLA-A29, -B44 and H63D are shown in geographically scattered Caucasian populations (Fig. 5.1(*a*)). For comparison, the frequencies of the alleles HLA-A3,- B7 and C282Y are also shown in the same populations (Fig. 5.1(*b*)). Although the C282Y mutation appears predominantly in populations of northern European descent independent of the distribution of HLA-A3 or -B7, the geographical distribution of the H63D mutation is different, and is significantly correlated with the distribution of the HLA antigens A29 and B44. No other combination of alleles showed a similar correlation. Further, correlations between independent case series support the hypothesis that there was, or is, a selective advantage for the association between the H63D mutation and the HLA alleles A29 and B44. All these alleles are relatively old in human evolution (see above), and therefore the strong association cannot be explained by a recent mutational event that did not have enough time for recombination, as seems to be the case for the linkage between the *HFE* mutation C282Y and the HLA alleles A3 and B7 in hemochromatosis patients[16].

Geographical variations in hemochromatosis prevalence

Although the hemochromatosis gene mutation is widely distributed among Caucasians, considerable variation in the prevalence of hemochromatosis occurs among different countries or even within the same countries. In France, the prevalence is much greater in Brittany than in other regions[4]. In Sweden, the prevalence in a north–central rural part of the country[17, 18] is much higher than the estimated prevalence in three main urban areas[19, 20]. Some restricted populations have even higher frequencies, such as the Afrikaner population of South Africa or populations in Sydney, Australia, the north of Portugal, or Saguenay-Lac-Saint-Jean in Canada[21]. In contrast, there are many European countries, especially the eastern or eastern Mediterranean countries, from which no reports of hemochromatosis are known.

Different patterns of migration and settlement of people are widely accepted as important factors determining the establishment of gene frequencies throughout the world.

Table 5.1. Frequencies of *HFE* mutations and of the most common HLA-A and B antigens

Population	Allele frequencies of *HFE* mutations			Allele frequencies of HLA-A antigens									
	Data source (*n*)	C282Y	H63D	Data source (*n*)	A1	A2	A3	A11	A23	A24	A28	A29	A30
Caucasians:													
USA:	(a) (645)	0.063	0.145	(h) (246)	0.169	0.283	0.122	0.055	0.016	0.096	0.045	0.026	0.026
Australia:				(h) (63)	0.203	0.263	0.078	0.016	0.008	0.078	0.056	0.040	0.032
Queenslanders	(a) (2375)	0.064											
Westerns	(a) (51)	0.059	0.176										
Europe:													
Icelanders	(b)[a] (90)	0.067	0.106										
Finns	(b)[b] (38)	0.000	0.118	(i) (10000)	0.081	0.224	0.186	0.038	0.004	0.083	0.057	0.006	0.004
Former USSR	(b)[b] (154)	0.010	0.104	(h) (328)	0.095	0.281	0.110	0.088	0.024	0.165	0.020	0.006	0.010
Norwegians	(b)[a] (94)	0.064	0.112										
	(a) (144)	0.063											
Danes	(b)[b] (37)	0.095	0.122	(h) (150)	0.130	0.303	0.136	0.056	0.020	0.083	0.057	0.033	0.013
British	(b)[c,b] (413)	0.064	0.128	(h) (117)	0.150	0.241	0.153	0.073	0.026	0.062	0.045	0.043	0.026
Germans	(b)[b] (115)	0.039	0.148	(h) (295)	0.180	0.278	0.141	0.046	0.027	0.075	0.051	0.022	0.024
Austrians	(c) (460)	0.048		(h) (98)	0.168	0.281	0.153	0.051	0.026	0.097	0.041	0.046	0.031
French	(a) (398)	0.031	0.166	(h) (321)	0.137	0.213	0.133	0.062	0.026	0.089	0.056	0.064	0.023
Spanish	(b)[b] (50)	0.030	0.240	(h) (264)	0.113	0.276	0.090	0.070	0.025	0.068	0.031	0.093	0.051
Basques	(b)[b] (28)	0.036	0.304	(h) (60)	0.127	0.246	0.070	0.075	0.050	0.100	0.080	0.113	0.113
Portuguese (North)	(a) (71)	0.030	0.227	(j) (71)	0.100	0.270	0.080	0.080	0.007	0.113	0.030	0.060	0.000
(Center)	(d) (195)	0.033	0.085	(h) (125)	0.103	0.266	0.127	0.058	0.020	0.103	0.048	0.048	0.036
Italians	(b)[d] (91)	0.005	0.126	(h) (551)	0.140	0.244	0.129	0.061	0.015	0.097	0.036	0.039	0.039
	(a) (50)	0.010	0.100										
Greeks	(b)[b,e] (139)	0.014	0.119	(h) (115)	0.092	0.261	0.086	0.088	0.030	0.113	0.041	0.030	0.019
Turks	(b)[b] (70)	0.000	0.136										
Global Caucasians		**0.039**	**0.158**		**0.133**	**0.264**	**0.120**	**0.062**	**0.021**	**0.094**	**0.047**	**0.045**	**0.029**
Saudi Arabians	(b)[d] (118)	0.000	0.085										
Askenazi Jews													
Europe	(b)[b] (35)	0.000	0.086	(k) (40)	0.188	0.108	0.025	0.013	0.013	0.138	0.113	0.013	0.013
USA	(e) (381)	0.013	0.097										
Africans:													
Zambians	(b)[f] (76)	0.000	0.007										
Nigerians	(b)[b] (80)	0.000	0.019										
Kenyans	(b) (78)	0.000	0.013										
Gambians	(b)[g] (39)	0.000	0.013										
Senegalese	(b)[b](f) (224)	0.000	0.000	(h) (99)	0.051	0.195	0.061	0.000	0.183	0.010	0.068	0.051	0.143
Zimbabweians				(h) (90)	0.017	0.171	0.033	0.000	0.092	0.006	0.078	0.056	0.316
Zairians				(h) (61)	0.044	0.138	0.070	0.000	0.193	0.016	0.101	0.034	0.135

Table 5.1. (*cont.*)

Population	Allele frequencies of *HFE* mutations			Allele frequencies of HLA-A antigens									
	Data source (*n*)	C282Y	H63D	Data source (*n*)	A1	A2	A3	A11	A23	A24	A28	A29	A30
West Africans				(h) (70)	0.053	0.206	0.015	0.000	0.097	0.038	0.126	0.023	0.118
Ethiopians/Algerians	(f) (276)	0.000	0.090										
Global Africans		**0.000**	**0.010**		**0.037**	**0.178**	**0.045**	**0.000**	**0.141**	**0.018**	**0.093**	**0.041**	**0.178**
African Americans	(g) (48)	0.019	0.010	(h) (447)	0.053	0.167	0.089	0.023	0.081	0.047	0.109	0.037	0.095
Asians:													
Chinese	(b)[b,e] (72)	0.000	0.028	(h) (144)	0.018	0.310	0.014	0.273	0.000	0.166	0.004	0.011	0.007
Indonesians	(b)[b] (90)	0.000	0.028	(h) (119)	0.034	0.129	0.008	0.127	0.000	0.414	0.000	0.000	0.000
Taiwan Aboriginals	(b)[b] (80)	0.000	0.000	(h) (165)	0.000	0.181	0.000	0.113	0.000	0.610	0.000	0.000	0.003
Global Asians		**0.000**	**0.019**		**0.017**	**0.212**	**0.009**	**0.182**	**0.000**	**0.354**	**0.001**	**0.004**	**0.005**
Indians:	(b)[e] (215)	0.020	0.084	(h) (99)	0.111	0.121	0.079	0.159	0.010	0.181	0.061	0.030	0.011
Australasians:													
Papua New Guinea	(b)[g] (139)	0.000	0.000	(h) (51)	0.000	0.000	0.000	0.121	0.000	0.657	0.000	0.000	0.000
Australia Aboriginals	(b)[b] (93)	0.000	0.000	(h) (99)	0.020	0.157	0.066	0.045	0.061	0.318	0.030	0.025	0.010
Vanuatuans	(b) (90)	0.000	0.006										
Americans (mixed):													
Mexicans	(b)[b] (54)	0.000	0.065	(h) (114)	0.053	0.232	0.070	0.048	0.039	0.167	0.096	0.039	0.057
Jamaicans	(b) (90)	0.011	0.022										
Colombians	(b)[b] (47)	0.000	0.000										
Brazilian				(h) (351)	0.099	0.260	0.069	0.047	0.029	0.096	0.064	0.046	0.060
Global Americans		**0.004**	**0.029**		**0.076**	**0.246**	**0.070**	**0.048**	**0.034**	**0.132**	**0.080**	**0.043**	**0.059**

Population	Allele frequencies of HLA-B antigens															
	Data Source (*n*)	B7	B8	B13	B14	B18	B27	B35	B39	B44	B45	B51	B58	B60	B61	B62	B70
Caucasians:																	
USA:	(h) (246)	0.100	0.128	0.030	0.041	0.049	0.041	0.085	0.018	0.104	0.006	0.037	0.010	0.045	0.033	0.055	0.016
Australia:	(h) (63)	0.103	0.100	0.034	0.040	0.051	0.032	0.071	0.000	0.156	0.008	0.024	0.000	0.024	0.040	0.067	0.008
Europe:																	
Icelanders																	
Finns	(i) (10000)	0.122	0.083	0.031	0.003	0.048	0.072	0.122	0.038	0.071	0.001	0.044	0.002	0.057	0.018	0.111	0.000
Former USSR	(h) (328)	0.088	0.040	0.043	0.022	0.023	0.041	0.069	0.023	0.072	0.010	0.075	0.033	0.041	0.026	0.033	0.006
Norwegians																	
Danes	(h) (150)	0.092	0.089	0.020	0.014	0.040	0.030	0.060	0.033	0.129	0.007	0.057	0.013	0.086	0.017	0.068	0.007
British	(h) (117)	0.085	0.137	0.013	0.056	0.034	0.038	0.085	0.021	0.103	0.013	0.051	0.009	0.038	0.017	0.060	0.009
Germans	(h) (295)	0.107	0.098	0.032	0.037	0.034	0.046	0.076	0.019	0.092	0.008	0.049	0.034	0.046	0.025	0.049	0.014

Austrians	(h) (98)	0.158	0.092	0.050	0.020	0.061	0.031	0.117	0.020	0.096	0.005	0.076	0.010	0.045	0.010	0.051	0.005
French	(h) (321)	0.075	0.068	0.030	0.072	0.048	0.036	0.084	0.025	0.108	0.006	0.069	0.019	0.030	0.027	0.053	0.006
Spanish	(h) (264)	0.076	0.064	0.025	0.069	0.055	0.028	0.084	0.006	0.170	0.014	0.078	0.019	0.019	0.017	0.037	0.004
Basques	(h) (60)	0.092	0.058	0.025	0.033	0.167	0.067	0.050	0.008	0.217	0.000	0.067	0.025	0.008	0.000	0.050	0.008
Portuguese (North)	(j) (71)	0.050	0.070	0.007	0.080	0.040	0.120	0.080	0.020	0.130	0.010	0.100	0.070	0.030			0.007
(Center)	(h) (125)	0.079	0.075	0.016	0.056	0.028	0.028	0.128	0.020	0.143	0.012	0.114	0.016	0.016	0.008	0.044	0.024
Italians	(h) (551)	0.058	0.068	0.039	0.036	0.096	0.022	0.154	0.033	0.092	0.010	0.084	0.018	0.005	0.014	0.028	0.003
Greeks	(h) (115)	0.059	0.036	0.026	0.028	0.063	0.024	0.183	0.022	0.094	0.002	0.144	0.018	0.017	0.018	0.017	0.009
Turks																	
Global Caucasians		**0.089**	**0.082**	**0.026**	**0.040**	**0.054**	**0.044**	**0.095**	**0.020**	**0.121**	**0.008**	**0.070**	**0.019**	**0.036**	**0.018**	**0.052**	**0.009**
Askenazi Jews																	
Europe																	
USA	(k) (40)	0.032	0.037	0.000	0.100	0.025	0.025	0.207	0.012	0.072	0.000	0.050					
Africans:																	
Senegalese	(h) (99)	0.045	0.063	0.000	0.017	0.047	0.020	0.131	0.030	0.056	0.010	0.021	0.086	0.000	0.000	0.000	0.094
Zimbabweians	(h) (90)	0.078	0.035	0.017	0.039	0.070	0.000	0.022	0.006	0.058	0.100	0.006	0.118	0.000	0.000	0.000	0.135
Zairians	(h) (61)	0.062	0.028	0.010	0.049	0.041	0.008	0.033	0.025	0.057	0.028	0.025	0.049	0.000	0.000	0.008	0.168
Global Africans		**0.063**	**0.039**	**0.007**	**0.034**	**0.045**	**0.012**	**0.081**	**0.015**	**0.050**	**0.046**	**0.031**	**0.083**	**0.000**	**0.000**	**0.004**	**0.120**
African Americans	(h) (447)	0.083	0.032	0.009	0.030	0.033	0.016	0.077	0.008	0.062	0.038	0.032	0.069	0.013	0.002	0.014	0.082
Asians:																	
Chinese	(h) (144)	0.028	0.035	0.084	0.000	0.007	0.043	0.028	0.022	0.018	0.000	0.042	0.114	0.138	0.025	0.056	0.007
Indonesians	(h) (119)	0.018	0.000	0.064	0.000	0.042	0.050	0.090	0.000	0.035	0.000	0.047	0.023	0.013	0.005	0.296	0.000
Taiwan Aboriginals	(h) (165)	0.003	0.000	0.094	0.000	0.000	0.034	0.000	0.158	0.003	0.000	0.003	0.009	0.323	0.028	0.042	0.000
Global Asians		**0.018**	**0.012**	**0.081**	**0.000**	**0.016**	**0.042**	**0.039**	**0.060**	**0.019**	**0.000**	**0.031**	**0.049**	**0.158**	**0.019**	**0.131**	**0.002**
Indians:	(h) (99)	0.095	0.038	0.010	0.020	0.025	0.028	0.120	0.015	0.071	0.000	0.076	0.025	0.025	0.129	0.056	0.000
Australasians:																	
Papua New Guinea	(h) (51)	0.000	0.000	0.127	0.000	0.000	0.056	0.000	0.020	0.010	0.000	0.000	0.000	0.155	0.029	0.283	0.000
Australia Aboriginals	(h) (99)	0.015	0.010	0.075	0.030	0.000	0.066	0.035	0.015	0.066	0.000	0.025	0.010	0.100	0.196	0.071	0.000
Americans (mixed):																	
Mexicans	(h) (114)	0.066	0.039	0.009	0.061	0.057	0.018	0.145	0.066	0.114	0.013	0.039	0.022	0.031	0.044	0.009	0.031
Jamaicans																	
Colombians																	
Brazilian	(h) (351)	0.051	0.049	0.017	0.050	0.053	0.031	0.137	0.049	0.106	0.021	0.071	0.010	0.024	0.019	0.042	0.001
Global Americans		**0.059**	**0.044**	**0.013**	**0.056**	**0.055**	**0.025**	**0.141**	**0.058**	**0.110**	**0.017**	**0.055**	**0.016**	**0.028**	**0.032**	**0.026**	**0.021**

Note:

Data Source: (a) Data collected from control groups used in studies of hemochromatosis (see Table 5.2 for more details). When more than one independent study was available for a given population, the mean frequency was considered. (b) [8] originated from: [a] = blood donors; [b] = anthropological community-based surveys; [c] = family studies of collagen disorders and polycystic kidney disease; [d] = community-based surveys of hemoglobinopathies; [e] = referrals for diagnosis of hemoglobinopathies; [f] = neonatal survey of hemoglobinopathies; [g] = community-based malarial survey. (c) [58] (d) [9] (e) [10] (f) [11] (g) [6] (h) [59] (i) [12] (j) [13] (k) [14.] (*n*) = Sample size.

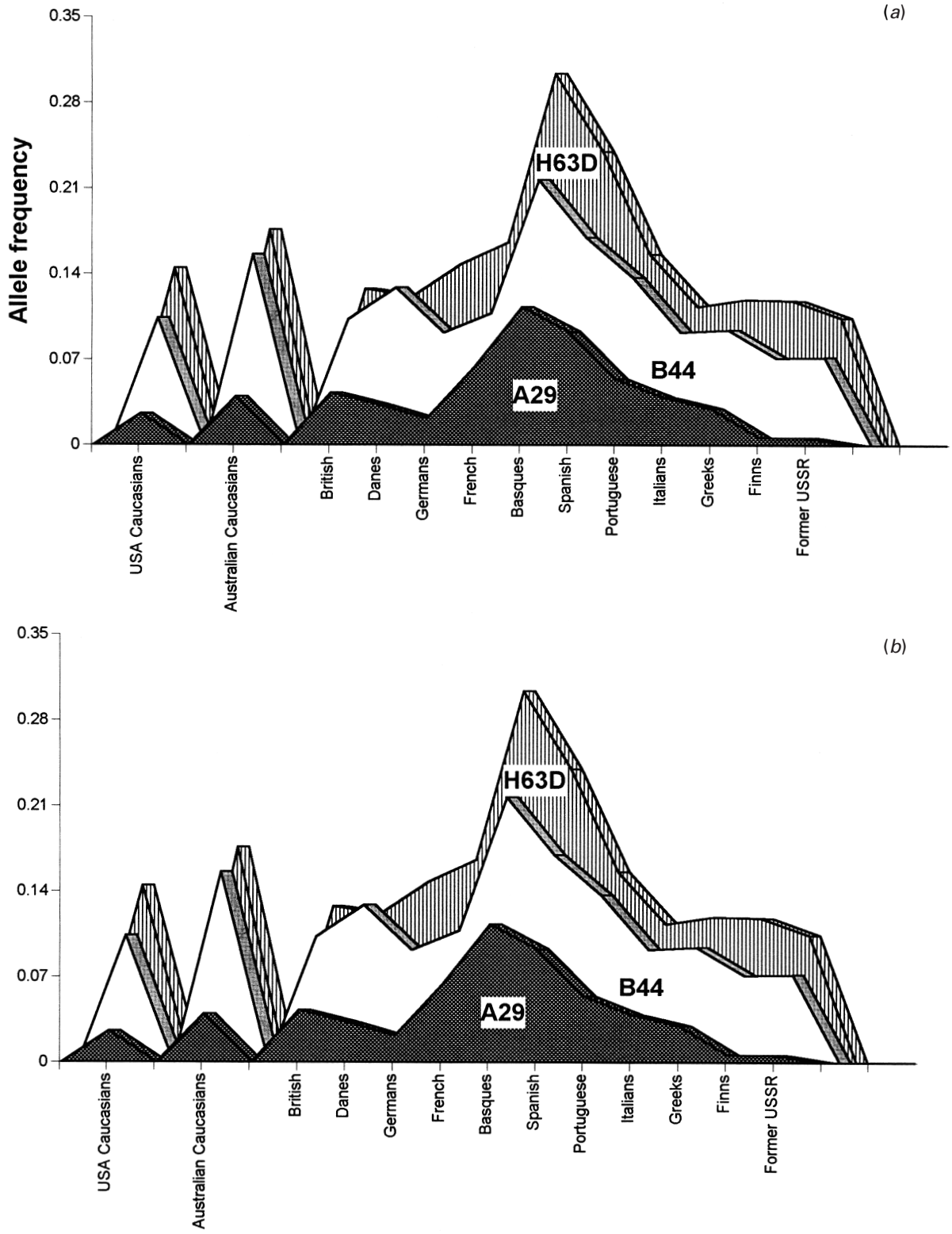

Fig. 5.1. Frequencies of the alleles H63D, HLA-A29 and HLA-B44 in geographically spread Caucasian populations (*a*) in comparison to the frequencies of the alleles C282Y, HLA-A3 and HLA-B7 in the same populations (*b*).

However, other factors should not be neglected when interpreting divergent results in estimations of hemochromatosis prevalence among different populations. The general awareness of hemochromatosis by clinicians and by the population is an important factor in the clarification of the true frequency of the disorder. The highest frequencies of hemochromatosis have been found in populations in which effective screening programs were implemented or family studies were systematically performed with HLA typing. The methods and cutoff values used in the screening programs may also affect estimations. This is the case for transferrin saturation, the best screening method for the biochemical expression of hemochromatosis, wherein informative threshold values in the range of 45–70% were computed in different studies[2]. Significant differences in the reference values also existed, even among different regions within the same country. In Portugal, for example, significant differences were found in reference values for transferrin saturation and serum ferritin concentration between the populations from the north or from the south of the country[22]. However, the reference values themselves may be influenced by the frequencies of the hemochromatosis trait in the control populations from which they derived, as demonstrated by McLaren and co-workers[23]. In addition, some demographic characteristics of the populations sampled may affect gene frequencies in particular regions. Besides the population density, other social, religious, or communication factors may influence the choice of partners in the direction of non-random matings. These arguments have been used to explain the high frequency of hemochromatosis in the rural population of central Sweden in contrast with urban areas, or in other isolated regions like in Saguenay-Lac-Saint-Jean or some rural communities in the north of Portugal. Finally, variations of prevalence may exist if there are variations in the clinical expression of the disorder, even with similar gene frequencies. Hemochromatosis is clinically very heterogeneous, and genetic factors other than the *HFE* gene mutation influence the expression of the disorder[24, 25]. HLA phenotype influenced the severity of iron loading[26–29]. Other immunological parameters also contribute to the clinical heterogeneity of the hemochromatosis phenotype, namely the relative proportions of $CD8^+$ T-lymphocytes and the CD4/CD8 ratio[29]. The levels of these parameters are themselves genetically determined[30]. In this context, one could speculate that the clinical expression of the disorder may also have a geographical distribution in relation to the distribution of other genetic traits, especially other MHC genes.

Geographical distribution of HLA haplotypes marking the *HFE* gene

A major characteristic of the mode of transmission of hemochromatosis is the haplotypic nature of the trait, which is transmitted in a very strong linkage with other genes within the MHC complex. This characteristic has permitted the definition of an ancestral haplotype, containing the HLA antigens A3, B7 and the microsatellite alleles D6S265–1 and D6S105–8, which prevail in all parts of the world where the disorder is described[31, 32]. Other linked haplotypes, however, seem to have particular geographical distributions. For example, haplotype A3/B14 is dominant in central Sweden, England, Ireland, and Denmark[33]; the haplotype A3/B35 is found in northeast Italy[34], and the haplotypes A11/B35 and A11/B5 are found in Brittany[4]. With the exception of A3/B14, these haplotypes are also found in linkage disequilibrium in the normal control populations from the same geographical areas, and are assumed to be the oldest hemochromatosis allele markers in the respective areas. In contrast, they are neither found frequently nor in linkage disequilibrium in non-Caucasian populations[59]. The haplotype A3/B14, which is not found in linkage disequilibrium in normal populations, could correspond to a more recent recombination between the HLA-A and -B loci. The remaining haplotypes associated with hemochromatosis, reflecting relatively recent recombinations between the *HFE* gene and the HLA-A locus, show, in general, the same distributions as in the respective control populations. An exception is the haplotype A1/B8 that is in linkage disequilibrium in most Caucasian populations, but that is not significantly associated with hemochromatosis except in central Sweden, where it was found in 14/50 unrelated hemochromatosis patients[18]. The fact that this haplotype is not associated with hemochromatosis in other adjacent countries led to the suggestion that its finding in central Sweden could represent a recent recombination event that has not yet spread beyond that confined region[35]. Certain patterns of HLA haplotypes associated with hemochromatosis within particular geographical areas may be influenced by the extension of the pedigrees studied. This was demonstrated in a population of central Sweden, wherein Ritter and co-workers showed that six apparently unrelated patients with the haplotype A3/B14 were indeed assigned to the same pedigree traced back to the end of the seventeenth century[18].

Table 5.2. Allele frequencies of the *HFE* mutations (C282Y and H63D) and wild-type alleles (wt) in hemochromatosis patients and controls

			Patients					Controls			
Country (region)	Reference	Sample size (*n*)	C282Y	H63D	wt	H63D/ (H63D+wt)	Sample size (*n*)	C282Y	H63D	wt	H63D/ (H63D+wt)
USA:	[3]	(178)	0.857	0.053	0.090	0.37	(310)	0.032	0.166	0.802	0.17
	[36]	(147)	0.857	0.027	0.116	0.19	(193)	0.075	0.122	0.803	0.13
(Alabama)	[25]	(74)	0.696	0.108	0.196	0.36	(142)	0.081	0.144	0.775	0.16
Australia:											
(Queensland)	[37]	(112)	1.00	0.00	0.00	—					
	[38]						(2375)	0.064			
(Western)	[39]	(41)	0.866	0.00	0.134	—	(51)	0.059	0.176	0.765	0.19
Norway:	[40]	(70)	0.843				(144)	0.063			
Sweden:											
(Central)	[41]	(38)	0.921								
(Stockholm)	[42]	(92)	0.897	0.033	0.070	0.32					
Germany:	[43]	(32)	0.953								
France:											
(Brittany)	[44]	(65)	0.947	0.034	0.019	0.64	(139)	0.029	0.165	0.806	0.17
	[45]	(207)	0.952	0.019	0.029	0.40					
(Paris)	[46]	(47)	0.926	0.032	0.042	0.43	(104)	0.029			
(Montpellier)	[47]	(70)	0.821	0.057	0.122	0.32	(60)	0.025	0.175	0.820	0.18
(Toulouse)	[48]	(94)	0.766	0.085	0.149	0.36	(95)	0.042	0.158	0.800	0.16
Italy:	[49]	(75)	0.687	0.067	0.246	0.15	(50)	0.010	0.100	0.899	0.10
Portugal:	[13]	(24)	0.896	0.042	0.062	0.40	(71)	0.030	0.227	0.743	0.23
	Mean ± SD					**0.36 ± 0.12**					**0.17 ± 0.03**

Geographical variations in hemochromatosis genotypes

After the description of the candidate gene for hemochromatosis[3], many research groups examined their patient material for the *HFE* mutations, building a strong body of evidence that indeed hemochromatosis may be caused by the presence of the C282Y mutation. However, the gene frequency for the mutation shows some variation among the different studies (Table 5.2). This could be explained either by the existence of other unidentified mutations in different geographical areas, or by the use of different clinical criteria in the patients' selection. Hemochromatosis is a clinically heterogeneous disease and even the application of strict definitions may not distinguish true genetic hemochromatosis from other forms of iron overload or from heterozygotes with phenotypic expression. The severity of iron overload is variable and may be influenced by several factors, including sex, age, the HLA phenotype, and the relative proportions of $CD4^+$ and $CD8^+$ T-lymphocytes[29]. In addition, the clinical expression may also be affected by the genotype. It is now becoming evident that the C282Y and H63D mutations may have some synergistic positive effect on iron storage and forms of minor or moderate iron overload exist associated with genotypes involving the H63D mutation[50].

The gene frequencies for the two *HFE* mutations in hemochromatosis patients from different geographical areas and the frequencies in the respective control populations are displayed in Table 5.2. For all series, the proportion of alleles carrying the H63D mutation among chromosomes not carrying C282Y was calculated by dividing the allele frequency of H63D by the sum of frequencies of H63D and wild-type alleles (H63D ÷ (H63D + wt)). Two results come to attention with inspection of Table 5.2. The first is that the frequency of the C282Y mutation is $< 100\%$ in most series, therefore implying that other determinants, genetic or not, may be involved in the disorder. This is most clear in Italy in which hemochromatosis seems to be prevalent, but in which the frequency of C282Y is among the

Table 5.3. Differencies in *HFE* mutations and wild-type alleles (wt) frequencies according to differing clinical criteria: example of selecting a population sample for estimating the C282Y and H63D gene frequencies in hemochromatosis patients

		HFE gene frequencies			
	Sample size (*n*)	C282Y	H63D	wt	H63D/ (H63D+wt)
Criterion A					
Step 1 = All patients with at least two of the following criteria: (a) hepatic iron concentration >100 μmoles/g dry weight; (b) liver Perls' Prussian blue stain >III; (c) hepatic iron index >2; (d) iron removed by phlebotomies >5 g (or 25 units of phlebotomy)	(44)	0.52	0.25	0.23	0.52
Step 2 = Exclude: (a) evidence of chronic hemolysis or other hematologic disorder; (b) serological evidence of HCV or HBV infection; (c) non-alcoholic steatohepatitis	(34)	0.68	0.13	0.19	0.41
Step 3 = Exclude all patients with iron removed by phlebotomy <5 g	(24)	0.90	0.04	0.06	0.40
Criterion B					
Select only the probands of genetically well characterized families with evidence of HLA linked transmission of the disease	(15)	0.93	0.01	0.06	0.14

lowest in Europe. Regional differences also exist in Italy; the C282Y mutation is more common in hemochromatosis patients from the north than from the south[51]. The second observation is that there is an obvious enrichment of the H63D mutation in chromosomes not carrying C282Y in hemochromatosis patients relative to their normal subjects from their respective populations. Whether the H63D mutation has any effect on iron overload per se or it is marking another yet non-identified gene associated with iron overload requires further investigation. The hypothesis that other forms of hemochromatosis, genetically distinct from the 'classical' C282Y-linked hemochromatosis, may be confounding the results in different series deserves to be considered. In Table 5.3, an exercise is presented of how a sample population can be selected to determine the gene frequencies in patients with hemochromatosis and illustrate how different clinical criteria used may influence the actual estimations. The sample is a population of patients referred to one health center in Porto, Portugal, and the diagnostic criteria were established by the same clinician. Using the 'classical' strict definition of hemochromatosis (Table 5.3, step 3: criterion A or B), the frequency of the C282Y mutation was similar to the high frequencies generally found in other European countries (see Table 5.2 for comparison). A lower frequency was found, however, if the strict criteria of a total body iron storage greater than 5 g was not obligatory (Table 5.3, step 2: criterion A). In this case series, the inclusion of patients with other forms of hemochromatosis (Table 5.3, step 1: criterion A) increases dramatically the frequency of the H63D mutation.

Conclusions

Two issues deserve special consideration after this presentation of the world-wide distribution of hemochromatosis and its linked alleles. The first is the observation that a mutant allele that causes hemochromatosis is so frequent in most Caucasian populations. The second is the tight linkage of the mutated *HFE* gene and other genes in the MHC class I complex, namely the HLA genes. The maintenance of human genetic disorders in high frequencies in certain populations interests evolutionary biologists who suggest that tight linkage may be maintained by compensating advantages of inheriting the linked traits. To explain the high frequency of hemochromatosis in most Caucasian populations, it was postulated that the *HFE* gene could have conferred a selective advantage to heterozygotes in the past, because they would absorb iron more efficiently in conditions of low iron supply[52]. Heterozygote advantage is usually considered the most likely factor balancing a

polymorphism. However, that kind of selective advantage has never been proven to occur in hemochromatosis. The only known fertility study addressing this question was done in Saguenay-Lac-St-Jean, a geographically isolated region of northeastern Quebec in which there is one of the highest frequencies of hemochromatosis found so far[53]. There was no demonstrable selective advantage for heterozygotes in this study. An alternative model of selection could be a differential selection for the sexes, in which females have greater fitness than males, as proposed by Livingstone in 1992[54]. Because the iron overload associated with hemochromatosis is generally less severe in women, homozygous and perhaps heterozygous females could have a selective advantage in conditions of low iron supply. However, the results of various genetic models of fitness investigated by Livingstone, including the differential selection for sexes, have shown that the extremely slow approach to equilibrium does not support any recent evolution of the hemochromatosis trait in humans[54]. Therefore, a founder effect and genetic drift continue to be the most likely explanations for the geographical distribution of the *HFE* gene. A founder effect may be responsible for the temporary maintenance of some traits in high frequencies in a given population, as long as they are relatively protected from the effects of natural selection[55, 56]. In this case, however, it must be assumed that the populations are of recent origin, a hypothesis that is consistent with the relatively recent age estimated for the C282Y mutation in the *HFE* gene[16].

Extreme genetic diversity is a hallmark of the MHC and some forms of selection are required to explain that diversity[15]. The most widely accepted selective force acting on MHC selection is resistance to infectious disease. The polymorphism of the HLA-A, -B, and -C genes determines the T-cell receptor repertoire of $CD8^+$ cytolytic T-cells and the response of natural killer cells, therefore modulating the immune responses in persons with different HLA types. In general, heterozygosity for any HLA locus is advantageous because it may enhance immunocompetence by increasing the number of possible peptide-binding specificities. If diversity is a goal to be achieved from the point of view of response to pathogens, it is clear that some haplotypes in the HLA system are maintained in strong linkage in both normal persons and in those with mutant alleles. There is no evolutionary explanation for that finding, unless essential functions are maintained in relation to particular haplotypes. In the case of hemochromatosis, linked haplotypes could have been conserved through recombination suppression, or any other evolutionary selection related to iron metabolism. That a major function of the immunological system is protection against the toxicity of iron was postulated 20 years ago by De Sousa[57], but only now is becoming accepted. The recent knowledge that the *HFE* gene encodes for an MHC class I protein[3] is of particular relevance. The entire MHC complex is a cluster of related genes, and the possibility that functional interactions exist favoring certain haplotypes to be selected together is now emerging. Examples include the strong world-wide association between the H63D mutation and the HLA antigens A29 and B44 and iron overload[13]. Evidence that the *HFE* gene may contribute to the presentation of some antigen to the cells of the immunological system is presumptive at present, and there is still no formal demonstration that it has any modulating effect on the T-cell repertoire. However, the fact that the H63D mutation affects a putative peptide-binding site in the molecule is relevant to those considerations. In human evolution, a balance must be found between diversity and homogeneity of the MHC, either enlarging the immunocompetence in response to the external environment, or favoring some fundamental endogenous functions. It may be that the *HFE* gene and the constancy of its first reported mutations correspond to a fundamental regulatory function of the MHC.

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6

Human leukocyte antigen (HLA) association and typing in hemochromatosis

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Introduction

After Sheldon's initial suggestion that hemochromatosis might be an 'inborn error of metabolism'[1], the question of whether environmental or genetic factors contribute to the familial clustering of the disorder was long debated. By 1970 a genetic causation was generally accepted, but the mode of inheritance was still controversial, despite cogent family data supporting recessive transmission of Saddi and Feingold[2]. Simon discovered the association of hemochromatosis with HLA antigens[3], confirmed its inheritance as an autosomal recessive disorder[4], and assigned the hemochromatosis gene to the short arm of chromosome 6 by demonstrating linkage to the Major Histocompatibility Complex (MHC)[5]. Simon also initiated a positional cloning strategy based on the search for maximal allelic association and the analysis of hemochromatosis-associated haplotypes, both ancestral and recombinants, under the assumption of a strong founder effect[6]. The latter approach, termed 'recombinant haplotype mapping'[7], permitted the prediction that the hemochromatosis gene(s) was distal to HLA-A[6]. These locations on Ch6p correspond to the critical region in which an HLA class-I like gene (*HFE*), the best candidate gene for hemochromatosis so far identified, was identified and cloned[8]. This chapter focuses on allelic and haplotypic associations described with serological and molecular markers located at the telomeric end of the MHC with respect to the mapping of the *HFE* gene. Their practical use in genetic counseling is discussed.

Association studies with serologically defined HLA markers

The MHC chromosomal region was first suggested as a possible genetic region for the hemochromatosis gene by Simon[3], who discovered a greater frequency of the HLA-A3 antigen among hemochromatosis patients than controls from the region of Brittany, France. An extension of the original series further revealed an increase in HLA-B14 and -B7 antigens[9]. Soon, nine independent series from Ireland[10], Scotland[11], the UK[12], Australia[13], Germany[14–16], France[17] and Belgium[18] confirmed the correlation between the disorder and the A3–positive and B7–positive phenotypes. This finding that distinct populations with different histories uniformly revealed HLA associations with hemochromatosis reduced the possibility of false-positive results due to population structure (recent admixture, heterogeneity, stratification). A further argument favoring the presence of a disease gene in gametic disequilibrium with the MHC complex was given by Simon[5], who examined the distribution of HLA-A3 phenotypes among unrelated patients and concluded that the excess of homozygosity for HLA-A3 was consistent with the occurrence of a recessive gene close to HLA-A. A mathematical formulation of the approach confirmed this interpretation and rejected a dominant model[19]. Finally, formal proof of genetic linkage as the cause of the disequilibrium was provided by the co-segregation of HLA markers and the disorder within affected sibships[5] and multiplex families[20, 21].

Numerous studies from various geographical areas settled by populations of north European origin have confirmed and extended much of the early data on HLA associations with hemochromatosis. These include population-based studies comparing phenotypic data in unrelated cases and controls, and family-based studies that gave direct estimates of allele and haplotype frequencies in hemochromatosis-associated and normal chromosomes segregating in the corresponding populations.

Associations with HLA antigens

Many research groups have compared HLA antigen frequencies in hemochromatosis patients with the general population or local controls drawn from hospital staff or blood donors[22]. Some have periodically extended their previous datasets, in which case only the original dataset or the one with the largest sample size is included in Table 6.1, that summarizes 21 independent case-control studies from different populations.

All the studies uniformly reveal an excess of A3 and B7 antigens among patients with hemochromatosis (Table 6.1). The mean frequency of positivity for A3 is 75.1% (range 53–83%) in hemochromatosis patients and 24.9% (range 19–31%) in control subjects. Expression of A3 is associated with an overall relative risk (RR) of 6.6 (95% confidence interval (CI) 5.7–7.6). The increased frequency of B7, although not statistically significant in some series, is a constant trend (mean value 48.8% vs. 21.6%, RR = 3.0, 95% CI 2.6–3.4). A small group of Afrikaner patients also had a high positivity rate for A3 and B7 (7/8) or both (5/8) antigens[39]. The frequency of B14 is greater than that in control subjects in one-half of the series, but the association is statistically significant in only three[12, 33, 22]. This undoubtedly reflects different evolutionary events in the populations studied, as illustrated by the heterogeneous results obtained in the two French series (Table 6.1), one from the well-defined population of Brittany[22] and the other from a mixed population living in Paris[24]. A further specific association with A11 is described in Brittany[6], and probably exists in other areas, such as Ireland and north-eastern Italy, where patients also exhibit an equal or even increased frequency compared to control subjects (Table 6.1). A similar pattern of distribution for the B35 antigen is found in the same series (data not shown).

As for other disorders associated with antigens at two or more loci, an important problem has been to detect which locus was primarily involved in the association. Interpretations based on the highest relative risk (RR) estimate (odds ratio in case-control studies) could be erroneous, because the magnitude of RR depends on both the amount of disequilibrium and the antigen frequencies[40]. For example, rare series show a lower RR for HLA-A3 than for -B14[33] or -B7[31]. The possibility of a preferential association with A3 was more convincingly supported by stratified analyses showing an excess of A3 among B7-negative patients, but no increase of B7 among A3-negative patients[24, 37]. This was later confirmed by haplotype analyses[6].

Associations with HLA alleles and haplotypes

Although positive associations are usually taken to imply genetic linkage between the loci under consideration, a number of factors can create gametic disequilibrium between distant or even unlinked loci[41,42]. Thus the excess of haplotype sharing among affected sib-pairs[5, 17] and high lod-score values obtained in multiplex pedigrees using the class I complex as the segregating locus[20, 21, 43] have been crucial to confirm the presence of a recessive gene closely linked to the HLA loci. In contrast, attempts to elucidate the gene position through conventional linkage analyses soon became unrealistic. The principal caveat was the rarity of informative recombinant events that led to an estimated recombination fraction of < 0.01 between *HFE* and class I genes[20, 21]. Accordingly, Simon[6] empirically focused on both disequilibrium and haplotype data to infer the gene location and order of markers (discussed below). This approach, simultaneously described by Bodmer[44], has become a popular gene mapping tool[42] because it exploits recombinants that have occurred over the entire history of a population, rather than the few directly available in the present living families. In fact, there is less dichotomy than may appear, because examination of unrelated pedigrees is the most convenient way to assess multilocus haplotypes segregating with the disorder and to obtain a control group perfectly matched for ethnic ancestry[41].

Association data and linkage disequilibrium mapping

Allelic association data derived from HLA haplotypes have confirmed the previous phenotypic results favoring a stronger association with A3. Formal proof was given by Simon[6] who showed that A3 haplotypes carrying non-B7 or non-B14 alleles were significantly more frequent on hemochromatosis than on control chromosomes, although the reverse was not true. Subsequent studies consistently indicated that HLA-B alleles were associated with the *HFE* gene only through their own disequilibrium with specific HLA-A alleles[29, 45, 46]. Similarly, the increased frequency of DR2[37, 47] and DR6[6] was attributable to a high degree of conservation of two specific haplotypes: A3, B7, DR2 and A3, B14, DR6, respectively. It was thus concluded that HLA-A3 was the only independent marker, and this placed the hemochromatosis gene closer to HLA-A than to HLA-B[6].

In the light of the cloning of a candidate gene (*HFE*) far distal to the MHC, an analysis of HLA haplotypes segregating in the present-day population of Brittany predicted a gene position telomeric to HLA-A (Table 6.2)[6]. Haplotypes showing the strongest association with *HFE* and a higher linkage disequilibrium between HLA-A and HLA-B loci on

Table 6.1. HLA-A, and HLA-B antigen associations in 21 case-control studies from 13 countries

		A3			A11			B7			B14		
Population–region study	Cases/controls (*n*)	FAD-FAP	RR	$p <$	FAD-FAP	RR	$p <$	FAD-FAP	RR	$p <$	FAD-FAP	RR	$p <$
Europe													
Belgium[18]	12/40	83–25	15.0	10^{-3}	?			75–15	17.0	10^{-3}	0–8	nc	nc
Denmark[23]	70/1967	80–27	10.9	10^{-12}	4– 10	0.4	NS	60–27	4.1	10^{-7}	10–5	2.4	0.02
England[12]	35/95	69–31	4.9	10^{-3}	17–21	0.8	NS	34–20	2.1	NS	20–6	3.7	0.03
France–Paris[24]	66/444	78–26	10.6	10^{-12}	14–10	1.4	NS	53–20	4.5	10^{-7}	9–9	1.0	NS
France–Brittany[22]	390/1005	74–29	7.0	10^{-12}	13–12	1.1	NS	46–27	2.3	10^{-10}	29–10	3.6	10^{-12}
Germany–West[15]	22/5046	73–30	6.2	10^{-3}	?			47–27	2.3	NS	0–5	nc	NS
Germany–West[16]	35/100	74–21	10.9	10^{-6}	3–9	0.3	NS	60–22	5.3	10^{-5}	3–6	nc	NS
Germany–West[25]	35/964	57–26	3.8	10^{-3}	9–10	0.9	NS	46–27	2.3	0.02	9–7	1.3	NS
Hungary[26]	10/1963	60–22	5.2	0.02	?			40–15	3.9	0.05	0–?	nc	nc
Ireland[27]	20/143	60–24	4.6	10^{-2}	10–8	1.2	NS	60–28	3.9	0.01	20–13	1.7	NS
Italy–Lombardy [28]	20/128	75–22	10.7	10^{-4}	5–9	0.6	NS	30–9	4.6	0.01	0–4	nc	nc
Italy–Veneto + South[29]	67/700	63–23	5.8	10^{-10}	8–10	0.8	NS	27–9	3.6	10^{-4}	5–5	1.0	NS
Italy–Veneto[30]	16/1348	81–22	15.1	10^{-4}	13–13	1.0	NS	25–11	2.6	NS	0–5	nc	nc
Portugal[31]	17/203	53–22	4.1	10^{-2}	?			35–9	5.6	0.01	0–?	nc	nc
Spain[32]	9/450	67–19	8.6	10^{-2}	?			56–16	6.6	0.01	0–10	nc	nc
Sweden[33]	50/500	66–32	4.1	10^{-5}	4–10	0.4	NS	40–28	1.7	NS	22–2	13.8	10^{-8}
North America													
Canada[34]	18/253	61–25	4.7	10^{-2}	?			50–22	3.5	0.02	17–8	2.3	NS
US–Utah[35]	23/201	56–22	4.6	10^{-3}	?			35–34	1.0	NS	4–4	1.0	NS
US–Minnesota[36]	56/1208	70–28	5.9	10^{-8}	?			50–24	3.2	10^{-4}	?		
Australia													
East[13]	10/700	60–20	6.0	10^{-2}	10–12	0.8	NS	50–21	3.8	0.05	20–7	3.3	NS
East[37]	73/522	70–26	6.7	10^{-11}	15–13	1.2	NS	60–27	4.1	10^{-7}	10–8	1.3	NS
Combined data	1054/17980	75.1–24.9[a]	6.6	10^{-12}	10.8–11.1[a]	1.0	NS	48.8–21.6[a]	3.0	10^{-12}	16.7–6.1[b]	2.6	10^{-12}

Note:

The antigen frequencies in the disease group (FAD) and in the general population (FAP) are rounded off to the nearest whole number for clarity. Relative Risk (RR) estimates for each series, combined estimates of RR for the overall series (which cannot be derived by directly using the frequencies obtained for the pooled data), and statistical significance were calculated by applying the Woolf–Haldane method[38]. The Bonferroni's correction for multiple tests is not required, because the associations have already been described. NS = not significant. nc = not calculated. χ^2 test for heterogeneity between series: [a] NS, [b] $p < 10^{-3}$.

Table 6.2. HLA-A, B haplotypes on hemochromatosis (H) and control (C) chromosomes in various populations

	Population-based controls					Family-based controls			
	Sweden [c 33] (100 H/190 C)	Italy [a 28] (42 H/5638 C)	Portugal [b 31] (16H/203 C)	Germany [b 48, 49] (76 H/1784 C)	Denmark [b 47] (39 H/1719 C)	Brittany [c 6] (609 H/475 C)	Australia [b 45] (98 H/63 C)	Denmark [b 46] (39 H/51 C)	Utah [c 50] (345 H/835 C)
A3[a]	42.0–**17.5**(d)	**56.4–11.7**(d)	**31.4–11.6**(b)	51.3–**16.5**(d)	56.4–**15.1**(d)	49.6–15.6(d)	41.1–17.5(b)	56.4–13.7(c)	37–11.5(d)
A3, B7[a]	19.0–6.8(b)	9.8–0.7(b)	17.6–1.8(a)	35.5–5.9(d)	25.6–6.6(c)	21.2–6.2(d)	22.4–7.9(a)	25.6–2.0(b)	24–5.0(d)
A3, B14[a]	11.0–0.5(c)		0–0	1.3–0.3	2.6–0.6	13.8–1.5(d)	4.1–3.2	2.6–0	4.4–0.6(c)
A3, B5(51)		9.8–1.4(a)		2.0–0.4	10.3–0.3(b)		2.6–0	10.3–0.0(a)	2.0–0.3(a)
A3, B12(44)	11.4–3.9(a)			2.0–1.0		5.1–1.4(b)	5.3–0		3.6–0.8(b)
A3, B15(62)	1.4–?			4.1–0.3	6.9–1.1	2.5–0.7(a)		6.9–4.0	2.8–0.4(b)
A3, B27	1.4–?				6.9–1.0		1.3–3.4	6.9–0.0	
A3, B35	1.4–?	21.9–1.9(c)		6.1–3.3	3.4–2.2	3.5–1.6	6.6–1.7	3.4–4.0	
A3, B40				0.0–0.7		2.5–1.4			
A3, B47					6.9–0.2(b)			6.9–0.0	
A1, B8	20.0–7.9(b)			8.2–7.3	13.8–10	4.6–8.9	3.9–8.6	13.8–10	7.3–7.9
A1, B17(57)	4.3–?			4.1–1.2			5.3–6.9		3.2–0.6(b)
A2, B7	2.8–?			2.0–5.1	1.4–5.3	3.0–5.0	2.0–0	1.4–2.0	6.5–4.1
A2, B12(44)	10.0–5.1		11.8–11.8	2.0–4.7	6.5–7.3	10.9–11.9	11.8–5.2	6.5–0	6–5.5
A2, B5(51)					3.4–1.8	2.0–2.7		3.4–2.0	0.8–1.8
A2, B15(62)	8.6–4.5	4.8–1.0		2.0–3.1	0.0–6.6	3.0–2.5	2.6–5.2	0.0–6.0	4.4–2.7
A2, B40(60)	2.4–?			0.0–2.8	0.0–4.7	2.3–2.3	1.3–1.7	0.0–10.0	0.8–2
A9, B5			11.8–2.7						
A9, B7	1.4–?			8.2–1.6(a)		2.5–3.0			
A9, B27				6.1–0.6(b)					
A11, B5	1.4–?			0.0–0.5	0.0–0.7	2.5–0.5(a)		0.0–2.0	
A11, B35					0.0–1.4	3.8–1.1(a)	1.3–1.7	0.0–2.0	0.8–2.3
A28, B12(44)	2.8–?			1.3–0.9			2.6–0		2.8–1.4
A29, B12(44)					10.3–0.9(b)	2.5–4.6	1.3–8.6	10.3–4.0	2.8–1.9

Notes:

The studies are classified according to the sampling strategy of control chromosomes (see text). The total number of H and C chromosomes is given in parentheses at the top of each column. To allow comparison between studies, haplotypes reported in recent studies are renamed according to previous broad designations of HLA specificities (the newly designed splits are given in parentheses). The A3 allele frequencies (p) in bold characters are derived from the antigen frequencies (FAg) given by the authors, as $p = 1 - (1 - FAg)^{1/2}$

[a] absolute (or 'uncorrected') frequencies for A3, B7 and A3, B14 haplotypes in all series, and for all haplotypes in the Italian series.

[b] and [c] conditional (or 'corrected') frequencies of the other haplotypes are calculated as in (ref. 6) by removing haplotypes A3, B7 [b] or both A3, B7 and A3, B14 [c] from the analysis. This method, like those referred to in (ref. [41]), detects secondary associations that can be masked by a strong primary association.

Nominal level of significance for the association: (a) $p < 0.05$ or 0.01, (b) $p < 10^{-2}$ or 10^{-3}, (c) $p < 10^{-5}$ to 10^{-7}, (d) $p < 10^{-8}$ to 10^{-10}.

HFE than on control chromosomes (viz. A3,B7; A3, B14; A11, B5; A11, B35) were interpreted as founder *HFE* chromosomes. Among these, the most prevalent (A3, B7) was thought to represent the oldest or ancestral configuration on which a hemochromatosis mutation first occurred. Other *HFE* haplotypes revealed either an increased frequency in patients (A3, B12 and A3, B15), or the same high degree of disequilibrium as in controls (A1, B8; A2, B12; A9, B7; A29, B12). Under the hypothesis of a unique founder mutation, all non-A3 haplotypes bearing *HFE* could be derived from the ancestral one by single historical recombinations either centromeric or telomeric to the HLA-A, B haplotype. Given previous results indicating a closer linkage to HLA-A, it was concluded that *HFE* should be sought in a location telomeric to HLA-A[6].

Geographic distribution of HLA haplotypes

The main HLA haplotypes segregating in various populations of European ancestry are listed in Table 6.2. The studies differ, in essence, by the sampling strategy used to assess the control (or normal) haplotype frequencies. Some authors have derived control haplotypes from the general population; others have applied affected family-based methods that use haplotypes not transmitted to affected subjects as controls. For a given disease group the two methods can give discordant results, a problem encountered by Summer[45] and Milman[46] who both observed less heterogeneity between the distributions of hemochromatosis-associated gene and normal haplotypes when family-based controls were used. In this regard, the Danish data[46] are illustrative, because the overrepresentation of three haplotypes (A3, B5; A3, B47; and A29, B44) was statistically significant only when the disease group is compared to population-based controls (Table 6.2). Such discrepancies may reflect sampling or statistical variations, but also artefacts of unrecognized population stratification inherent in case-control designs[51].

Although the above methodological differences hamper a precise analysis of population genetics, there is substantial evidence for a common HLA chromosomal background with additional geographically specific haplotypes (Table 6.2). The most striking feature is the widespread distribution of A3, B7, the strongest and preferential haplotype of *HFE* world-wide (except in Italy; see below). The A3, B14 haplotype is significantly increased in hemochromatosis patients from Brittany, Utah, and, central Sweden, but is uniformly rare in control subjects. Additional haplotypes in significant disequilibrium with *HFE* can be detected by applying the method of Simon[6]. These include A3 haplotypes carrying B5, B12 or B15 alleles found in different countries, and non-A3 haplotypes that are restricted to specific regions: A1, B8 in central Sweden, A1, B17 in Utah, A11 haplotypes carrying B5 or B35 in Brittany, and, to a lesser extent, A29, B12 in Denmark, A9 haplotypes carrying B7 or B27 in Germany, and A2, B12 in Australia.

The current geographical distribution of HLA markers in Caucasian hemochromatosis patients (Fig. 6.1) fits the hypothesis that the majority of affected chromosomes descended from a remote or a few European ancestor(s)[6, 47]. Numerous additional factors, including recombinations with the original haplotype(s) followed by local founder effects and genetic drift, possibly associated with natural selection, in addition to migrations and mixing of populations could account for the observed variations of haplotypic associations with *HFE*. The haplotype A3, B7 (or at least an association of A3 and B7) is ubiquitous, and thus may represent an ancestral *HFE* chromosome pre-dating migrations and population divergence. The second major *HFE* haplotype, A3, B14, is less commonly distributed, and is thought to be an ancient recombinant introduced into Brittany, Ireland, and England by 'Celtic' colonizations and spread by migrations towards North America. Its high frequency in central Sweden is the result of a founder effect, because most of the A3, 14 chromosomes in apparently unrelated patients have been traced to a common ancestor living eight generations ago[33]. Other *HFE* haplotypes carrying A3 are interpreted as A/B recombinants.

Non-A3 haplotypes are found in limited geographic areas (see above), and are presumed to have arisen from recent mutation(s) or recombination events with ancestral haplotype(s)[6, 45, 46]. The most particular pattern is seen in Italy, where four HLA haplotypes (A3, B7; A3, B35; A3, B5; and A11, B35) are unevenly distributed across the country[28, 52]. The HLA profile of Italian patients is characterized by the presence of A3, B35, the most frequent and primarily associated haplotype (uncorrected frequencies: 21.9% vs. 1.9%, $p < 10^{-6}$) (Table 6.2). This haplotype seems particular to northeastern Italy[28, 30], and accounts for 54% of *HFE*-bearing chromosomes in that region and only 8.1% in other Italian regions[29]. In contrast, a low prevalence of the A3 antigen (39%) and a specific haplotype A9, B21 have been reported in patients originating from southern Italy[52]. This is consistent with the well-known heterogenous genetic background of the Italian population[53] and could support the hypothesis of a second independent mutation, possibly A9 associated.

Association studies with molecular markers from 6p21 to 6pter

Since 1988, numerous association data have been accumulated, attributable in part to the availability of an

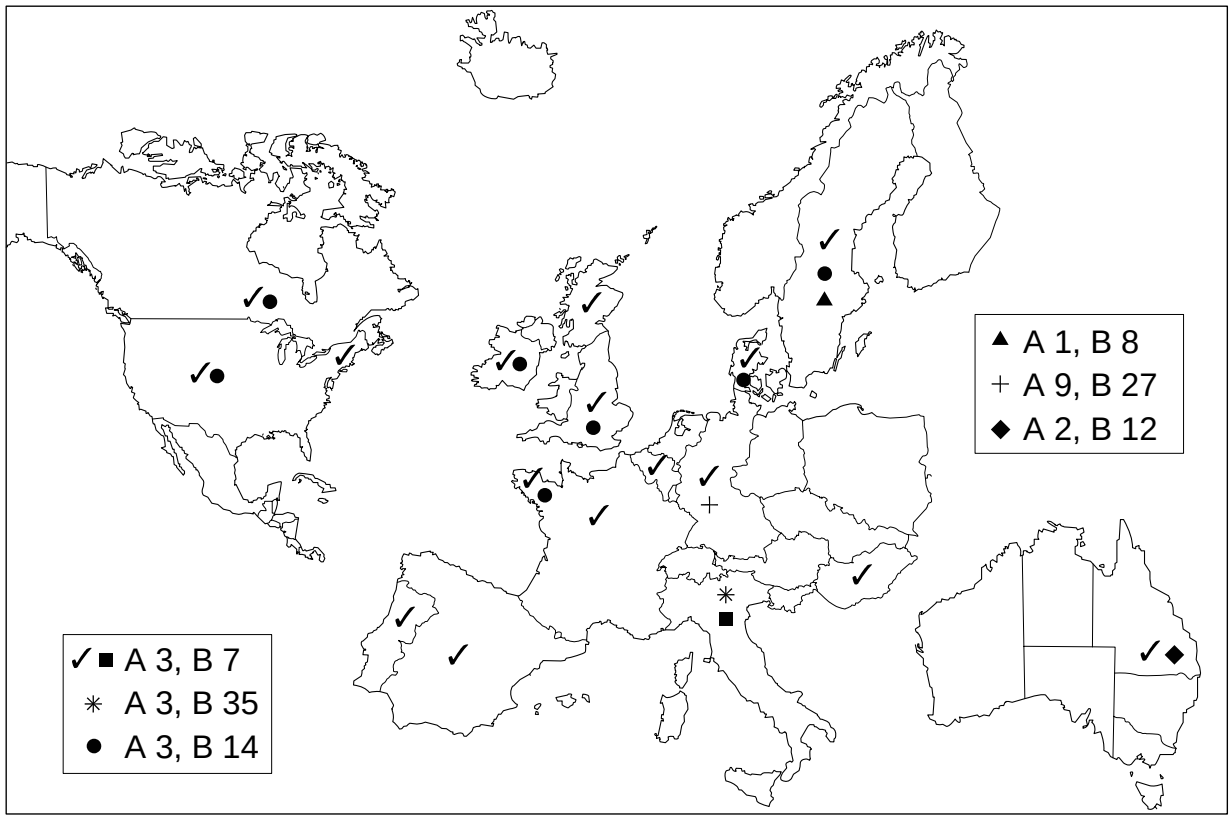

Fig. 6.1. Geographical distribution of the main HLA haplotypes associated with hemochromatosis. Adapted from ref. [43] and updated with data reported in Tables 6.1 and 6.2. Primary (✔, ✳) and secondary (■, ●) associated A3 haplotypes. Secondary associated non-A3 haplotypes (▲, ✚, ◆) with a corrected frequency >10% and/or a higher disequilibrium on *HFE* than on control chromosomes may represent local founder effects.

increasing number of markers within and beyond the telomeric region of the MHC. On the basis of standard linkage analyses placing the disease gene <1 cM from HLA-A[20, 21], molecular studies first focused on the MHC class I region and the rare markers identified at the time, and merely mapped in the vicinity of HLA-A. The unexpected finding of a disease association with one allele at the D6S105 locus[54, 55] and the continued isolation of microsatellite markers from 6p21.3 to 6pter has resulted in a progressive extension of the candidate region towards the telomere and the cloning of a candidate gene situated 4 Mb distal to the MHC class I region[8].

Genotyping with markers of the 6p21.3 region

Initial molecular studies with cDNA and genomic probes of the class I region did not yield substantial information. Most of the restriction fragment length polymorphisms (RFLP) correlated with HLA serological specificities, and none appeared specifically associated with hemochromatosis after adjustment on HLA-A3[56–58]. Further DNA analysis with pulsed-field gel electrophoresis and a panel of probes between TNFa and F13A also revealed haplotype-specific variations, but did not identify a disease-specific pattern[59]. Then, several groups embarked upon a systematic characterization and analysis of markers physically mapped within a region of 2 Mb surrounding HLA-A. These included RFLP loci recognized with anonymous probes (P3A, P3B, P5, i97, i82, 6.7: D6S128), microsatellite loci D6S510 and D6S265, and several genetic markers at HLA-F.

Using the first available RFLP markers covering the 1–Mb interval from P3A (close to HLA-B) to HLA-A, Boretto[60] concluded that *HFE* did not reside within the 900–kb region centromeric to i82 (100 kb centromeric to HLA-A). An extensive study of multilocus haplotypes comprising markers from P3A to HLA-F (250 kb telomeric to HLA-A) confirmed these results and firmly established i82 as the most centromeric marker in disequilibrium with

HFE[61]. On the telomeric side of HLA-A, interpretation of the data was first confused because the HLA-F/Hind III polymorphism initially used was poorly informative and had a predominant allele (F1) in both hemochromatosis and control chromosomes. Moreover, disequilibrium and recombinant haplotype analyses produced two mutually exclusive positions for *HFE*: closer to HLA-A than to HLA-F from the apparent random allelic association of *HFE* and HLA-F, or telomeric to HLA-F if the two specifically *HFE*-associated haplotypes (viz. HLA-A3, D6S128–2, F1 and HLA-A11, D6S128–2, F2) in the Breton population were assumed to be related ancestrally by a unique recombination event[61]. Three independent studies later demonstrated that HLA-F was included in the disequilibrium zone. A strong *HFE* association with HLA-F was found in a case-control study examining the XbaI RFLP pattern[62] and a highly variable locus[63] of the 5' untranslated region of HLA-F, and this observation was confirmed in two family-based association studies using microsatellite markers derived from the 3' end of the HLA-F gene[64, 65].

Despite the strong disequilibrium between *HFE* and markers within the i82 to HLA-F interval, it is now known that the HLA-A subregion does not contain *HFE*[8]. The allelic associations of *HFE* with markers around HLA-A reflect the high degree of disequilibrium between these markers, and mainly result from the high frequency of the most common molecular variant of HLA-A3 (A*0301) on *HFE* chromosomes. As pointed out by Worwood[55, 66] and Dorak[67], HLA-A*0301: (i) is strictly correlated with allele 1 of the microsatellite locus D6S265 (~70 kb centromeric to HLA-A); (ii) is characterized by its association with i82–2 and D6S128–1 in the normal population; and (iii) belongs to the *HFE* ancestral haplotype since homozygosity for its equivalent (D6S265–1) confers a high risk for hemochromatosis (relative risk (RR) = 30.3, 95% CI 9–102). Thus the 'i82–2, D6S265–1, D6S128–1' haplotype defines the invariant configuration of chromosomes carrying HLA-A*0301 rather than a disease-specific genomic segment. Similarly, all alleles involved in the ancestral haplotype extended to HLA-F seem to be associated with *HFE* through their own disequilibrium with each other[61, 65, 66].

Genotyping with microsatellite markers from 6p21.3 to 6pter

Jazwinska[54] was the first to examine the microsatellite marker D6S105 in hemochromatosis. She took as a starting point her genetic analysis of 13 affected pedigrees that mapped the three loci *HFE*, HLA-A and D6S105 within less than 1 cM, and indicated a co-segregation of HLA-A3 and D6S105–8 alleles. Then focusing on cases and controls, she found that positivity for D6S105–8 (in the heterozygous or homozygous state) conferred a higher RR for hemochromatosis than positivity for the A3 antigen (RR = 48.4, 95% CI 18–130, and RR = 4.8, 95% CI 2.4–9.3, respectively), and concluded that D6S105 should be the closest associated marker. This interpretation could appear weakened by the case-control sampling strategy and the use of RRs that sometimes give misleading indications on the strength of association. In this respect, a similar study of Worwood[66] did not distinguish between RR values associated with D6S105–8 and HLA-A3 positivity (RR = 13.0, 95% CI 4.4–30 and RR = 9.1, 95% CI 4–21, respectively), because the confidence intervals overlapped. Alternative methods using family-based controls and the correlation coefficient (termed Δ or r) as a measure of gametic association yielded no more consistent information. D6S105–8 was found either less strongly associated with *HFE* than HLA-A3 ($\Delta = 0.32$, $p < 10^{-5}$ and $\Delta = 0.43$, $p < 10^{-3}$, respectively)[68], or equally strongly associated ($r = 0.453$, $p < 10^{-12}$ and $r = 0.426$, $p < 10^{-12}$)[65]. Such an extent of disequilibrium over a region as large as 3 Mb could appear contrary to the theoretical inverse relationship between disequilibrium and physical distance. However, the HLA-A3 and D6S105–8 associations with *HFE* are mainly attributable to a special haplotype 'A3, 8' that accounts for ~30% of *HFE* chromosomes in Australia[64] and Italy[68], and 50% in Wales[69] and Brittany[65], although it is rare or absent in normal populations.

Although the precise location of *HFE* relative to D6S105 remained questionable from the above results, Raha-Chowdhury[62] suggested that the highest P_{excess} value she obtained at D6S105 (Table 6.3) would indicate the potential residency of *HFE*. In comparison, if different datasets are considered from Australia, Brittany, and Italy in which P_{excess} values can be calculated from the reported allele frequencies at i82 to D6S105 (Table 6.3), similar conclusions could have been reached except in Italy. This emphasizes the potential pitfalls of disequilibrium analyses when applied to populations with a complex history and evolution[42]. However, in many realistic settings, P_{excess} which corresponds to δ[40] is the best estimator of the marker nearest the disease locus. Unlike other measures it is unaffected by the sampling strategy and yields a unimodal pattern with a peak 'at' the disease locus[73]. Its pursued application to newly defined markers spanning the 6.5 Mb region telomeric to HLA -A revealed a progressive increase of P_{excess} values from D6S105 towards D6S1260 (Table 6.3)[71, 72], and even beyond with a peak around D6S2241[8]; a rapid drop towards D6S276 fixed the telomeric limit of the candidate zone[8, 72]. Taken together, the association data consistently indicated a high degree of disequilibrium ($P_{excess} > 0.5$) across a region spanning ~5 Mb from HLA-A

Table 6.3. Allele frequencies on hemochromatosis (H) and control (C) chromosomes and linkage disequilibrium with *HFE* for various markers from i82 to D61260

		Alleles of markers ordered in the direction 6cen-6pter					
Population [study]	Number of H/C chromosomes	I82-2 FD–FP (δ)	D6S265-1 FD–FP (δ)	HLA-A3 FD–FP (δ)	D6S105-8 FD–FP (δ)	D6S464-9 FD–FP (δ)	D6S1260-4 FD–FP (δ)
Family-based controls							
Brittany[61, 65]	151/182	82–63 (**0.52**)(b)		52–14 (**0.44**)(e)	64–19 (**0.56**)(e)		
Australia[64]	64/43		44–3 (**0.42**)(c)	45–3 (**0.43**)(c)	63–9 (**0.59**)(c)		
Italy[70]	38/40	69–48 (**0.40**)	39–7 (**0.38**)(b)		42–12 (**0.34**)(a)		
Population-based controls							
Wales[62]	76/126	74–52 (0.46)(a)	52–10 (0.46)(d)	55–12 (0.50)(d)	78–28 (0.72)(d)		
Wales[71]	136/186		45–12 (0.37)(d)		66–26 (0.54)(e)	70–45 (0.46)(b)	87–47 (0.76)(e)
US[72]	122/88		39–13 (0.30)(b)		54–28 (0.36)(b)	81–60 (0.53)(b)	76–42 (0.59)(c)

Notes:

The marker allele frequencies in the disease (FD) and normal (FP) groups are rounded off to the nearest whole number. δ (= P_{excess} = (FD–FP)/(1–FP)) values not given by the authors are displayed in bold characters.

Nominal level of significance for the association: (a) $p < 0.05$ or 0.01, (b) $p < 10^{-2}$ or 10^{-3}, (c) $p < 10^{-5}$ to 10^{-7}, (d) $p < 10^{-8}$ to 10^{-10}, (e) $p < 10^{-12}$.

to D6S276, but could not define the precise boundaries of the search gene area.

Several authors have expressed doubts about the value of statistical measures of association to infer the location of disease-predisposing genes in terms of genetic and physical distance[42]. The difficulties encountered in positional cloning of the hemochromatosis gene through allelic associations illustrate this issue well. What has hampered a more rapid localization of the gene is the unexpected extent of disequilibrium telomeric to the MHC. There is now convincing evidence that non-uniform recombination rates from HLA-A towards the telomere explain the apparent inconsistencies between physical[8] and genetic mapping data in both normal[74] and hemochromatosis families[21, 54]. In particular, the very high conservation of *HFE* ancestral haplotypes over many megabases supports the possibility that they have remained fixed throughout past generations as a result of cross-over suppression[65], perhaps due to structural variations. These conserved haplotypes include the ancestral haplotype 'HLA-A3, D6S105–8' that comprises invariant alleles at many loci in all the populations studied, and other haplotypes, e.g., 'HLA-A11, D6S105–6' that seems confined to the Brittany population[65]. Such complex patterns obviously complicate simple disequilibrium mapping, because the strength of association of a particular allele is a function of its relative frequency on the relevant haplotype (vs. irrelevant haplotypes), rather than proximity to the actual disease gene[75]. That is why other linkage disequilibrium approaches such as recombinant haplotype mapping may more accurately predict the true position of a gene. This strategy combining the search for a core ancestral haplotype and for historic recombination events has allowed Feder[8] to reduce the critical zone to a 250–kb interval in which a MHC class I-like gene, 'HLA-H' (now defined as *HFE*) that is mutated in 85% of disease chromosomes, was isolated.

Applications of association data in genetic counseling

Since 1977[5], linkage between the disease gene and MHC markers is widely used to identify siblings who are genetically at risk (two haplotypes shared with the proband), or at no risk for the disease (no parental haplotype shared with the proband). Genetic counseling of obligate heterozygotes (i.e., siblings, offspring, and remote relatives sharing one haplotype with the proband) is more complex. Their risk of having hemochromatosis is, on average, equal to the gene frequency in the population but varies from low to high according to the probability of the unshared-haplotype to carry *HFE*. Therefore, knowledge of high-risk haplotypes in each population allows one to detect those obligate heterozygotes who have a higher risk of being *HFE* homozygotes and thus require intensive follow-up. This approach, initially described on a theoretical basis[76] and refined by Simon[22], can be applied to family members originating from any given population where the distribution of marker haplotypes on both normal and disease chromosomes is well defined.

This approach based on population association data may well remain a useful tool for genetic counselors of unaffected relatives of a proband. Indeed, while *HFE* genotyping now provides a direct test for predictive diagnosis in a family setting, there is evidence that the test lacks sensitivity. The C282Y mutation seems strongly specific for hemochromatosis, but the predictive value of the H63D variant is undefined, and 15–25% of disease chromosomes are negative for both mutations in certain areas[8, 77, 78]. Consequently, obligate heterozygotes whose unshared chromosome with the proband does not carry C282Y cannot be considered at no risk for hemochromatosis. In turn, a precise identification of high-risk molecular haplotypes beyond the ancestral one (D6S265–1, HLA-A3, D6S105–8) in each given population could help to identify otherwise unsuspected hemochromatosis genes introduced into the family by spouses. This should improve the accuracy of the prediction and the management of family members, because the molecular defects causing the disease in non-C282Y carriers are unknown.

Conclusions

The cloning of *HFE* is a major advance in the study of hemochromatosis, because direct testing for its major mutation C282Y should facilitate early diagnosis and prevention of the disease in a significant number of persons. Functional studies should soon clarify whether the *HFE*-encoded protein is directly involved in the disease process. The next challenge will be the elucidation of the molecular defect(s) in patients without the ancestral haplotype or in affected persons who are negative for C282Y. The constant observation that *HFE* genotype distribution in patients deviates from that expected under Hardy–Weinberg equilibrium[8, 77, 78] supports locus heterogeneity. Mutation(s) at a second HLA-linked locus, rather than recombinations between A3 and C282Y, could explain some regional disease associations with non-A3 haplotypes that are rare in controls, especially those carrying A11 in Brittany and A9 in Germany (Table 6.2) or southern Italy[52]. The recent suggestion that two susceptibility regions within the

disequilibrium zone from HLA-A to D6S276 may contribute to the phenotype[75] lends support to the possibility that duplications of MHC genomic segments have clustered disease genes on certain haplotypes. Thus, the molecular basis for hemochromatosis may ultimately reveal to be much more complex than previously thought.

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7

Localization, allelic heterogeneity, and origins of the hemochromatosis gene

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Introduction

Hemochromatosis is a common autosomal recessive disorder, with a prevalence varying from 1 in 2000 to 1 in 200 in several European populations[1]. It is characterized by excessive iron absorption. Since the body has no effective way of ridding itself of the excess, the resulting iron accumulation eventually leads to organ damage[2]. There is variable age of onset and clinical heterogeneity. Full expression of the disease is usually seen in men during the fourth or fifth decade of life. Symptoms in women generally do not develop until after the menopause, as iron losses associated with menstruation and childbirth often compensate for the enhanced iron absorption. Although hemochromatosis is generally considered a disease of adults, juvenile and childhood onset have been described as well. Neonatal hemochromatosis is a different disease that is unassociated with the inheritance of hemochromatosis.

Association with HLA antigens

Simon et al.[3] described an association between the HLA antigens A3 and B14, and the hemochromatosis gene (*HFE*), localizing it to the major histocompatibility complex (MHC) on the short arm of chromosome 6 (6p21.3). This association with HLA class I antigens was confirmed by several investigators[4–6]. Pedigree analysis placed the hemochromatosis gene within 1cM of HLA-A[7–9]. Beyond the initial studies linking HLA and hemochromatosis, further progress in defining the critical region was slow because of the lack of informative chromosomal translocations and recombinants, and the lack of ordered polymorphic markers telomeric to HLA-A.

The HLA association confirmed the recessive nature of hemochromatosis and made it possible to trace the inheritance of an affected chromosome within a family once a proband was discovered. The HLA haplotypes linked to hemochromatosis were consistent within a family, but showed inter-family variation. Simon et al.[10] and Edwards et al.[11] reviewed early association studies. In Brittany, the most frequent hemochromatosis haplotypes were HLA-A3, B7 and HLA-A3, B14, but other haplotypes were more common in other parts of Europe. Simon and colleagues[7] proposed that the hemochromatosis mutation was a 'rare if not unique event' that may have occurred on a chromosome carrying the HLA-A3, B7 haplotype (the 'ancestral' haplotype). They suggested that subsequent recombinations involving both HLA-B and (more rarely) HLA-A alleles and population movements produced the varied haplotype associations that were described.

The search for candidate genes

The association with HLA and subsequent localization to human chromosome 6p also spurred the search for regional candidate genes. Attempts to infer the metabolic nature of the hemochromatosis defect, combined with advances in gene mapping suggested several candidate proteins (Table 7.1). Human ferritins, the major intracellular iron storage proteins, are made up of two types of subunits in varying proportions, the heavy (H) subunit of 21 000 MW and the light (L) subunit of 19 000 MW[11]. In liver and spleen ferritin, the L subunit predominates. The H subunit predominates in the more acidic isoferrritins found in heart and red cells. There is about 55% homology between L- and H-subunit sequences. The gene for the L subunit is located on chromosome 19 (19 q13.3–q13.4) and the gene for the H subunit is found on chromosome 11(11q13)[12]. Two ferritin H subunits were discovered on the

Table 7.1. Chromosome location of candidate genes for hemochromatosis[a]

Name	Gene symbol	Map location
Ferritin (H)	FTH1	11q13
	FTHL1-4	1p31-p22; 1q32.2-q42; 2q32-q33; 3q21-q23
	FTHL7,8	13q12; xq26-q28; 5; 8;
	10-16	9; 14; 17; 6p; 11q13
	FTHP1	6p21.3-p12
Ferritin (L)	FTL	19q13.3-q13.4
	FTLLI	20q12-qtr
	FTLL2	Xp22.3-p21.2
Transferrin	TF	3q21
	TFP	3
Transferrin receptor	TFRC	3q26.2-qter
Lactoferrin	LTF	3q21
Iron regulatory binding protein	IREB1	9
Aconitase (soluble)	ACO1	9p22-q32
δ-aminolevulinate synthase 1 and 3 (liver)	ALAS1 and 3	3q21
δ-aminolevulinate synthase 2 (erythroid)	ALAS2	Xp11-q12
Haptoglobin	HP	16q22.1
Hemopexin	HPX	11p15.5-15.4
Ferrochelatase	FECH	18q21.3
Heme oxygenase (inducible)	HMOX	22q12
Heme oxygenase	HMOX2	16p13.3

Note:
[a] From Worwood et al., 1996.

short arm of chromosome 6, but neither is expressed and one, FTHP1, was later mapped to a region centromeric of the hemochromatosis locus[13]. Southern blotting of ferritin H subunits revealed restriction fragment length polymorphisms (RFLP), but none were shown to segregate with the phenotype in affected families[14–16]. Other non-expressed ferritin sequences (especially H subunit sequences) were eventually mapped to 11 other chromosomes.

From a genetic point of view, the hypothesis that the metabolic defect is due to mutations of the transferrin or transferrin receptor gene is also unlikely, because both genes are found on human chromosome 3. The genes encoding the IRE-binding proteins are located on chromosomes 9 and 15 (see Table 7.1), thus eliminating these proteins as candidates for the primary defect in GH. Although advances in the Human Genome Project have been substantial over the last several years, the large remaining gaps in the gene map, and especially in the telomeric portion of the HLA region provided few viable candidates for GH[17].

Mapping of the 6p21.3 to 6p22 interval

Genetic mapping

The critical interval for the hemochromatosis gene was substantially expanded with the analysis of the simple sequence repeat (SSR) marker, D6S105[18], which mapped 1–2 cM telomeric to HLA-A[19–21]. An informative recombinant analyzed with D6S105 supported a position telomeric of the known HLA genes including HLA-F[22]. Lack of additional recombinants in this region hindered the physical ordering of additional regional SSR markers. The precise position of the *HFE* gene relative to D6S105 was unknown, but the telomeric limit was considered to be D6S299 which mapped 2cM telomeric of D6S105[23]. Eventually, an analysis of new markers, including D6S1260[24] suggested that the gene mapped telomeric to D6S105 and possibly telomeric to D6S1260. This work confounded those who still believed that the hemochromatosis gene would be found closer to HLA-A. However, the new data suggested a map location that was at least 3 Mb telomeric of the original candidate search area, near HLA-A.

Physical mapping

As no positional candidate for the disease had been identified and the cellular phenotype was unknown, positional cloning appeared to be the most reasonable approach to identifying the *HFE* gene. For this approach to prove successful the region containing the gene needed to be cloned and mapped in detail. One of the main hindrances to identifying the *HFE* gene was the paucity of markers and mapping resources in the telomeric HLA region. In order to generate additional markers for fine mapping, several groups generated a redundant contig, spanning this region with yeast artificial chromosome (YAC) and bacterial artificial chromosome (BAC) clones[25–27]. From these clones, additional SSR markers were derived and physical locations assigned.

The marker locations shown in Figure 7.1 are based on physical map locations[17, 25] and on information derived from two yeast artificial chromosome (YAC) contigs centered on HLA-F and D6S105[19, 26, 27]. D6S265 mapped within 100 kb and on the centromeric side of HLA-A[8]. Alleles of D6S265 correlate with HLA-A alleles[23]. D6S258 mapped approximately 700 kb telomeric of HLA-F. Markers D6S306, CS3, D6S105, D6S464, D6S1260 (CS5) mapped telomeric to HLA-F. D6S1558 mapped telomeric to, and within a very short distance of D6S1260[28]. In situ hybridization studies of YAC clones[26] provided the following order: CEN-HLA-A-D6S306–D6S1260–pTER. Marker D6S1621 (AFMa207wh2) mapped approximately 600 kb telomeric of D6S1260 andD6S1281, telomeric of D6S1558, and centromeric of

D6S461[28a]. Both D6S299 and D6S461 were genetically mapped approximately 5cM telomeric of HLA-A[29]. The genetic distance between HLA-A and D6S276 is 1–2cM but the physical distance is 6 Mb, confirming earlier observations that this region of chromosome 6 appears to be subject to low rates of recombination[30].

Identification of the *HFE* gene

Feder et al.[27] constructed a YAC and BAC clone contig spanning the region from HLA-A to D6S276. They then used these clones to saturate the region with SSR polymorphic markers for linkage disequilibrium studies, and eventually sequenced a 250kb interval that correlated with a maximum disequilibrium value (for details see Chapter 9 by Wolff et al.). A single base substitution in an MHC class I-like gene was found that consistently segregated with the haemochromatosis phenotype. This mutation, a G to A transition at nucleotide 845 of the open reading frame, resulted in a cysteine to tyrosine substitution at amino acid 282 of the open reading frame (C282Y). Analysis of DNA from unrelated hemochromatosis patients showed that this mutation was present in 85% of all hemochromatosis bearing chromosomes and in 3.2% of controls, consistent with earlier predictions of carrier frequency. A second variant was found elsewhere in the gene, a C to G change in exon 2 resulting in a histidine to aspartic acid substitution at position 63 (H63A). The second mutation was found in 4% of GH patients, who were compound heterozygotes for C282Y and H63A[27]. This HLA class I-like gene was originally dubbed HLA-H, but has since been named, *HFE*[31].

Linkage analysis: *HFE* and markers spanning 6p21.3 to 6pter

Linkage analysis has proven successful in locating genes for several genetic disorders. Linkage is the cosegregation of alleles from distinct markers (or phenotypes) at a frequency that is greater than predicted by random assortment (>50%). It is the contradiction of independent assortment. Markers used for linkage analysis are defined as unique segments of the genome that map to a particular chromosomal location. They can be unique non-coding sequence segments, or unique restriction fragments, or unique coding sequences that define an expressed antigen within a protein. Marker alleles can be sequence length polymorphisms (i.e., SSRs), or restriction length polymorphisms (i.e., RFLPs), or antigenic variations of a protein domain (i.e., HLA seroantigens).

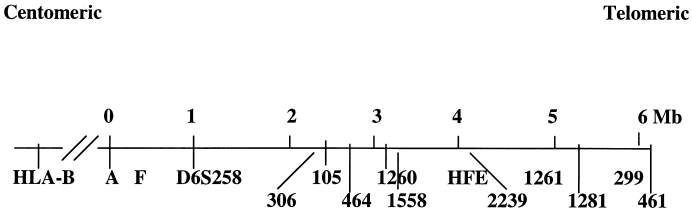

Fig. 7.1. Genetic map of the region analyzed. The most centromeric marker is D6S265 which lies approximately 70 kb telomeric to HLA-A (shown as A). Mapping information from refs. [20, 24, 26, 27, 28a, 71 and 73].

The physical phenomenon that determines the assortment of alleles is meiotic recombination. The greater the distance between markers along the chromosome, the more likely a recombination event can occur between them. This distance is quantified as the recombination fraction, θ, expressed as Morgan units. A recombination fraction of 0.01 (1% = 1centiMorgan = 1cM) is defined as 1 recombination per 100 meioses. A recombination event tends to inhibit others in the vicinity so that recombination fraction and sequence length are not necessarily linearly related over long distances.

The likelihood that an estimated recombination fraction is accurate is dependent on the number of meioses that can be analyzed, or in which phase can be determined. The number of meiosis observed in typical studies does not allow sufficient recombination events to differentiate between loci spaced less than 1 cM from each other[32]. This likelihood is expressed as a lod score, that is, the probability that the markers are linked divided by the probability that they are unlinked, and is recorded as the logarithm of the odds ratio ('lod'). In practice a lod score is calculated at a range of values for the recombination fraction and is plotted producing a curve showing likelihood versus genetic distance (recombination fraction). A lod score of >3 has been arbitrarily set as reasonable evidence for linkage (odds 1:1000), and a lod score of -2 or less excludes linkage[33]. Lod scores are computed using Liped[34], Linkage[35] or similar applications.

Early linkage studies using HLA serotyping, which were the only 6p markers available at the time, showed unusually strong linkage to the HLA-A3 allele, with a cumulative lod score of greater than 60[11]. Later studies using multiple markers including HLA serotypes, DNA restriction fragment length polymorphisms (RFLP), and simple sequence repeat (SSR) markers, suggested that the *HFE* gene mapped to a more telomeric location. Gasparini et al.[9] examined 25 families for a total of 136 subjects including 27 hemochromatosis patients. Individuals were classified under seven liability classes defined by age and transferrin

saturation. The marker loci included HLA-DQ_α, HLA-B, I82, HLA-A, HLA-F, D6S105, D6S109 and D6S89 (CEN ->pTER). Data were analyzed with the MLINK, ILINK and LINKMAP programs. Pairwise linkage showed a maximum lod score of 6.27 for *HFE* vs. HLA-B at a recombination fraction of 0.004. Multipoint linkage analysis gave the most likely position for the *HFE* locus as very close to I82. The authors also described a double recombinant, one occurring between HLA-DQ_α and HLA-B, and the second between HLA-A and HLA-F. They suggested that this placed the boundaries of the *HFE* locus between HLA-DQ_α and HLA-F and supported the results of the linkage analysis. Later however, the double recombinant was retracted. Jazwinska et al.[8] used many of the same markers in the analysis of 13 large pedigrees from Australia: HLA-B, HLA-A, D6S105, D6S109, D6S89 and F13A. No recombinants were found between *HFE* and HLA-A or D6S105 and two-point linkage analysis placed *HFE* within 1cM of HLA-A and D6S105. A multipoint map (HLA-B, HLA-A, D6S109) gave a gene location within 1 cM of HLA-A. The precise location of *HFE*, centromeric or telomeric to HLA-A, could not, however, be determined on the basis of linkage analysis alone because of the lack of recombinants in this region.

Linkage disequilibrium analysis

Linkage disequilibrium analysis can provide a powerful and often rapid approach to locating a disease gene within a few centimorgans[36], and may also be useful for high resolution mapping in isolated founder populations[37, 49]. It was successfully used to narrow the genomic location of the Wilson's disease gene[38], the Batten disease gene[39], and the diastrophic dyslplasia gene[37]. If hemochromatosis was spread by multiplication of a founder mutation through successive generations then linkage disequilibrium analysis could be used to precisely locate the causative gene.

In contrast to genetic linkage, linkage disequilibrium analysis examines allelic associations in populations, not individual families. Linkage disequilibrium holds that among a collection of chromosomes carrying the same ancestral mutation, genetic markers nearest the mutation will recombine less often and thus show the highest degree of allelic association. To apply this method the haplotypes of disease-bearing chromosomes for a dense collection of genetic markers are determined. Then the haplotypes are used to identify a subset of chromosomes that are likely to carry a common ancestral mutation. Among these chromosomes the genetic markers that show the strongest allelic association with the disease will be the closest. In effect, linkage disequilibrium mapping attempts to glean information from all recombination events that have occurred during the history of a population rather than simply those in existing families[37, 40].

Linkage disequilibrium analyses were completed with HLA-B, HLA-A, D6S105, D6S109, D6S89, and F13A by Jazwinska et al.[8]. Surprisingly, that study showed a significant peak of disequilibrium with D6S105, which mapped 1–2cM telomeric of HLA-A. At Cardiff, a larger study was undertaken with more markers and using a new and powerful method for computing likelihood of linkage disequilibrium with markers D6S265, HLA-F, D6S306, CS3, D6S105, D6S464, D6S1260 (CS5), D6S1558, D6S1261, GATAp-19326, D6S461 and D6S299[24, 41, 49]. This new approach described by Terwilliger[42], computes linkage disequilibrium for several polymorphic loci. Haplotypes found on hemochromatosis and control chromosomes were also compared using the same markers[24]. Feder et al.[27] and Ajioka et al.[43] later used similar approaches.

Allele frequencies among UK populations

Individuals with a single allele at any marker were assumed to be homozygous. Allele frequencies for hemochromatosis chromosomes for each marker were calculated for up to 68 unrelated patients (Table 7.2). Controls consisted of 60 blood donors and 15 control families for which only the chromosomes from the parents were included. At every locus except D6S299 there was at least one allele with an increased frequency in hemochromatosis chromosomes[24, 44]. Allele sizes were given in Table 7.3.

Linkage disequilibrium (λ)

The likelihood method of Terwilliger[42] is not limited by the number of alleles at any locus or by the number of markers to be considered in a multipoint test for linkage disequilibrium. Unlike conventional likelihood analyses it is not necessary to reduce the allelic distributions to a diallelic system for analysis. The parameter λ is the proportion of the maximum possible association between a marker and disease, where the frequency of the associated allele is $p+\lambda(1-p)$ where p is the allele frequency in the general population. The likelihood ratio test statistic, -2In[L (HO)/L (H1)], was computed for two-point analysis (each marker and hemochromatosis) and for multipoint analysis using all markers jointly.

The calculated values of λ are presented in Table 7.4. Hemochromatosis chromosomes were compared with the control group of 60 blood donors[44]. All the markers were in significant linkage disequilibrium with the haemochromatosis gene. Our previous report showed the maximum value for λ was 0.74 for D6S1260[24]. At that time there were no telomeric markers close to D6S1260. Values of λ remain

Table 7.2(*a*). Allele frequencies on hemochromatosis (H) and control (C) chromosomes

	D6S265		RF		D6S258		D6S306		CS3	
Allele no.	H	C	H	C	H	C	H	C	H	C
1[a]	60	21	2	3	1	3	5	3	4	10
2	0	0	6	14	2	2	10	23	3	3
3	30	67	4	11	1	6	3	8	90	69
4	10	12	1	16	3	2	13	38	4	6
5	23	50	6	14	8	19	94	84	0	2
6	9	23	9	14	85	74	1	19	1	2
7	2	1	3	11	10	32	0	5	0	1
8	0	1	6	12	2	3			0	5
9	0	1	2	11	0	1			3	20
10			28	5	0	0			1	1
11			22	13	10	36				
12			10	9						
13			12	17						
14			2	8						
15			2	5						
16			2	1						
17			5	12						
18			10	9						
19			0	2						
Total	134	176	132	187	122	178	126	180	106	119

	D6S105		D6S464		D6S1260		D6S299		D6S461	
	H	C	H	C	H	C	H	C	H	C
1	0	2	4	10	0	0	15	22	0	0
2	0	5	3	5	2	3	25	48	0	0
3	4	6	8	28	7	52	17	28	0	1
4	8	13	1	5	113	85	7	26	1	6
5	8	29	1	3	1	24	10	30	14	15
6	21	63	4	10	3	3	8	15	7	5
7	4	14	4	7	0	1	2	5	10	29
8	90	49	8	6	4	10	0	0	67	42
9	0	2	87	72	0	1	2	0	13	13
10	1	2	4	11	0	2			0	1
11	0	0	0	4	0	1			0	3
12	0	1							0	1
Total	136	186	124	161	130	181	86	174	112	116

Note:

[a] See Table 7.3 for allele sizes.

high for D6S1558 and D6S1621 and are reduced for D6S1281. D6S1281 lies approximately 1.2Mb from D6S1260 (Fig. 7.1). The peak of association is between markers D6S1260 and D6S1621. D6S1621 has the strongest association with hemochromatosis ($p < 10^{-15}$) which is the closest marker to the *HFE* gene (approximately 600 kb telomeric).

Linkage disequilibrium was also calculated using P_{excess}, described by Hastbacka et al.[38]. By taking advantage of a founder effect in the relatively stable Finnish population, Hastbacka et al.[40] predicted that the gene causing diastrophic dysplasia should lie within 64 kb of the marker, CSFIR (colony stimulating factor 1 receptor). This prediction proved to be correct. Similarly, if hemochromatosis

Table 7.2(*b*). Alleles on hemochromatosis (H) and control (C) chromosomes (additional markers)

	D6S1558		D6S1621		D6S1281	
Allele no.	H	C	H	C	H	C
1	2	7	96	12	1	2
2	1	5	3	0	0	0
3	141	87	5	12	0	1
4	2	6	8	29	0	1
5	0	1	2	5	43	24
6	6	4	20	53	19	32
7	0	6	0	0	3	3
8	0	1	0	0	0	1
9	0	1	0	0	0	0
10	0	0	0	0	0	0
11	0	0	0	2	0	0
Total	152	118	134	114	66	64

Table 7.3. PCR conditions for the markers used in this study

Locus	Annealing Temp. (°C)	Cycle	GH associated allele no.	Size (base pairs)
D6S265	55	27	1	122[a]
HLA-F(RF)	64	30	10	285[b]
D6S258	62	35	6	199[c]
D6S306	55	35	5	238[c]
CS3	65	35	3	180[a]
D6S105	55	27	8	124[c]
D6S464	54	35	9	206[d]
D6S1260	65	30	4	150[a]
D6S1554	65	30	3	251[a]
D6S1621	54	35	5	286[c]
D6S1281	58	35	5	196[e]

Notes:

[a] Size increases by 2 base points with increasing allele number.

[b] Complex trinucleotide repeat, allele sizes decrease with increasing allele number.

[c] Size decreases by 2 base points with increasing allele number. For D6S258 allele 10 = 191 bp, allele 11 = 181 bp.

[d] Allele 2 is 220 bp then size decreases by 2 base points with increasing allele number.

[e] Complex tetra-nucleotide repeat, allele sizes increases by 4 base points with increasing allele number.

Partial or complete hemochromatosis haplotypes are shown with a 'dot' background and the 'A1–B8' haplotype on a hatched background. At least five consecutive markers must carry the required alleles to define a haplotype. The subtotals refer to haplotypes with D6S265 allele 1, 3, 4, 5 or other.

Table 7.4. Two locus (marker and hemochromatosis) linkage disequilibrium analysis λ for selected markers (see text)

Locus	λ	*p*
D6S265	0.359	2×10^{-9}
D6S306	0.483	4×10^{-6}
D6S105	0.462	2×10^{-7}
D6S1260	0.771	1×10^{-13}
D6S1558	0.725	2×10^{-3}
C282Y	0.901	1×10^{-18}
D6S1621	0.68	1×10^{-15}
D6S1281	0.36	0.05

The patient and control chromosomes are those described in Table 7.2 with a maximum of 59 patients and 60 control subjects. Note that some of the control chromosomes carried the C282Y mutation of the *HFE* gene (7/120).

Table 7.5. Linkage disequilibrium (P_{excess}) in European hemochromatosis patients

Locus	UK $n=160$	Germany $n=64$	Norway $n=66$
D6S265	0.36	0.38	0.23
D6S105	0.54	0.34	0.22
D6S1260	0.77	0.75	0.68
D6S1558	0.72	0.71	0.58
C282Y	0.90	0.94	0.73
D6S1621	0.68	0.63	0.40
D6S1281	0.36	0.20	0.18

Controls were selected from 120 unaffected family members from above countries.

was spread by multiplication of a founder mutation through successive generations, then a similar analysis may be valuable in locating the gene[23] (Table 7.5). $P_{Excess} = (P_{affected}–P_{normal})$ / $(1–P_{normal})$ in which $P_{affected}$ and P_{normal} denote the frequency of the disease-associated allele on disease-bearing and normal chromosomes, respectively[46]. Raha-Chowdhury et al.[23] calculated P_{excess} for several markers from HLA-B to D6S105 but could not localize the *HFE* gene in this haplotype. Although the value of P_{excess} for D6S105–allele8 was 0.72 compared with 0.46 for HLA-A3, the 95% confidence interval (CI) overlapped. Later this group again showed that P_{excess} for CS5 (D6S1260) was 0.75 (95% CI: 0.61–0.84). This finding suggested for the first time that the *HFE* gene lies telomeric of D6S105. Table 7.5 lists P_{excess} values for GH patients from the UK, Germany, and Norway.

Feder et al.[27] isolated a total of 29 new simple sequence repeat markers, which they used to identify a peak of linkage disequilibrium in hemochromatosis patients from the USA. The maximum value for P_{excess} was 0.81, suggesting that over 80% of disease bearing chromosomes carry a common mutation. The marker that gave this maximum value, D6S2239, mapped over 1Mb telomeric of D6S1260, the marker that had previously been shown to be closest to the gene with a P_{excess} of 0.75[24].

Haplotype analysis

Haplotypes were constructed by analysis of pedigrees (Table 7.6(*a*)). Control chromosomes were those from spouses of patients where it could be shown that they were not associated with inheritance of hemochromatosis in the children. Control chromosomes were also obtained from 15 control families (Table 7.6(*b*)) but it should be noted that these families were not screened for hemochromatosis and there is a 5% chance that any chromosome carries the *HFE* gene[24].

The HLA-A1–B8 haplotype

The haplotype HLA-A1–B8 is very frequent in Northern European populations[47]. This may be due to both population migration and a selective pressure. A haplotype extending from HLA-B8 to D6S1260–3 was found on several control and haemochromatosis chromosomes carrying HLA-B8 and HLA-A1: HLA-B8, D6S265–3, D6S258–11, D6S306–4, CS3–9, D6S105–8, D6S464–3, D6S1260–3 (Table 7.6(*a*) and 7.6(*b*)). These associations were confirmed by the analysis of linkage disequilibrium in 60 blood donors[44].

A hemochromatosis specific haplotype

Haplotypes were constructed from 40 hemochromatosis families (Table 7.6(*a*)) as well as the 15 control families (Table 7.6(*b*)). With the 8 markers on the centromeric side of D6S1260 and D6S1558 (which show maximum values of λ), there are over 6×10^9 possible haplotypes. The following loci are in significant positive linkage disequilibrium with hemochromatosis: D6S265–1, HLA-A3, D6S258–6, D6S306–5, D6S105–8, D6S464–9, D6S1260–4, D6S1558–3, C282Y, D6S1621–5 and D6S1281–5. Thirty out of a total of 100 hemochromatosis chromosomes carry this exact haplotype (Table 7.6(*a*)). These vary at the highly polymorphic HLA-F locus[44], which has therefore not been included in this analysis. There is no example of this haplotype among the control chromosomes. Forty-one hemochromatosis chromosomes out of a total of 100 carry the haplotype from D6S265–1 to D6S1621–5. Again, there are no examples among the control chromosomes. The haplotype D6S258–6, D6S306–5, D6S105–8, D6S464–9, D6S1260–4, D6S1558–3, C282Y and D6S1621–5 occurs on 57 chromosomes, with none among the control chromosomes. Sixty-eight chromosomes carry D6S464–9, D6S1260–4, D6S1558–3 and D6S621–5 with three examples among the control chromosomes. The only other haplotypes which occur more than once are either a variant of the 'common' haplotype (6–5–5–9–4–3 *HFE*-5, Table 7.6(*a*), No. 43) or an exact copy of the HLA-A1–B8 haplotype (3–1–11–4–8–3–3–3 *HFE*-5) of which there are 4 hemochromatosis examples (Table 7.6(*a*), Nos. 26–28) as well as 5 control chromosomes (Table 7.6(*b*)).

The only haplotypes found more than once were variants of the HLA-A1–B8 extended haplotype which includes D6S265–3, D6S258–11, D6S306–4, D6S105–8 and D6S1260–3[44]. There were 6 copies of a haplotype including all these alleles (Table 7.6(*a*), Nos. 29–32) and 5 of the 6 had D6S1621–5. There were three other haplotypes that differed only by having D6S258–6 instead of allele 11 (Table 7.6(*a*), Nos. 16–18). Haplotypes No. 35 and No. 36 (Table 7.6(*a*)) may also be derived from the HLA-A1–B8 haplotype. Thus the HLA-A1–B8 haplotype extends almost to the *HFE* gene. For the control chromosomes ($n = 74$) 72 different haplotypes were identified. Some of the normal families were not screened for hemochromatosis and 6 of the 83 chromosomes originally studied also carried the C282Y mutation including two HLA-A1–B8 haplotypes (data not shown). The frequency of the C282Y mutation in the general UK population is approximately 8%[47, 48] so this finding is not surprising. These chromosomes have not been included in Table 7.4 (linkage disequilibrium analysis. Thus, when haplotypes extending about 2 Mb around the *HFE* locus (D6S464–D6S1621) are examined, over 68% of chromosomes from patients are haploidentical. However, it is difficult to compare the haplotypes described in earlier papers with the 'ancestral' haplotype described by Feder et al.[27], as different markers and a different description of alleles were used. Here an extended haplotype analysis is provided including the *HFE* gene and markers covering about a 5 Mb span of DNA in order to provide data from the UK which may be of value in comparing hemochromatosis haplotypes in various countries. The various historical recombinations are also discussed that have introduced new alleles into the ancestral haplotype.

Table 7.6(*a*). Hemochromatosis haplotype

Haplotype	265	HLA-A	258	306	105	464	1260	1558	C282Y	H63D	1621	1281	No. haplotype	Subtotal
1	1	3	6	5	8	9	4	3	2	1	5	5	30	
2	1	3	6	5	8	9	4	3	2	1	5	6	5	
3	1	3	6	5	8	9	4	3	2	1	5	4	4	
4	1	3	6	5	8	9	4	3	2	1	5	1	1	
5	1	3	6	5	8	9	4	3	2	1	5	8	1	
6	1	3	6	5	8	9	4	3	2	1	9	6	1	
7	1	3	6	5	8	9	4	3	2	1	10	4	1	
8	1	3	6	5	8	9	4	3	2	1	8	5	2	
9	1	3	6	5	8	9	4	3	2	1	10	6	2	
10	1	3	6	5	8	9	4	3	2	1	4	5	2	
11	1	3	6	5	5	9	4	3	2	1	5	4	1	
12	1	3	6	5	8	7	8	3	2	1	6	5	1	
13	1	3	6	2	7	3	4	3	2	1	8	6	1	
14	1	3	5	4	8	9	4	3	2	1	10	7	1	
15	1	3	11	5	8	9	4	3	2	1	10	6	1	
														n = 54
16	3	1	6	5	8	8	4	3	2	1	5	5	3	
17	3	1	6	5	8	9	4	3	2	1	5	4	2	
18	3	1	6	5	8	9	4	3	2	1	5	6	2	
19	3	1	6	5	6	9	4	3	2	1	5	7	1	
20	3	1	6	5	5	9	4	3	1	1	6	4	1	
21	3	1	6	4	6	7	4	3	2	1	8	6	1	
22	3	1	7	5	6	9	4	3	2	1	5	5	1	
23	3	1	7	5	6	1	4	3	2	1	10	6	1	
24	3	1	7	5	8	9	4	3	2	1	10	5	1	
25	3	1	11	5	8	9	4	3	2	1	5	5	2	
26	3	1	11	4	8	3	3	3	2	1	5	6	2	
27	3	1	11	4	8	3	3	3	2	1	5	5	1	
28	3	1	11	4	8	3	3	3	2	1	5	4	1	
														n = 19
29	4	11	6	5	8	9	4	3	2	1	5	6	1	
30	4	11	6	5	8	9	4	3	2	1	5	5	1	
31	4	11	6	5	8	9	4	3	2	1	10	6	2	
32	4	11	6	5	8	9	4	3	2	1	9	6	1	
33	4	11	7	5	8	9	4	3	2	1	5	5	1	
34	4	11	7	5	5	9	4	3	2	1	5	4	1	
35	4	11	7	2	4	9	4	3	2	1	10	6	1	
														n = 8
36	5	2	3	5	5	9	4	6	2	1	5	6	1	
37	5	2	5	5	6	9	4	3	2	1	5	6	2	
38	5	2	5	5	6	8	4	3	2	1	5	6	1	
39	5	2	6	5	8	9	4	3	2	1	5	5	2	
40	5	2	7	1	3	8	2	3	2	1	4	6	1	
41	5	2	7	5	6	9	4	3	2	1	5	7	1	
42	5	28	6	5	8	9	4	3	2	1	5	6	1	
43	5	28	6	5	5	9	4	3	2	1	5	3	2	
44	5	29	6	5	8	9	4	3	2	1	5	5	2	
45	5	30	6	5	8	9	4	3	2	1	10	6	1	
46	5	24	5	5	6	8	4	3	2	1	4	6	1	
														n = 15
47	6	30	6	2	4	9	4	3	2	1	5	5	1	
48	31	6	5	8	9	9	3	3	2	1	10	4	1	
49	6	X		2	6	9	4	3	1	1	10	6	1	
50	7	25	7	5	8	9	4	3	2	1	10	8	1	
														n = 4
													Total = 100	

■ An ancestral A3 haplotype or a partial haplotype is conserved in all GH patients.
■ A second haplotype A1B8 is present in patients and in controls.

Table 7.6(*b*). Control haplotype

Haplotype	265	HLA-A	258	306	105	464	1260	1558	C282Y	H63D	1621	1281	No. haplotype	Subtotal
1	1	3	1	5	5	9	4	6	1	1	5	5	1	
2	1	3	1	2	8	10	4	6	1	1	10	4	1	
3	1	3	5	6	4	9	3	7	1	1	10	4	1	
4	1	3	5	8	5	5	4	7	1	1	10	4	1	
5	1	3	5	5	9	4	3	10	1	1	10	5	1	
6	1	3	6	5	8	9	4	6	1	1	10	5	1	
7	1	3	6	5	8	9	4	3	1	1	10	6	1	
8	1	3	6	1	5	9	4	3	1	1	5	6	1	
9	1	3	11	5	6	1	10	1	1	1	8	6	1	
10	1	3	X	4	4	3	3	3	1	1	5	5	1	$n=10$
11	3	1	8	5	6	10	6	3	1	1	5	6	1	
12	3	1	2	4	6	9	6	3	1	1	5	5	1	
13	3	1	5	4	2	10	5	4	1	1	8	6	1	
14	3	1	5	4	6	1	2	5	1	1	5	6	1	
15	3	1	6	4	8	5	4	3	1	1	10	5	1	
16	3	1	6	4	8	3	3	3	1	1	10	5	1	
17	3	1	6	4	8	3	3	3	1	1	5	5	1	
18	3	1	6	5	8	3	3	3	1	1	8	7	1	
19	3	1	6	4	6	10	4	3	1	1	8	7	1	
20	3	1	7	5	8	3	7	3	1	1	X	X	1	
21	3	1	7	6	5	1	4	1	1	1	10	5	1	
22	3	1	7	5	6	X	6	3	1	1	10	5	1	
23	3	1	7	2	7	9	3	1	1	2	5	1	1	
24	3	1	7	4	7	10	4	2	1	1	10	5	1	
25	3	1	7	5	8	3	7	3	1	1	10	4	1	
26	3	1	11	5	6	9	4	3	1	1	5	4	1	
27	3	1	11	6	8	9	4	3	1	1	5	4	1	
28	3	1	11	4	5	9	4	3	1	1	5	5	1	
29	3	1	11	4	8	3	3	3	1	1	5	4	2	
30	3	1	11	4	8	3	3	3	1	1	5	5	1	
31	3	1	11	4	8	3	3	3	1	1	5	7	2	
32	3	1	11	7	8	3	3	3	1	1	10	3	1	
33	3	1	11	2	8	9	3	3	1	1	10	4	1	
34	3	1	11	6	7	7	8	1	1	1	10	6	1	
35	3	1	11	6	5	3	3	3	1	1	8	6	1	
36	3	1	11	3	5	3	3	3	1	1	5	5	1	
37	3	1	11	5	3	6	4	7	1	1	8	5	1	$n=29$
38	4	11	5	5	5	9	5	4	1	1	5	7	1	
39	4	11	6	4	4	10	4	2	1	1	10	1	1	
40	4	11	6	5	6	9	4	1	1	1	10	6	1	
41	4	11	6	2	6	6	6	3	1	1	10	5	1	
42	4	11	7	X	1	1	4	4	1	2	8	7	1	
43	4	11	7	11	6	1	5	3	1	1	10	5	1	
44	4	11	11	5	3	X	3	3	1	1	6	4	1	$n=7$
45	5	24	1	2	6	9	4	1	1	1	8	5	1	
46	5	2	5	2	5	1	4	1	1	1	4	5	1	
47	5	2	5	6	5	7	4	3	1	1	6	7	1	
48	5	2	5	1	8	7	4	6	1	1	10	4	1	
49	5	2	6	4	1	1	4	4	1	1	5	6	1	
50	5	2	6	5	6	4	4	4	1	1	10	6	1	
51	5	2	6	4	5	9	4	1	1	1	10	6	1	
52	5	2	6	5	3	9	4	1	1	1	10	4	1	
53	5	2	6	5	4	6	4	3	1	1	10	6	1	

Table 7.6(*b*). (*cont.*)

Haplotype	265	HLA-A	258	306	105	464	1260	1558	C282Y	H63D	1621	1281	No. haplotype	Subtotal
54	5	2	6	5	8	9	4	6	1	1	10	6	1	
55	5	2	6	3	6	7	5	3	1	1	10	6	1	
56	5	2	7	3	5	9	9	3	1	1	5	5	1	
57	5	2	7	5	5	2	8	3	1	1	8	4	1	
58	5	28	8	5	6	9	5	7	1	1	8	4	1	
59	5	28	11		2	7	3	3	1	1	8	7	1	
				2										*n* = 15
60	6	29	5	6	6	9	4	1	1	1	10	1	1	
61	6	29	6	5	8	4	4	3	1	1	10	1	1	
62	6	30	7	7	6	8	3	3	1	1	10	6	1	
63	6	30	7	5	8	6	4	5	1	1	8	5	1	
64	6	30	7	5	5	3	5	8	1	1	4	4	1	
65	6	31	9	5	5	9	5	8	1	1	10	4	1	
66	6	31	9	5	6	9	5	4	1	1	9	6	1	
67	6	26	11	6	4	10	5	3	1	1	8	5	1	
68	6	25	11		5	8	10	3	1	1	6	5	1	
				5										*n* = 9
69	7	23	5	4	4	9	5	3	1	1	10	5	1	
70	8	23	6	5	6	9	3	1	1	1	10	7	1	
71	8	24	11	5	8	9	3	1	1	1	5	6	1	
72	9	24	11		5	10	5	3	1	1	6	7	1	
														n = 4
													Total = 74	

■ An ancestral A3 haplotype or a partial haplotype is conserved in all GH patients.

Allelic heterogeneity and C282Y mutations within the hemochromatosis haplotype

Jazwinska et al.[8] and Worwood et al.[1] demonstrated that the telomeric marker D6S105 also shows a highly significant allelic association with hemochromatosis. D6S105 is located approximately 2.5Mb from HLA-A towards the telomere[20]. Allele frequencies and haplotypes have now been examined for HLA-A, D6S105 and several intervening markers for families with GH from Queensland[50], the UK[23,24], Brittany[51], Italy[52] and the USA[28]. Several measures of linkage disequilibrium have been applied. In Queensland and the UK, D6S105–8 is more strongly associated with hemochromatosis than HLA-A3 (or the tightly associated marker allele D6S265–1), but in Brittany and the USA, D6S105–8 and HLA-A3 show a similar strength of association. In Italy, D6S105–8 shows less association than HLA-A3 or D6S265–1. In all populations studied, there is a predominant haplotype on hemochromatosis chromosomes involving D6S265–1, HLA-A3 and D6S105–8. In 1996, Feder et al.[27] identified a strong candidate gene. This is an HLA class I-like gene, originally called HLA-H but more properly named *HFE*[31], that is located approximately 4.5 Mb telomeric of HLA-A. In the USA, about 85% of chromosomes from patients with hemochromatosis carry a point mutation of the gene at amino acid 282 (C282Y) and about 83% of patients with haemochromatosis are homozygous for this mutation. The frequency of the mutation in the general population is 0.03. Subsequently, both epidemiological and functional studies confirm that this is the causative mutation in most patients. The mutation is confined to populations of European origin[47] and from 68 to 100% of patients have been found to be homozygous for C282Y. The highest value (100%) was reported for Queensland, Australia[50] and the lowest (68%) for Italy[53]. Intermediate values were reported for the UK (91%)[48], Brittany (92%)[54], the USA (83%)[55] and France (80%)[56]. Rhodes et al.[57] reported an interesting family where homozygosity for the C282Y mutation was detected in an asymptomatic male sibling, 50 years of age, whose genotype was identical to the proband. The finding of an asymptomatic homozygous C282Y subject, haploidentical to affected sibs, indicates that clinical expression of symptomatic disease is variable, even in a middle age C282Y homozygote. However, whether or not a second more frequent mutation (H63D) causes any significant degree of iron overload remains unclear.

Table 7.7. The ancestral haplotype observed in hemochromatosis and control (C) chromosomes

	No. of chromosome	
Haplotype	GH ($n=100$)	C ($n=100$)
A.1 The ancestral haplotype observed in HLA-A3 patients		
1–10–**6–5–8–9–4–3–2–5**	54	0
Several different non-A3 haplotype often found in GH patients		
B. Haplotype observed in HLA-A1 patients		
3–13–**6–5–8–9–4–3–2–5**	7	0
3–13–11–**5–8–9–4–3–2–5**	2	0
C. Haplotype observed in HLA-A1-B8 subjects		
3–13–11–4–8–3–3–**3–2–5**	10	6[a]
D. Haplotype observed in HLA-A11 subjects		
4–18–**6–5–8–9–4–3–2–5**	8	0

Note that there are several changes in the ancestral haplotypes shown in Table 7.6. Haplotype B is found with HLA-A1 and the D haplotypes are observed in HLA-A11 patients. Haplotype C is the HLA-A1-B8 haplotype. [a]HLA-A1-B8 is present in six control subjects who did not have the C282Y-2 mutation . All six controls were C282Y-1 allele.
The marker order is D6S265-RF-258-306-105-464-1260-1558-C282Y-D6S1621.

The ancestral haplotype: origin of the first mutation

The haplotype studies above suggest that hemochromatosis is associated with a strong founder effect. Several authors have speculated about an ancestral chromosome where the first mutation may have occurred. Simon et al.[10] suggested that haemochromatosis is found only in populations of Northern European origin, and that its distribution could be explained by the migration of the Celts. Olsson and colleagues studied 50 unrelated patients from central Sweden[10] and found that the frequency of the HLA-A3 and B14 antigens was significantly higher than in controls. Milman et al.[6] studied 70 unrelated Danish hemochromatosis patients and found very high frequencies of A3 (80%), B7 (60%) and B14 (10%), especially in comparison to the Swedish study[5]: A3 (66%), B7 (40%) and B14 (22%). This prompted Milman to conclude: 'The geographic distribution pattern of HLA-antigens suggests a common ancestral source of the haemochromatosis gene, which might have originated in the Danish area and subsequently been spread through the conquering and settling activities of the Vikings, who extended their settlements both towards the north and east to Norway and Sweden towards the Southwest to Brittany and Normandy, and towards the east of England'[6].

Why should a gene that appears to have originated from a single mutation spread throughout Northern Europe? Is there any heterozygous advantage? Were population migrations responsible for spreading the mutation? The genetic analysis of patients with hemochromatosis from various populations may reveal valuable information about the number of different mutations causing the disease and the origin of the disease. An extended haplotype was identified that represents the ancestral haplotype, in which the mutation may have occurred: D6S265-RF-D6S258-D6S306-CS3-D6S105-D6S464-D6S1260-D6S1558-C282Y-D6S1621 (1-10-6-5-3-8-9-4-3-2-5; Table 7.7). This haplotype is present in 54% of hemochromatosis chromosomes but no control chromosomes. A haplotype involving D6S306-5, D6S105-8, D6S464-9, D6S1260-4, D6S1558-3 and C282Y-2 was found in 62% of *HFE* chromosomes but only 1% of control chromosomes. Changes in the different haplotypes were also shown (Table 7.7: A, B, C, D). Haplotypes A and B are very similar and represent a cross-over event changing D6S265-1-RF-10 (the HLA-A3 haplotype) to D6S265-3-RF-13 (the HLA-A1 haplotype). In the case of haplotype D, D6S265-1-RF-10 changed to D6S265-4-RF-18. This haplotype is found in HLA-A11 subjects. The C haplotype is very different from the rest of the hemochromatosis haplotypes, and is the HLA-A1–B8 haplotype that is also found in control chromosomes. This may be another ancestral GH haplotype that reaches its highest frequency of 12.1% in Scandinavia[46], followed by the Celts (Scottish, Welsh and Irish) with a frequency of 11%[44]. Most of these chromosomes have the common D6S1260-4, D6S1558-3, C282Y-2 alleles (note control chromosomes C282Y-1, instead of C282Y-2).

In summary, about 70% of hemochromatosis chromosomes are haploidentical from D6S306 to D6S1621 and

may carry the same mutation (Table 7.7). Another 5% may carry a different mutation associated with the common, Northern European HLA-A1–B8 haplotype. Patients in Sweden, Germany and Norway share the same ancestral haplotype (HLA-A3, D6S105–8, D6S1260–4, C282Y-2) as that found in the UK[58–60]. A detailed analysis of hemochromatosis haplotypes may provide information about the geographical spread of the mutation.

Organization of the *HFE* gene

Feder et al.[27] identified an MHC class 1-like gene, originally dubbed 'HLA-H' and later, *HFE*. The high degree of sequence similarity with other HLA class 1 molecules suggests structural homology. The crystal structure of several class 1 molecules is known and extrapolation to HLA-H suggests some interesting features. Class 1 molecules interact non-covalently with beta2-microglobulin. The groove formed by the α1 and α2 domains is responsible for peptide binding in class I molecules. *HFE* differs from other class I proteins in that two of the four tyrosine residues forming the classical class I groove are missing and there is a proline side chain blocking one end of the groove. These features are found in a related molecule, the Fc receptor (hFcRn), which forms a heterodimer with beta2-microglobulin and lacks a peptide binding groove, interacting with an altogether larger molecule, IgG.

The His63Asp mutation is predicted to occur in the α1 domain located on the loop between the third and fourth β strands of the peptide binding domain. There are four conserved cysteine residues in class I molecules and the second forms a disulphide bridge giving rise to the Ig like α3 domain, which interacts with beta2–microglobulin. The common Cys282Tyr mutation loses one of these conserved residues and is predicted to inactivate the protein by preventing correct folding of the α3 domain and hence interfere with its interaction with beta2–microglobulin. Beta2–microglobulin deficient mice display a progressive iron overload[61], although the mechanism by which this occurs has not been defined. These mice have been proposed as an animal model for hemochromatosis[62].

Age of mutation and conclusion

The discovery of the *HFE* gene has already provided a valuable diagnostic tool and will advance our understanding about the control of iron absorption. Moreover, knowledge of the gene, the physical map of the immediate region and haplotypes found on chromosomes carrying the C282Y mutation will make it possible to estimate the time when the first mutation occurred and to follow the spread of the gene throughout Europe and other parts of the world.

The localization of the *HFE* gene by Feder et al.[27] following earlier linkage disequilibrium and haplotype analyses provides a good example of the power and limitations of both linkage disequilibrium and high resolution haplotype analysis. Although linkage disequilibrium analysis provided a clear indication that the gene was located in a 1 Mb region considerably telomeric of HLA-A, the variability of the values of P_{excess} obtained for adjacent markers reduced the ultimate power of the analysis. A study of closely spaced markers on hemochromatosis chromosomes provided a more precise guide to the gene location. However it is difficult to compare the haplotypes described in earlier papers with the 'ancestral' haplotype described by Feder et al.[27], since different markers and a different description of alleles were used. The markers applied here will make it possible to compare results of earlier studies with new studies of hemochromatosis and control haplotypes. Linkage disequilibrium analysis should provide the necessary data to calculate the time of the original ancestral mutation using the formula $\lambda = \alpha(1-\theta)^n$ where α is the proportion of hemochromatosis-bearing chromosomes traceable to the common ancestor, θ is the recombination rate between the *HFE* locus and the marker, and n is the number of generations since the occurrence of the ancestral mutation.

Unfortunately, the variability of λ values and the conserved haplotype on the centromeric side of the *HFE* gene mean that available data do not permit a valid estimation of n. The analysis only applies to an isolated population and neither the USA nor UK data fit this condition. Ajioka et al.[43] estimated the recombination rate between *HFE* and HLA-A (6 recombinants in 433 informative meioses) and calculated the age of the *HFE* mutation at 69 generations (95% CI 27–161). A value of 69 generations would place the original mutation at about 300 AD – after the Celtic migrations (up to 500 BC) but before the Viking invasions of much of Europe's coastal areas. Olsson and Ritter[63] proposed that Viking excursions were responsible for the spread of the *HFE* gene and that calculation is compatible with this proposal. However, using the same formula as that applied by Ajioka et al.[43] and the recombination rate between HLA-A and *HFE* calculated by Malfroy et al.[64], a best estimate of 113 generations (2800 years) is obtained. This would be more compatible with a Celtic origin.

The principal value of the haplotypes presented here is that, in combination with the analysis of the *HFE* mutations it will be possible to trace the spread of the mutation through Europe. In 1987 Simon and colleagues described

the hemochromatosis gene as a mutation originating on a chromosome carrying the haplotype HLA-A3 and B7. This mutation would have spread through successive generations with recombinations replacing HLA-B7 and, less commonly, HLA-A3 with other alleles. Even this limited analysis permitted some speculation about the origin of hemochromatosis. Simon et al. suggested that it may have originated in Germany and spread throughout Europe with mass migrations of the Celtic people. As the Celts were themselves displaced to the western fringes of Europe so hemochromatosis became more concentrated in these areas. There have been further founder effects that have brought the gene to South Africa[65] and Queensland, Australia[50]. However, initial calculations of the age of the mutation do not provide strong evidence for a Celtic origin. The archaeological evidence about a mass, Celtic, population migration is not impressive[66]. The old haplotypes, and the historical recombinations revealed in them, will form a useful way of comparing hemochromatosis chromosomes from various European populations and tracing the spread of the gene through the continent. Of particular note is the region between HLA-A and D6S258. Twenty chromosomes show evidence of historical recombination in this region (Table 7.6(*a*)). Patients in Sweden, Germany and Norway share the same ancestral haplotype (HLA-A3, D6S105–8, D6S1260–4) as that found in the UK[58–60]. From these three studies and our English, Scottish, Irish and Welsh populations, we have identified 23 mutations in 730 meioses. This finding suggests that the *HFE* mutation first occurred 200–250 generations ago, roughly at 4300 BC. A great change took place about 4000 BC (the Neolithic revolution). Klitz et al.[67] described a portion of the variation in allele frequencies from 26 polymorphic loci, including HLA-A and HLA-B, in populations sampled across contemporary Europe. Variations in allele frequencies have been correlated with archeological dates for the first appearances of agriculture in its spread across Europe from an origin in the near East 10 500 years ago[68]. It is implied that the change in the allele frequencies in the genetic systems, including HLA-A and HLA-B, correlate with the spread of agriculture. It can be explained by a simple function of admixture rates coincident with the dilution of Neolithic peoples into the Mesolithic European natives[69].

Neolithic behavior is, of course, not in the genes, but its practice must have genetic consequences for the practitioners. The agricultural revolution resulted in a sharp rise in population densities, which in turn supported a whole new suite of pathogens. Also accompanying the switch to farming is radical dietary change highlighted by greatly increased uniformity and low diversity of foods. Both of these aspects of the Neolithic environment would be expected to create conditions for the differential survival of individuals based on their genetic makeup. The hemoglobinopathies, adult lactase expression and even hemochromatosis are responses of human populations to these new environments. Evolution of HLA alleles might also be expected here. Could variation in HLA allele frequencies track both the movement of agriculture across Europe and balance selection in response to pathogens and possibly other causes? The low correlation coefficients of associations of HLA with the origin of agriculture make it clear that sufficient HLA variability is present (i.e., in the unexplained variation) to record selection pressures over the intervening 2–400 human generations during which agriculture has existed in Europe[67].

It may suggest that most of the Swedish GH patients have ancestors from the west coast of Sweden[63]. An ancestral GH mutation may have originated in a normal HLA-A3 haplotype some where in the West Coast of Sweden, mainly from the former Norwegian county of Boleustan[70]. This original HLA-A3 haplotype may have been present long before the Viking invasions. The people living on the West Coast of Sweden interbred more often and this haplotype became frequent in their population. The Viking invasion throughout Northern Europe would then explain the distribution of the hemochromatosis gene.

Ireland suffered 26 Viking attacks during the first period of 25 years until 830 AD. A Rune-stone reveals an Irish–Scandinavian marriage from 850. Second generation Vikings were given Celtic names[72]. This may be the reason why in the Irish population the haplotypes HLA-A3–B7 and HLA-A1–B8 are common[46].

Simon et al.[10] described hemochromatosis as a 'Celtic disorder'. Brittany suffered the effects of the Viking raids in a similar fashion to the empire (Louis the Pious), with fleets fighting at various times against the Bretons or in alliance with them in attacks on the Franks (914–936). Brittany was particularly unfortunate in that the main base of the Loire Vikings was situated at the mouth of the river on the island of Noirmoutier. This was the site of the once prominent monastery that was destroyed by the Scandinavians early in the ninth century, close to the Breton capital at Nantes. To prove this hypothesis of a 'Viking gene' it is necessary to study the various Scandinavian and other European populations. Certainly the widespread invasions by the Vikings provides more opportunity for distribution of the gene than the much more limited movement of the Celts. The strikingly high prevalence of the hemochromatosis gene appears to be due to an initial founder effect, later bottlenecks, and genetic drift in this population causing expansion in relative isolation, followed by the spread throughout Europe due to Viking invasions. In particular,

most hemochromatosis-bearing chromosomes would be expected to descend from a single ancestral chromosome present in the initial founding population.

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8

The ancestral haplotype in hemochromatosis

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Introduction

Many chromosomal loci occur in different forms in different individuals; these different forms are called alleles. A haplotype can be defined as the alleles at different loci that are inherited from one parent. The presence of an ancestral haplotype in hemochromatosis, concomitant with a common ancestral origin for the disorder, was first proposed by Simon et al. in 1987[1]. The aim of this chapter is to present the evidence that such a haplotype exists, and to describe its geographical distribution and likely origin. Because the *HFE* gene has now been identified, the characterization of the ancestral haplotype postgene identification will be discussed, together with the implications this has on our understanding of the apparent correlation between the presence of the ancestral haplotype and severe clinical expression of hemochromatosis.

Haplotype analysis in hemochromatosis

A significant step towards understanding the pathogenesis of hemochromatosis came in 1975 with the demonstration that the gene for hemochromatosis was clearly linked to HLA-A3 in the Brittany population in France[2]. Subsequent studies confirmed the HLA-A3 association in other countries: for example, the UK[3], Canada[4], Australia[5], Germany[6] Sweden[7], and the US[8]. The HLA-A gene is a classical class I MHC gene, which lies close to the other classical class I genes of the MHC on the short arm of chromosome 6 at 6p21.3. These genes are highly polymorphic and are thus represented by many allelic forms. Alleles at HLA-A and HLA-B are usually inherited together in an HLA haplotype, their joint inheritance being a function of their close physical proximity. Studies of HLA and hemochromatosis also identified a significant association with the HLA-B alleles, B7[4] and B14[9]. The location of the gene for hemochromatosis in close linkage with the HLA-A and -B genes was serendipitous and provided the following: (i) the first unequivocal demonstration that hemochromatosis is a genetic disorder; (ii) the first important step in the positional cloning of the gene by its unequivocal location on 6p, and (iii) the implementation of tracking for the hemochromatosis gene by HLA-typing, enabling early diagnosis and preclinical assessment of risk in sibs of a proband.

After these initial observations of HLA linkage, Simon et al.[1] compared the relative strengths of the HLA-A and HLA-B allelic associations with hemochromatosis. Results demonstrated that HLA haplotypes carrying -A3, with or without -B7 or -B14, were significantly increased in patients compared to controls; whereas HLA haplotypes with -B7 or -B14 but without -A3 were found with the same frequency in patients as in controls. Simon et al.[1] concluded that the association between hemochromatosis and HLA-B was purely a function of the positive linkage disequilibrium between the Class I genes[1]. Alleles in a haplotype are said to be in positive linkage disequilibrium when the observed frequency of a haplotype is greater than the frequency expected from random encounter of the alleles, thus -B14 and -B7 were found more frequently in association with -A3 than would be expected by chance. The important conclusion from this analysis was that HLA-A3 was the only independent marker significantly associated with hemochromatosis.

Proposal for an ancestral haplotype in hemochromatosis

Several studies of HLA haplotype associations with hemochromatosis were subsequently reported[3–12] and it was evident that hemochromatosis is a common disorder

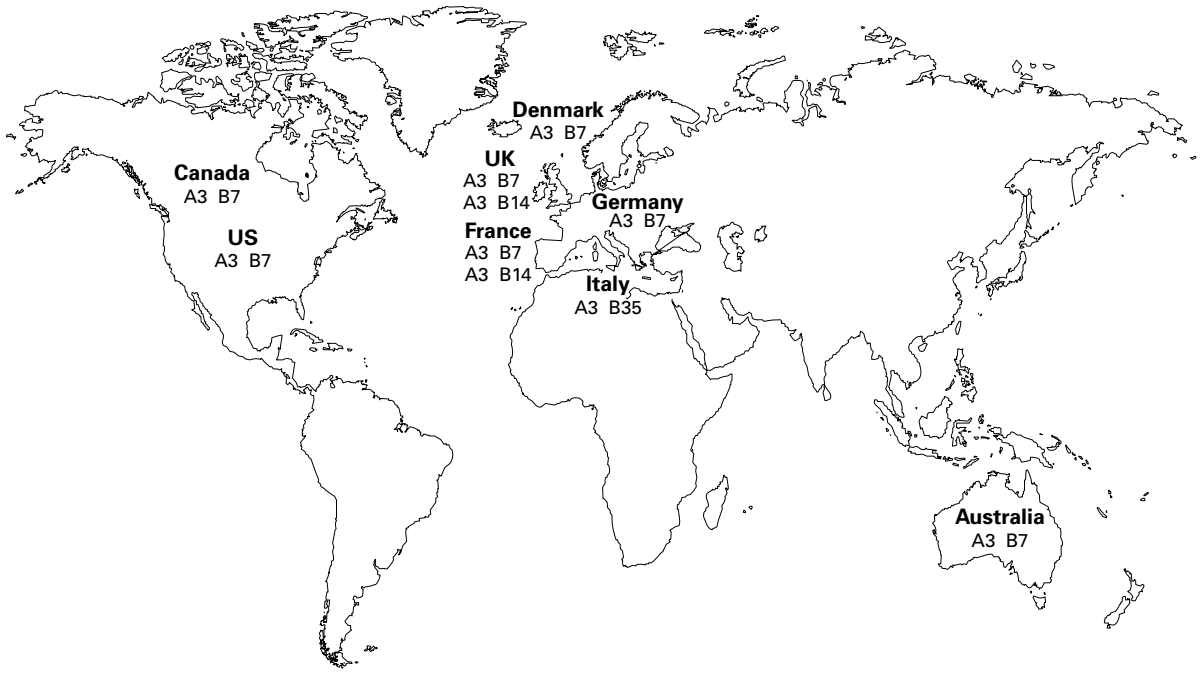

Fig. 8.1. The early characterization of the HLA haplotype association with HC indicated the disorder was associated predominantly with HLA-A3, B7, and also that it was present exclusively in Causasian populations of Northern European origin[1].

of Caucasian populations and that the same, or similar, HLA haplotypes were found significantly associated with hemochromatosis in all populations examined (see Fig. 8.1). For example, the HLA-A3, -B7 haplotype was reported in France[1], Canada[4] and Germany[6]; the HLA-A3, -B14 haplotype was present in France[9], England[3], Ireland[10] and Scotland[11], and a HLA-A3, -B35 haplotype was reported in Northeast Italy[12]. The predominance of the HLA-A3 associated haplotypes on hemochromatosis chromosomes, and the pattern of their distribution in the world, led Simon et al.[1] to propose the founder hypothesis, postulating that the hemochromatosis mutation was a rare event that occurred once on a particular chromosome that was subsequently modified by recombination and geographical migration. Under this assumption, the founder or ancestral chromosome was defined as one carrying the HLA-A3, -B7 haplotype, because this haplotype was common and ubiquitous in its association with hemochromatosis. After the introduction of hemochromatosis into the Caucasian population, Simon et al.[1] proposed that a series of recombinations had occurred, leading to the stepwise introduction of hemochromatosis onto chromosomes carrying different HLA haplotypes (see Fig. 8.2). Simon et al.[1] took this schema as providing the first key evidence that the hemochromatosis gene lay telomeric to HLA-A.

Extension of the ancestral haplotype

Following the characterization of the HLA haplotype on the ancestral chromosome, further genetic characterization of the ancestral chromosome was hampered for many years by lack of additional highly polymorphic markers at 6p21.3, particularly in the proposed vicinity of the gene for hemochromatosis, telomeric to HLA-A. This situation changed dramatically with the discovery of microsatellites[13], the highly polymorphic markers distributed abundantly throughout the genome. Using this new series of markers, it became possible to saturate 6p with highly informative markers to define further the ancestral haplotype beyond HLA-A and HLA-B. Microsatellite characterization of hemochromatosis chromosomes in several populations for example, Australia[14], the UK[15], the US[16], France[17], and Italy[18], indicated that (i) the gene for hemochromatosis was associated with a number of different haplotypes but the predominant (ancestral) one carried HLA-A3; (ii) the ancestral haplotype extended at least 4 Mb telomeric to HLA-A; (iii) the same ancestral haplotype was recognized in all hemochromatosis populations examined; and (iv) the ancestral haplotype was exclusively associated with hemochromatosis. These data provided convincing evidence in support of Simon's earlier proposal that

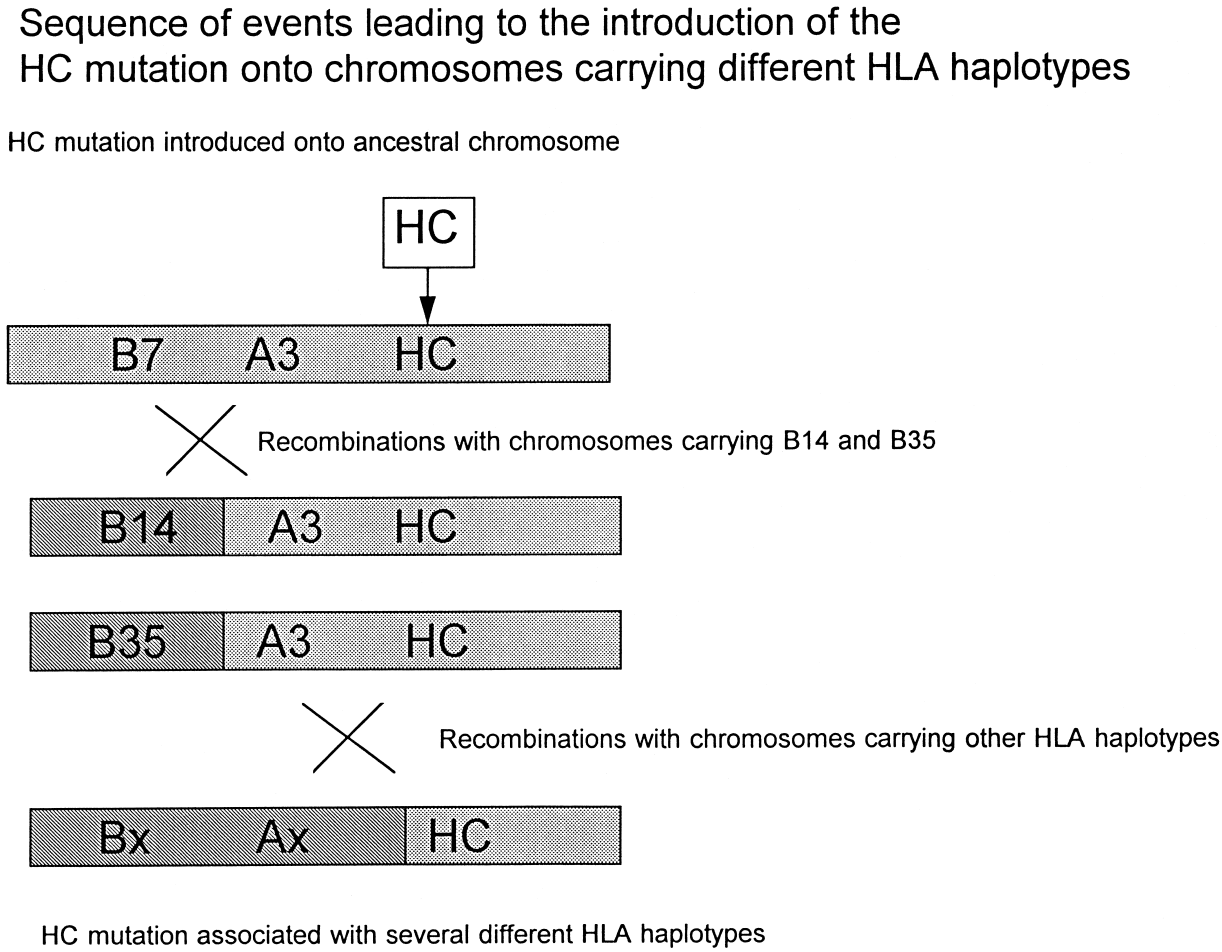

Fig. 8.2. Simon et al.[1] proposed a founder hypothesis for the introduction and spread of the HC mutation. The schema comprising a series of recombinations between and within HLA and HC is represented in this diagram.

hemochromatosis arose by a single mutation in a single ancestral individual[1].

Chromosomes carrying a gene mutation, descended from a common ancestor, often show a particular genetic profile in close proximity to the mutated gene[19]. This observation can be exploited to refine gene location by a haplotype comparison examination, termed parsimony analysis. This analysis involves aligning all of the haplotypes on chromosomes carrying the mutated gene. The region of the haplotype common to most affected chromosomes can then be defined; it is proposed that this conserved region has been protected from recombination separating it from the mutated gene by its proximity to the gene. Parsimony analysis in hemochromatosis indicated that whilst the ancestral haplotype including HLA-A3 was present on 33% of hemochromatosis chromosomes in Australia[14], 39% in the UK[15], and 50% in France[17], alleles at D6S105 and D6S1260 were conserved on most affected haplotypes and were present on at least 70% of HC chromosomes[15, 16, 20]. D6S105 and D6S1260 are several megabases telomeric to HLA-A and the conservation of their alleles in association with hemochromatosis indicated the gene for hemochromatosis was likely to be located close to these markers and some distance telomeric to HLA-A[15, 20].

The ancestral haplotype postgene – identification

Prior to the identification of the gene for hemochromatosis, the characterization of the ancestral haplotype and subsequent parsimony analysis in hemochromatosis was focused in a 3 Mb region telomeric to HLA-A[15] and surrounding the markers D6S105 and D6S1260. Feder et al.[21]

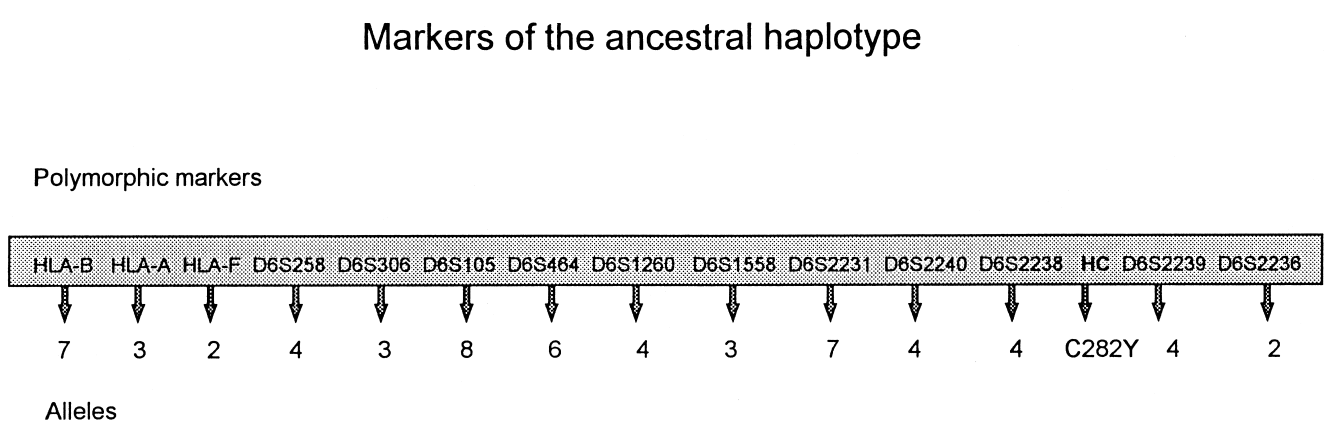

Fig. 8.3. The markers covering approximately 6 Mb at 6p21.3 and their alleles which comprise the ancestral haplotype (data taken from refs 21,38 and the genome data base). Haplotype includes the *HFE* gene marked HC and its causative mutation C282Y.

began their search for the gene for hemochromatosis by extending the genetic characterization and parsimony analysis to a region covering some 5–6Mb telomeric to HLA-A[21]. Their haplotype analysis of 45 chromosomes carrying the hemochromatosis defect enabled them to identify a highly conserved region of the ancestral haplotype comprising 250 kb, 5 Mb distal of HLA-A. Subsequently, the gene for hemochromatosis, first named HLA-H and now termed *HFE*, was identified in this specific 250 kb interval, and all 45 chromosomes displaying the common 250 kb region carried the main causative mutation in *HFE* (C282Y)[21]. Feder et al.[21] defined the conserved 250 kb region as the ancestral haplotype. However, there had been extensive literature prior to this report that described the ancestral haplotype as extending to HLA-A (A3)[14–18]. Therefore, it is more accurate to describe the entire 5–6 Mb region, immediately telomeric to HLA-A, as the ancestral haplotype (see Fig 8.3) and the 250 kb region containing the *HFE* gene as the conserved core of the haplotype.

Population genetics of the ancestral haplotype

It has long been widely recognized that hemochromatosis is found in Caucasian populations, predominantly those of Northern European origin, and that it is predominantly associated with the same ancestral haplotype. The identification of *HFE* and a highly penetrant single causative mutation, C282Y, was a tremendously exciting development. The definition of the C282Y mutation gave considerable credence to the proposal that the *HFE* defect was the result of a single mutational event. Furthermore, detailed analyses of the population genetics of hemochromatosis became immediately possible. For example, although it was clear that hemochromatosis was common in Caucasian populations, it was not known if this was a true reflection of the distribution of the genetic defect, or whether the expression of the disorder in different populations was being modified by environmental factors such as diet. Recently, an extensive analysis of the world-wide distribution of the C282Y mutation has shown clearly that it is a Caucasian defect, and that the highest frequency of this defect is found in Ireland where it is present on 1 in 10 chromosomes[22].

One of the specific difficulties in the positional cloning of the gene for hemochromatosis had been the lack of reported recombinants impeding the fine localization of the gene. Analysis of CEPH families had shown that recombination in the region of the MHC was one-fifth of the expected rate[23], and it was thus predicted that this suppression of recombination extended telomeric to HLA-A. After the identification of *HFE*, Ajioka et al.[24] analysed all hemochromatosis haplotypes and found that recombination over the *HFE* region was indeed suppressed and was approximately 28% of the expected rate. These same authors carried out a phylogeny analysis of hemochromatosis chromosomes, and were able to estimate that the C282Y mutation arose 60–80 generations ago. Assuming an average generation time of 20 years, this estimate places the age of the origin of the mutation at 800–1200 years ago. It is possible that the maintenance of the ancestral haplotype, and the persistence of strong linkage disequilibrium between hemochromatosis and markers in a 5 Mb region, is a function of the relatively recent age for the appearance of the mutation, combined with the suppression of recombination in the region.

There has been an on-going debate on the population origin of the hemochromatosis defect. Given the geographical origin of the defect, it had been generally proposed that the single mutational event occurred in an

ancient Celtic population[1]. The Celts arose in Middle Europe and their civilization then expanded throughout Northern Europe in a pattern consistent with the appearance of the hemochromatosis defect (see Fig. 8.4). Given the recent data on the age of the mutation, however, the geographic origin of hemochromatosis is once again thrown into debate. It has been suggested that the Celts arrived in the area of the present United Kingdom circa 4 500 BC[25] although it is possible that a Celtic civilization, as such, never existed. This latter proposal is based on the interpretation of a civilization. For example, does 'Celtic' pertain purely to language, art, and tools? Is this related to features already present in a country that are then imposed upon, rather than brought with, the incoming migrants[26]?

If the age of the hemochromatosis mutation was so relatively recent, the timing of its introduction and spread throughout Northern Europe is consistent with the hypothesis that the hemochromatosis ancestor was a Viking. The main Viking invasions occurred *c.* 800 AD and involved the countries of Northern Europe[27] where hemochromatosis is now found in high frequency (Fig. 8.4). Perhaps the only unequivocal demonstration of the geographic/population origin of hemochromatosis will come from the PCR identification of the C282Y mutation in ancient remains that are either Celtic or Viking in origin.

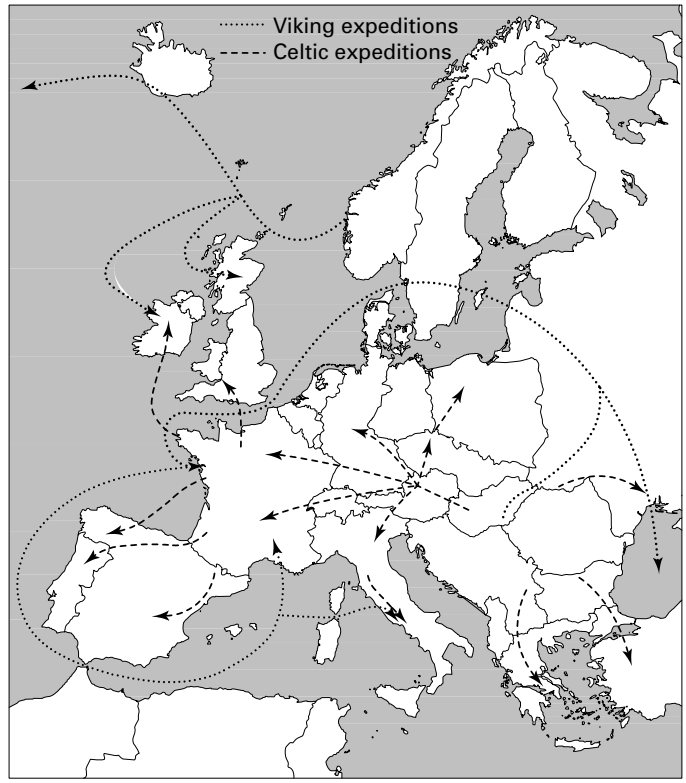

Fig. 8.4. The comparison between the pattern of Celtic and Viking Migrations (data taken from ref.[27] and from *The National Geographic* 1975).

Association between the ancestral haplotype and clinical expression of hemochromatosis

The disorder in iron metabolism which we recognize as adult–onset hereditary hemochromatosis, referred to as hemochromatosis throughout this chapter, results in an apparent lack of regulation of iron absorption, such that iron is absorbed at a high rate irrespective of body iron stores or requirements. It is thus recognized that, with adequate iron intake, and in the absence of pathological or excess physiological blood loss, virtually all subjects homozygous for hemochromatosis will develop clinical expression of hemochromatosis[28]. The clinical phenotype in hemochromatosis, however, is highly variable, and variation occurs especially in the age of onset and the degree of iron loading evident in different patients. The standard diagnosis of hemochromatosis involves assessment of iron indices. If these are elevated, a hepatic biopsy is carried out to permit a more precise diagnosis of hemochromatosis to be made by histological and biochemical analysis of the iron-loaded tissue. Biochemical analyses of the hepatic biopsy specimen involve the measure of hepatic iron concentration (HIC) which represents the amount of iron per gram dry weight of tissue, and hepatic iron index (HII), which is calculated from the HIC divided by age. The HII measure, which takes into account both the level of iron loading and the age of onset of hemochromatosis, is regarded by many as the most precise definition of the clinical expression of the disorder[29].

Analyses of haplotype/genotype in several clinical disorders demonstrate that different haplotypes are correlated with variants of the clinical phenotype; for example, in Wilson disease and cystic fibrosis. These correlations are due to the fact that different haplotypes carry different mutations, thus providing a direct molecular link with different phenotypes[30–33].

Prior to the identification of *HFE*, an analysis of the hemochromatosis clinical phenotype in Australian families demonstrated that the HIC in siblings of the same sex showed a significant correlation irrespective of environmental factors[34]. This was considered to represent prima facie evidence that the variation in hemochromatosis was primarily due to genetic factors. More recently, an analysis of haplotype/phenotype in Australian families with hemochromatosis found that hemochromatosis patients carrying two copies of the ancestral haplotype showed a significantly more severe expression of the disorder, as

measured by HII, than those with one copy or without[35]. In addition, results indicated that hemochromatosis heterozygotes can also show partial clinical expression of hemochromatosis and this expression is influenced by the presence of the ancestral haplotype in females, but not in males. Similar data, linking the presence of the ancestral haplotype to severe iron loading in hemochromatosis, has subsequently been reported in the Italian[36] and American[37] populations. In each of these latter studies, the level of severity of hemochromatosis was assessed by phlebotomy. In the Italian population, the amount of iron removed by phlebotomy was calculated and divided by age, the resulting value indicated either mild or severe hemochromatosis; haplotype analysis indicated the ancestral haplotype was significantly associated with the severe phenotype and that 7 out of the 8 patients in the severe phenotype category were actually homozygous for this haplotype[36]. In the American population, the phlebotomy history of patients was assessed and results indicated phlebotomy requirements were higher in hemochromatosis patients homozygous for HLA-A3 and D6S105 allele 8, two of the markers found on the ancestral haplotype[37].

Cumulatively, these data suggest that the phenotypic variation in hemochromatosis is associated with hemochromatosis haplotype and may thus be determined predominantly by genetic factors. The simplest explanation for a genetic association with phenotypic variation is that more than one causative mutation is present in a gene. There are two mutations in *HFE*: H63D and C282Y[21]. The C282Y mutation has a high penetrance and is always associated with the ancestral haplotype[21]. The role of the H63D mutation is less clear, but it is generally accepted that it has low penetrance. There are thus two possibilities. First, the analysis of the ancestral haplotype prior to *HFE* identification provided an indirect measure of the presence of the highly penetrant C282Y mutation. Other haplotypes may have carried the less penetrant H63D mutation or the wild-type *HFE*, and thus indicate hemochromatosis heterozygosity. The second possibility is that genetic modifiers of *HFE* function exist, and because all hemochromatosis is HLA-related[38, 39] such modifiers are present in the region of 6p21.3. It is clear that the characterization of the ancestral haplotype and its associations in hemochromatosis have not yet been exhausted, and their exploitation in the future may lead to a greater understanding of the factors governing the phenotypic variation of this common disorder.

Conclusions

Analysis of genetic markers in the hemochromatosis gene region has provided support for the presence of an ancestral haplotype in hemochromatosis. The identification of one predominant mutation in the hemochromatosis gene, unequivocally associated with expression of the disorder, supports the proposal that this mutation occurred as a single event on a Caucasian ancestral chromosome approximately 800–1200 years ago. The alleles on this ancestral chromosome define the original ancestral haplotype; this haplotype has been modified since by recombination such that the gene for hemochromatosis is now associated with a variety of haplotypes.

The core of the ancestral haplotype is comprised of approximately 250 kb around the gene. This region of the haplotype is highly conserved, is still found on the vast majority of chromosomes carrying the hemochromatosis defect, and its maintenance is likely to be a factor of the suppression of recombination telomeric of HLA-A, combined with the recent age for the arrival of the hemochromatosis mutation in the population. The identification and characterization of the ancestral haplotype has permitted analyses of the geographical distribution and population origin of the defect. It has enabled the important initial chromosomal localization and ultimately the identification of the causative gene. More recently, haplotype analysis in hemochromatosis has indicated the age of the ancestral C282Y mutation and reintroduced debate over its likely population origin. In the future, analysis of the association of the ancestral haplotype, the C282Y mutation and severity of clinical expression may provide a clear understanding of the factors controlling the variation in hemochromatosis phenotypic expression.

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9

Evidence for multiple hemochromatosis genes

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Introduction

The absorption of iron from the gastrointestinal tract is a complex, poorly understood process. Accordingly, it is not surprising that a variety of defined mutations are associated with a phenotype that includes excess iron storage and, presumably, increased iron absorption. Among these are β-thalassemia, atransferrinemia, X-linked sideroblastic anemia, and aceruloplasminemia. Although these disorders can be differentiated clinically without difficulty from hemochromatosis, the most common type of iron storage disease, they serve to emphasize that a variety of disorders can result in the accumulation of excess iron.

The successful search for a gene that causes hemochromatosis (*HFE*) was focused on chromosome 6 due to the known linkage of most cases of the disorder to the HLA-A locus. When the *HFE* gene was cloned from a region several megabases telomeric of HLA-A, mutations of the *HFE* gene were found to account for a majority of cases of hemochromatosis. Homozygosity for the 845G→A (C282Y) mutation was detected in 59%[1] to 100%[2] of patients with hemochromatosis, and an additional 4–8% of patients were heterozygous for 845A/187G, a genotype that also appears to cause hemochromatosis[3]. In all but one study[2], however, patients who appeared to have 'classical' hemochromatosis were encountered who carried neither the 845A or the 187G mutation. The questions addressed in this chapter concern these latter patients. Do they have hemochromatosis? If so, what mutation(s) are possibly responsible for their iron overload?

Hereditary iron overload syndromes not primarily caused by HLA-H mutations

β-Thalassemia

World-wide, β-thalassemia is probably the most important cause of clinically significant iron overload[4, 5]. Caused by mutations of the β-globin loci on chromosome 11, β-thalassemia causes markedly ineffective erythropoiesis associated with the accumulation of large amounts of iron. Death is usually due to cardiomyopathy secondary to iron overload. The iron overload that occurs in β-thalassemia is due partly to the transfusions that are necessary to sustain life. However, the accumulation of iron in most patients greatly exceeds that contributed by transfusion, and is due to increased absorption of iron, like that which occurs in persons with hemochromatosis. It is generally believed that the marked ineffective erythropoiesis characteristic of β-thalassemia sends inappropriate signals for iron absorption to the gastrointestinal tract, ultimately resulting in lethal iron storage.

Congenital dyserythropoietic anemias

Iron overload is a frequent accompaniment of the congenital dyserythropoietic anemias (CDA)[6–12]. Iron overload may be the presenting symptom, is the predominant clinical manifestation[8, 9], and occurs in type I and type II (HEMPAS) forms of the disease. CDA type II is due to a defect in erythrocyte membrane glycosylation, apparently due to a deficiency in α-mannosidase-II[13, 14]. The manner in which this underlying abnormality stimulates iron absorption is surely indirect.

Congenital atransferrinemia

Congenital atransferrinemia is an excessively rare disorder. Less than ten families have been reported[15, 16], but iron overload is a constant feature. This suggests that abnormalities in transferrin might be responsible for iron overload, a suggestion made in 1961 by Turnbull and Giblett[17] but not pursued actively, because the electrophoretic

mobility of transferrin is normal in hemochromatosis. With the demonstration that hemochromatosis is linked to chromosome 6 and that the transferrin gene is located on chromosome 3[18], it seemed unlikely that mutations of the transferrin gene were the cause for the most common form of hemochromatosis. However, the possibility that transferrin abnormalities cause hemochromatosis now deserves greater consideration (see below).

Ceruloplasmin deficiency

The relationship between copper and iron metabolism has long been appreciated[19], but the role of copper metabolism in iron metabolism is only recently beginning to be understood. The transport of iron into yeast has been dissected, and a multicopper oxidase designated FET3 catalyzing the conversion of Fe^{2+} to Fe^{3+} is required for high-affinity transport of iron to take place[20]. First described in the Japanese literature by Morita et al.[21] in 1992, a few cases of ceruloplasmin deficiency have been described in the past five years. The distribution of pathologic iron deposits in these patients is different than is found in hemochromatosis. There is a tendency for accumulation of iron in the brain, but hepatic iron overload is present and diabetes mellitus is a part of the syndrome. Like FET3 in yeast, ceruloplasmin is a ferrioxidase.

Sub-Saharan iron overload

The existence of siderosis among the Bantu natives in South Africa has been recognized for many years[22, 23]. Associated with hepatic cirrhosis and other stigmata of hemochromatosis, the ingestion of a local beer that is high in iron content has been regarded as playing a primary role in this disorder. Because not all drinkers of large amounts of traditional beer develop iron storage disease, a search for possible genetic factors has been carried out. There was no correlation between lifetime beer consumption and transferrin saturation, and only a relatively weak correlation between beer consumption and serum ferritin concentrations. However, mean transferrin saturation values were higher in first-degree relatives than in other family members, and among subjects with increased iron intake, bimodality of transferrin saturation values was demonstrated[24]. This led to the conclusion that there was a single-gene locus that influenced iron status, in addition to the effect of diet. There was no evidence of HLA-linkage, and thus this postulated locus was different from *HFE*. Similarly, HLA-linkage was not present among African Americans with iron overload[25].

Porphyria cutanea tarda

The association between this abnormality of porphyrin metabolism and iron storage has been known for many years. Notably, phlebotomy is a well-established treatment for porphyria cutanea tarda.

Candidate genes

Genes involved in HLA trafficking

The involvement of mutations of *HFE* in the etiology of hemochromatosis is now generally accepted, but the mechanism continues to be obscure. Because the mutation with the highest penetrance is one that prevents the binding of *HFE* to β_2-microglobulin, a protein involved in the transport of HLA class I molecules to the cell surface, the molecules involved in the transport of *HFE* deserve serious attention. This pathway is outlined in Fig. 9.1. Preliminary investigations of some of these proteins and of *HFE* itself have been performed in an attempt to find mutations that could account for hemochromatosis in patients who have not inherited the *HFE* mutations at nt 845 or at nt 187.

HFE

The author's group have sequenced the *HFE* gene in 18 chromosomes from persons with hemochromatosis who did not carry common mutations[26]; Carella et al. have sequenced another six chromosomes[27]. No mutations were found.

β_2-microglobulin

The possible involvement of β_2-microglobulin in the etiology of hemochromatosis was suggested by the development of iron storage in β_2-microglobulin 'knockout' mice[28]. The author's group has sequenced the coding region of this gene in 14 chromosomes from patients with hemochromatosis who did not have the common mutation, but did not find any mutations[26].

Calnexin

Calnexin–HLA complexes promote the association of HLA with β_2-microglobulin[29–31]. The HLA-β_2-microglobulin complex is then transferred from calnexin to a soluble calcium-binding protein, calreticulin[29] (see below). Although mutations affecting the function of calnexin could impair transport of *HFE* to the cell surface, this protein has not yet been examined in hemochromatosis to our knowledge.

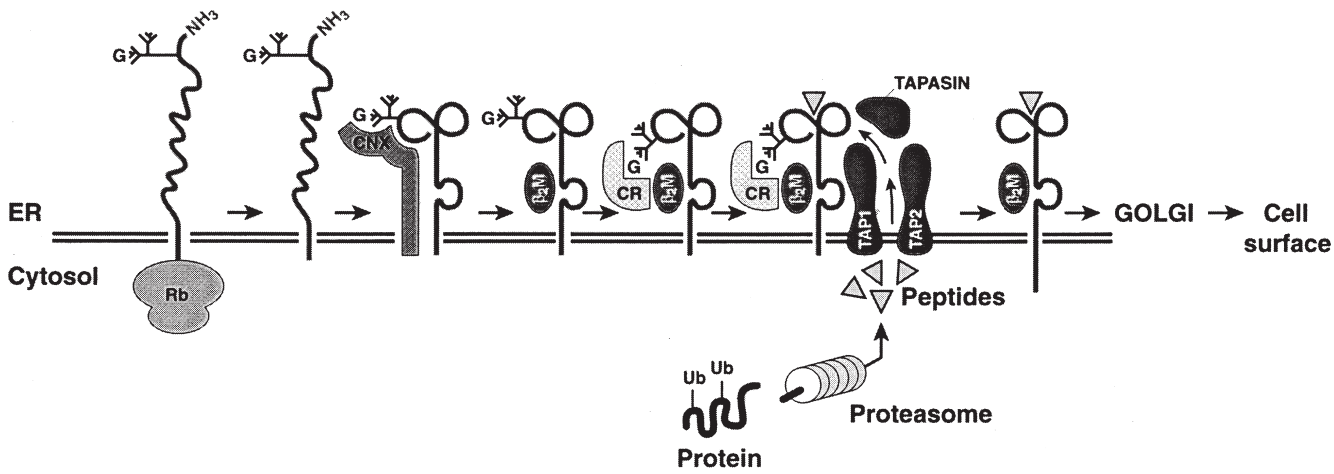

Fig. 9.1. Assembly and transport of class I major histocompatibility complex (MHC) molecules. After synthesis on ribosomes, class I MHC molecules are translocated to the endoplasmic reticulum (ER). After removal of the signal peptide and initial glycosylation (G) and deglucosylation, class I molecules bind to the ER resident chaperone molecule canexin (CNX). Calnexin assists in the proper folding of class I molecule, which is then able to bind to β_2-microglobulin ($ß_2$M). Calnexin then transfers the class I MHC molecule-β_2-microglobulin dimer to calreticulin, another chaperone molecule. This complex can then associate with the transporter molecules involved with antigen processing (TAP1 and TAP2) and tapasin. The complex formed between all of these components imports peptides from the cytoplasm into the ER, where they can bind to the peptide-binding site of the class I molecule. The ternary complex of class I molecule, β_2-microglobulin, and peptide is now fully assembled and is translocated to the surface of the cell.

Calreticulin

Calreticulin is of possible interest as a cause of hemochromatosis, not only because it is involved in the transport of class I HLA molecules, but also because the iron transport protein mobilferrin is probably identical to calreticulin[32]. Mobilferrin has been implicated as a major component of the intestinal iron transport pathway delineated by Conrad and his colleagues[33]. Mobilferrin (calreticulin) binds both iron and zinc with high affinity[34]. Calreticulin-HLA-β_2-microglobulin complexes associate with the transporter associated with peptide processing (TAP) and tapasin for subsequent peptide loading[29]. Calreticulin is returned to the endoplasmic reticulum by virtue of its carboxy-terminal KDEL sequence[35]. The author's group sequenced the calreticulin gene of five patients with hemochromatosis who were not homozygous for the nt 845 mutation of *HFE* and found no mutations[26].

Tapasin

Nothing is yet known about the possible association of *HFE* with tapasin, the protein that plays an important role in the final loading of HLA class I molecules with peptide.

Genes involved in iron transport, metabolism, and absorption

Many proteins have been known for decades to be involved in the transport and/or metabolism of iron, but mutations of the corresponding genes have not been considered viable candidates to cause hemochromatosis because they are not linked to the HLA locus.

Ferritin

For example, Zheng et al. carefully considered the possibility that ferritin mutations may cause hemochromatosis[36]. Finding only a processed pseudogene on chromosome 6, they appropriately discarded the possibility that ferritin was a candidate gene for the common form of hemochromatosis. Mutations of ferritin genes could still be causes of the less common, non-HLA-linked forms of the hemochromatosis.

Transferrin

Turnbull and Giblett[17] considered the possibility that transferrin variants might be responsible for hemochromatosis. Finding no correlation between electrophoretic

variation of the transport protein and iron storage disease, they abandoned the search for transferrin gene mutants in patients with hemochromatosis Yet, more subtle differences in transferrin might be responsible for some cases of hemochromatosis.

Transferrin receptor

Transferrin and its receptor are important links in the chain of iron transport. The transferrin receptor was not detectable in hepatocytes in hepatic biopsy specimens from most of 21 patients with hemochromatosis, but was present in each of 50 biopsy specimens from control subjects[37]. It seemed likely that this change was secondary to iron overload, because the receptor was present in patients who had been treated by phlebotomy. Serum transferrin receptors concentrations in patients with hemochromatosis are decreased[38, 39], but increase during phlebotomy, indicating that the low levels observed in the untreated patients were secondary to iron overload. The patients who were studied were presumably predominantly those with *HFE* mutations, and the role of the transferrin receptor in the subset of patients without *HFE* mutations remains unknown. The gene that codes for the transferrin receptor has been cloned[40], and examination of both the coding region and the iron-responsive element might be productive.

Nramp2

Nramp2, a gene that is homologous to *Nramp1*, a gene active in host defense, has been implicated recently as a participant in iron absorption in mice[41, 42]. A point mutation in this gene produces microcytic anemia that is assumed to be associated with decreased intestinal iron absorption. If this gene regulates iron absorption in the intestine, mutations could also result in increased iron absorption in man. Therefore, this is a new candidate gene for hemochromatosis.

Conclusions

This chapter briefly reviews some of the genes other than *HFE* that might be involved in the etiology of hemochromatosis. Determining which, if any, are actually involved will require sequencing of the coding regions of these gene and determining their rate of transcription. It is possible that variations in some of these genes play a role in determining the phenotype of patients who have the principal *HFE* mutations. Some may cause hemochromatosis by themselves. The task in identifying additional genes that cause the hemochromatosis phenotype is a difficult one. Even when the approximate location of the hemochromatosis gene was known, it required approximately ten years of extensive effort to identify the *HFE* gene. For those patients who do not have *HFE* mutations, no linkage relationship is known and our incomplete understanding of iron absorption and metabolism must indicate the direction for further search and investigation.

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Part III

Metal absorption and metabolism in hemochromatosis

10

Intestinal iron-binding proteins

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Introduction

The major disturbance in hemochromatosis is the excessive lifelong absorption of dietary iron that eventually results in excessive body iron loading. Under normal circumstances, iron absorption is carefully controlled according to the body iron stores. This control is disturbed in hemochromatosis and enhanced iron absorption continues despite the presence of expanded body iron stores[1–3].

The regulation of iron status is normally carried out at the level of the enterocyte in the upper small bowel[4]. Intrinsic regulation factors are undoubtedly present within the mucosal cell itself, and internal body regulatory factors are also at play, relaying body iron needs according to the whole body iron status. Until recently, there was no animal model of hemochromatosis, and in vivo studies of iron metabolism in humans were limited by the difficulty of obtaining direct access to small bowel mucosal cells. Significant progress has recently been made in these areas. The proteins involved in the different phases of non-heme and heme iron absorption at the level of the gastrointestinal tract are discussed below, emphasizing known abnormalities in hemochromatosis.

Phases of absorption

Iron absorption consists of a series of mechanistically distinct, temporarily synchronous, overlapping stages. These consists of iron presentation to cells and uptake by the mucosal cells, transcellular transport and handling of iron, and finally the transfer of iron out of the mucosal cell into the portal venous system[5]. The various proteins presumed to be involved in the different phases of absorption are depicted in Table 10.1. Absorption pathways differ for non-heme iron and heme iron in the initial phases of iron uptake and transcellular handling. Within the mucosal cell, iron is released from the heme molecule and joins the non-heme intracellular iron pool in a common pathway[6, 7].

Proteins involved in iron uptake by the mucosal cell

A number of mechanisms for iron uptake from the gastrointestinal tract lumen have been described in various animal models. These mechanisms for iron uptake by the brush border surface of the mucosal cell include binding of iron to both specific and non-specific low-affinity binders[8–10], and to specific high-affinity binders[11]. Few such mechanisms have been studied in man.

Mucus and mucins

Although not an integral part of the cell itself, elutable factors such as mucus and mucins coating the cells lining the gastrointestinal tract lumen undoubtedly play a role in non-heme absorption[12, 13]. These factors maintain iron in solution in the duodenum despite its alkaline milieu, keeping iron available for absorption by the mucosal cell surface. Iron bound to mucin may be taken up into the mucosal cell via a saturable pathway for iron uptake. This pathway's maximal capacity is greatly increased in iron deficiency in an animal model[9].

Lactoferrin

Specific lactoferrin receptors have been implicated in the uptake of lactoferrin-iron complexes present in breast milk or lactoferrin present in upper small bowel secretions[14–16]. The K_d of human fetal intestinal lactoferrin binding

Table 10.1. Intestinal iron binding proteins/compounds according to phase of absorption

Mucosal cell uptake	Intracellular iron transport/storage	Mucosal cell to plasma transfer
Mucus/Mucins	Transferrin	Transferrin receptor and transferrin
Lactoferrin	Mobilferrin	HLA-H protein
Brush border membrane vesicle fatty acids	Ferritin	β_2-Microglobulin
Glycoprotein of Teichmann and Stremmel	Iron regulatory proteins	Ceruloplasmin
Integrins		NF-E_2 protein

(3×10^{-6} M) is relatively low and is similar to that seen in other animal models[17]. However, adult intestine appears to lack these receptors. It is only in suckling animals or early neonatal life that these receptors play any significant role in iron absorption. After the neonatal period, no role has been demonstrated for iron uptake via this mechanism[18].

Transferrin and transferrin receptors

Although transferrin and the transferrin receptor were presumed to play a role in iron uptake from the gastrointestinal tract[19], neither protein has been detected in the apical microvillous brush border of the proximal small bowel[20–24]. Administration of ^{59}Fe-transferrin in vivo to achlorhydric subjects did not enhance iron absorption, suggesting that transferrin is not involved in iron absorption in man[25]. It is unlikely that transferrin and its receptor are major transporters of iron from the lumen into the musocal cell.

Fatty acid moieties

A variety of fatty acids present in lipid extracts of the brush border membrane vesicles bind adsorbed iron from the gastrointestinal tract. Lipids capable of iron binding include phosphatidic acid, phosphatidylserine, and oleic and stearic acids. Of these, oleic acid has the highest iron-binding affinity. There is evidence that these fatty acids may play a role in iron binding and transport into the mucosal cell[26]. However, further studies are required to delineate this mechanism.

Glycoprotein of Teichmann and Stremmel

A glycoprotein receptor with high affinity for ferric iron has been isolated from the microvillous membrane. This receptor is saturable, temperature-sensitive, and actively facilitates ferric iron transport across microvillous membrane vesicles. The receptor has a trimer structure with molecular weight 160 kDa, and consists of three 54 kDa monomeric peptides, probably linked by disulfide bonds[27]. Antibodies to this receptor inhibit iron uptake by mucosal cell membrane vesicles. The concentration of this protein in duodenal mucosal cells varies in inverse proportion to iron status, with the concentration being increased in iron deficiency, and reduced in secondary iron overload and hemochromatosis. Further studies are required to confirm the activities of this protein.

Integrins

An iron-binding transmembrane protein identified as an integrin has been located in rat duodenal mucosal cell microvilli[28]. This integrin protein consists of two protein chains, α and β, of 150 kD and 90 kD, respectively. This transmembrane protein coprecipitates with mobilferrin. It is postulated that the cytoplasmic end of the α-chain of the integrin docks mobilferrin to a perimembrane location, similar to the interaction between calreticulin and integrin[28]. Further confirmation of the role of these proteins in iron uptake is awaited.

There is little understanding of transmembrane iron transport after the initial adsorption to the microvillous membrane. Uptake into the brush border membrane vesicles occurs as part of the internalization process[29]. After attachment to the putative proteins and lipids mentioned above, it is unknown how iron is then shuttled internally. In the case of non-receptor mechanisms, simple diffusion, that may be an energy-dependent process[30] or non-energy dependent process[31], has been described. Other possible mechanisms that have not been studied include entry via iron channels, absorptive pinocytosis, receptor-mediated pinocytosis, or fluid-phase endocytosis.

Heme iron receptors

Heme is taken up by the mucosal cell via an entirely different pathway from non-heme iron. Heme binds to specific heme receptors on the mucosal cell surface[32, 33], and rapidly enters the mucosal cell as an unchanged porphyrin ring. The heme receptors have been poorly characterized. Studies in dogs using autoradiography and diaminobenzidine staining indicate that heme iron is

endocytosed in microendocytotic vesicles, especially at the base of the microvilli, and as tubulovesicular structures in the apical cytoplasm of mucosal cells[34]. The internalized heme is then split by heme oxygenase in the mucosal cell, releasing iron from the heme moiety, after which the iron enters the same pool as non-heme iron in the mucosal cell[5]. The intramucosal heme-splitting process is one of the rate-limiting steps in heme iron absorption[35].

Intracellular iron transport and handling

There are two separate compartments involved in intracellular iron handling: an iron transfer pathway and an iron storage compartment pathway. There is little understanding of the mechanisms involved in the transfer pathway. Iron moves rapidly through this pathway, so that absorption of iron from a meal occurs within 2–4 hours after intake[36], and is essentially complete within 24 hours. Transferrin and mobilferrin possibly play a role in this early phase of iron absorption, and may partly account for the unsaturated iron-binding capacity of small intestinal homogenates, that varies inversely with the degree of iron stores[37].

Transferrin

A role for transferrin in the transfer of iron across the cell is suggested in animal models. Significant amounts of iron are found in the transferrin fraction of mucosal cell homogenates early after iron installation into rat gastrointestinal tract[38]. The amount of iron found in the transferrin fraction varies according to iron status; increased transferrin iron levels are found in iron-deficient animals and decreased amounts in iron-loaded animals. There is a direct correlation between mucosal cell transferrin concentration and iron absorption[39, 40]. However, the role of transferrin in transcellular iron transfer has been questioned. Hypotransferrinemic mice[41] and humans develop iron overload due to enhanced iron absorption despite low serum transferrin concentrations, and hypoxemic animals with enhanced iron absorption do not have increased mucosal cell transferrin, in spite of having increased plasma transferrin concentrations[42]. Furthermore, rats with induced inflammation have reduced iron absorption despite raised levels of muscosal cell transferrin[40]. Transferrin isolated from mucosal cells in many studies may have been (at least partly) a contaminant from serum[43] and may have been modified by enzyme cleavage[44].

Other studies in man indicate that transferrin may not play a crucial role in transcellular iron transport. There is no significant difference in duodenal mucosal cell transferrin concentrations in iron-deficient patients and normal subjects[45]. The concentration of mucosal cell transferrin is not correlated with body iron status, or with non-heme or heme iron absorption. Only in severely iron-deficient patients does mucosal transferrin concentration rise, suggesting that transferrin is not important in transcellular iron transfer under normal circumstances. Furthermore, transferrin mRNA has not been identified in rat or human gastrointestinal mucosal cells[11, 46, 47], indicating that transferrin is not produced within the mucosal cell itself. Transferrin most likely enters the cell through transferrin receptors found on its basolateral surfaces[22]. The transferrin concentration within the mucosal cell is probably achieved as a consequence of the cell's iron requirements during cellular growth and development.

Mobilferrin

The iron-binding protein mobilferrin consists of 14 165 amino acids, with a molecular weight of 56 kd[48, 49]. This monomeric protein contains minimal amounts of carbohydrate. Each molecule of mobilferrin binds one atom of iron. Iron-binding is reversible, and dissociation is pH-dependent, with accelerated iron loss occurring in an acid milieu, such as that present within the mucosal cell. This protein has a lower iron-binding affinity than other proteins such as transferrin. Mobilferrin is located in the apical portion of the duodenal mucosal cell cytoplasm, in close association with integrins present in the microvilli. It may have a role in shuttling iron, at least in the apical segment of the cell. This protein also plays a role in the binding of other metals such as copper, cobalt, zinc, and lead, and may be responsible for the competitive inhibition by iron of absorption of these metals in the small bowel.

Ferritin

Ferritin's main function is that of a reservoir for excess iron within the mucosal cell. It was previously postulated to be the main protein accepting iron absorbed into the mucosal cell, thus acting as a 'mucosal block' to prevent absorption of unneeded iron[50, 51]. Excess iron that has been absorbed from the gastrointestinal tract and not transported into the portal system is deviated to the storage compartment of the cell, and ferritin is the main protein involved in this compartment. Only small amounts of iron taken up by the storage compartment re-enter the transport pathway. After a meal, $\leq 10\%$ of iron that has entered the late release storage compartment re-enters the transfer pathway[52].

Radio-iron absorption studies in rats indicate that progressively greater amounts of iron are diverted to the ferritin fraction in the latter phase of absorption, while progressively decreased amounts of iron are found in the transferrin fraction[38]. Iron-deficient animals have reduced iron incorporation into the ferritin fraction during absorption due to increased throughput of absorbed iron into the circulation. The concentration of mucosal cell ferritin varies in direct proportion to body iron stores; levels are low in iron deficiency and raised in secondary iron overload[39, 45, 53]. These observations suggest that mucosal cell ferritin may have some regulatory role in transport through the cell. More likely, as in other cells, ferritin concentration reflects its repository function of sequestering excess cellular iron that has not been absorbed into the circulation. Evidence for this is provided by measurement of ferritin concentration in feces, that for the most part, reflects the status of the mucosal cells that have been desquamated from the gastrointestinal villi[54]. Fecal ferritin concentration is approximately twenty-fold lower in iron-deficient patients compared to normals, reflecting the increased throughput of iron from the mucosal cell to the circulation in iron-deficient states. A close relationship exists between fecal L-rich ferritin and serum ferritin concentration, reflecting increased incorporation of iron into mucosal cell ferritin when stores are high[54].

Both L-rich and H-rich ferritin are present in the duodenal mucosal cell in humans[45, 53]. The L-rich ferritin concentration of the mucosal cell is correlated directly with whole body iron, bears an inverse relationship to both heme and non-heme iron absorption, and increases with oral iron ingestion. In this manner, it directly reflects excess iron taken up by the mucosal cell[53]. Further, there is a prompt increase in fecal L-rich ferritin after oral iron ingestion[54]. Excess iron taken up by the mucosal cell leads to the rapid assembly of ferritin molecules within the cell. The H-rich ferritin content of the mucosal cell is also correlated directly with whole-body iron, and bears an inverse relationship to both heme and non-heme iron absorption. Fecal H-rich ferritin also reflects iron stores and increases after iron ingestion, but to a lesser extent than L-rich ferritin. Although L-rich ferritin has an iron storage function in various cells, including mucosal cells, the precise function of H-rich ferritin is unclear. It may function as a protein for short-term iron storage, because iron is rapidly incorporated and released from H-rich ferritin. However, its role as a shuttle protein in the mucosal cell is unknown.

The mucosal cell shares some similarities with red blood cell precursors, in that it also has a high input and internal circulation of iron, and a similar isoferritin distribution. As in red blood cells, the L-rich to H-rich ferritin ratio is approximately 1.5 in normal iron status. The levels are approximately equal or reversed in iron deficiency, and the ratio is increased in secondary iron overload[45]. In normal subjects undergoing phlebotomy, fecal ferritin showed an L-rich to H-rich ferritin ratio of 1.2 in persons with normal iron stores, a reversed ratio of 0.3 in persons with depleted iron stores, and persistence of the ratio of 0.3 with the development of iron-deficiency anemia. The L-rich fecal ferritin concentration decreased approximately eight-fold, while H-rich ferritin decreased by three-fold[54].

Both L-rich and H-rich ferritin mRNA are present in the mucosal cells of the stomach and duodenum; higher mRNA levels are present in duodenal than gastric cells. The mRNA levels for both ferritin subunits are reduced in iron-deficiency and increased in secondary iron overload[47, 55]. The latter findings indicate that the transcription of intracellular ferritin subunits is regulated according to the iron content of or the amount of iron traversing the mucosal cell[56].

Iron regulatory proteins

Studies of iron regulatory proteins in the gastrointestinal tract mucosal cell are few. Iron regulatory proteins are involved in the internal regulation and control of iron metabolism, similar to the mechanism found in other organs involved with significant iron handling[55]. A labile intracellular iron pool is involved with the intracellular regulation of iron metabolism through these regulatory proteins. The iron content of this pool is reflected by the activity of two similar iron regulatory proteins, IRP-1 and IRP-2, of 90-kD and 105-kD respectively, that act as physiological sensors of iron[57, 58]. The absence of iron in the labile pool leads to the increased activity and binding of IRP-1 to specific mRNA motifs with stem-loop structures known as iron-responsive elements (IRE) on the 5´ untranslated regions of mRNA for H- and L-ferritin subunits, and on the 3´ untranslated regions of mRNA for transferrin receptor. This leads to inhibition of ferritin synthesis by blocking translation of the message, and a corresponding increase in transferrin receptor synthesis by increasing the stability of transferrin receptor mRNA. This results in increased translation and production of transferrin receptor for subsequent iron acquisition by the cell. When cells contain adequate iron in the labile iron pool, IRP-1 contains a 4Fe–4S cluster; in this form, IRP-1 is a cytoplasmic homologue of mitochondrial aconitase. IRP-1 in this form does not bind to the IRE for ferritin mRNA, to cause translation of ferritin mRNA and subsequent ferritin production, and iron sequestration by the cell. When cells become iron-depleted, there is disassembly of the 4Fe–4S cluster with

reversal of the protein's activity. IRP-2 lacks the aconitase activity of IRP-1 and the 4Fe–4S cluster. Human IRP-2 is 57% identical and 79% similar in amino acid sequence to IRP-1. Iron depletion leads to de novo synthesis of IRP-2; iron repletion leads to its degradation. IRP-2 regulates H- and L-ferritin and transferrin receptor synthesis in a manner similar to IRP-1, except that absence of the 4Fe-4S cluster results in less efficient regulation of this protein's activity. The relative activities of IRP-2 and IRP-1 can vary significantly in different cell types. It is likely (but unproven) that the activity of these proteins in the mucosal cell is also modulated by products of inflammation such as nitrous oxide[59], and oxidative stress such as reactive oxygen species[60].

Proteins involved in mucosal cell iron transfer to plasma

The final step in the absorptive process is the transfer of iron across the basal surface of the mucosal cell into the portal circulation. There is little information regarding this process. After exiting the mucosal cell, iron traverses the interstitial space and the endothelial cell wall and enters the portal venous system.

Transferrin receptors

Transferrin receptors are present on the basolateral surface of the mucosal cells. Consequently they were once presumed to be the pathway for iron transfer from the mucosal cell into the portal circulation. Transferrin receptor levels in this location increase in iron deficiency and are reduced in secondary iron overload states, and can be correlated with the amount of iron absorption. However, involvement of these receptors in iron absorption probably does not occur. Transferrin receptors are involved in iron acquisition for the cell's own metabolic requirements, and may also be involved in signalling mucosal cells regarding body iron status. Transferrin receptor numbers are regulated according to the cell's iron status: their concentration is down-regulated with normal iron status, and up-regulated with iron depletion. In individuals with normal iron status, transferrin receptors on mucosal cells are present in the crypts of villi and extend up the basal portions of the villi; the receptors are decreased or absent towards the apical portions of the villi[61–63]. The crypts are the progenitor regions for these epithelial cells, and cells in this location have high iron requirements for proliferation. In iron deficiency, transferrin receptors are expressed by cells at the apices of the villi[64, 65] as well. Although transferrin receptors on mucosal cells increase along the villi in iron deficiency with increases in iron absorption, induction of hemolysis (that leads to increased iron absorption) is not associated with increased receptor density or in binding affinity, indicating that there is no relationship between iron absorption and receptor numbers on these cells[64]. Similar changes in transferrin receptor levels related to iron stores occur in other non-absorbing areas of the gastrointestinal tract, such as the gastric mucosa and distal segments of the intestine[65].

In normal patients transferrin receptor mRNA is present in mucosal cells of the stomach and duodenum, and transferrin receptor mRNA levels do not differ between these two sites as is the case with mRNA for L-rich ferritin and H-rich ferritin[47]. In iron deficiency, however, transferrin receptor mRNA levels are increased only in duodenal cells. This is due to the rapid absorption of iron through iron-deficient duodenal mucosal cells, contributing to a perceived cellular iron-deficient state relative to mucosal cells in other sites of the gastrointestinal tract. This is further substantiated by the fact that reduced ferritin mRNA levels are found in these cells in iron deficiency[47]. In patients with secondary iron overload, transferrin receptor mRNA levels are reduced. This is not a uniform finding in rats, in which transferrin receptor mRNA levels were increased in iron-loaded animals[63]. Based on the observations in neonatal and adult rat models, the function of transferrin receptors on the basolateral surface of mucosal cells is to sequester iron from portal blood in order to meet iron requirements for cellular proliferation and metabolism[65]. Uptake of apotransferrin may also occur to satisfy growth of the crypt cell, because transferrin also acts as a growth factor[62].

Based on these observations, the iron content of the mucosal cell is partly self-regulated to ensure sufficient iron for its own relatively high metabolic requirements. This is achieved by the entry of iron from the gastrointestinal lumen or via transferrin receptors at the base of the cells, or, most likely, by both pathways. When the mucosal cell iron content is sufficient, transferrin receptor mRNA levels are down-regulated, and ferritin mRNA levels simultaneously increase, so that sufficient amounts of apoferritin are produced to sequester excess iron in the internal cellular environment[47]. This control occurs to a greater extent in younger cells at the bases of the crypts, and is probably not entirely lost in cells which have migrated towards the apical portions of the villi . Although these findings suggest control in the mucosal cell at a transcriptional level, further work is required to establish whether transcriptional regulation, which has been well described in other cell types, is also operative in these cells.

HFE protein and β_2-microglobulin

The putative gene linked to genetic hemochromatosis has been identified[66] and termed *HFE*. The *HFE* gene is telomeric to the major histocompatibility complex (MHC) on chromosome 6p, 1–2 cM from the HLA-A gene. The *HFE* gene is similar to other HLA class I genes. The structure of the *HFE* protein, for the most part, resembles that of the antigen-presenting HLA-A protein, but it also contains features of the F_c receptor[66]. mRNA expression of this gene is found in most tissues, including the small intestine, but not in brain or lymphoblasts. The structure of the *HFE* protein is presumed to be that of a single polypeptide composed of 343 amino acids, with three extracellular domains (α1, α2, and α3) analogous to other MHC class I proteins, a transmembrane region, and a short intracellular tail region. An intramolecular disulfide bridge is presumed to be present in the α2 and α3 portions of the polypeptide chain. A noncovalent bond is presumed to be present between β_2-microglobulin and the α3 domain of the protein. Evidence for involvement of this protein in iron metabolism is provided by the recent finding of deranged iron metabolism in a β_2-microglobulin knockout mice model, that accumulate excess iron in a manner analogous to hemochromatosis[67, 68]. β_2-microglobin is required for the normal surface expression of MHC class I molecules, and these mice do not express the MHC class I gene products.

Using antibodies against the presumed C-terminal portion of *HFE* predicted from the corresponding cDNA, immunohistochemical localization of this protein in the gastrointestinal tract has been performed[69]. *HFE* was detected in all segments of the gastrointestinal tract. The most abundant staining occurs in mucosal cells in the crypts of the small intestine, with more intense staining in the duodenum than in the jejunum and ileum. The staining is mainly intracellular and in the perinuclear region of the small bowel; no staining was apparent in the basolateral or apical portions of the cells. Staining of the gastric and colonic epithelial cells is restricted to the basolateral surfaces, and the stratified epithelial cells of the esophagus possess staining of the entire plasma membrane. These differences in immunoreactivity suggest that the *HFE* protein may have different functions in different locations of the gastrointestinal tract.

The exact function of the *HFE* protein is unknown, but it is likely that it is involved in internalization or ligand recycling via receptor mediated pathways, rather than in antigen presentation[66]. How this protein may be involved in iron metabolism is presently unknown. Possible mechanisms include receptor function for an iron-binding ligand on the basal cell surface. This activity could function as a negative feedback control, limiting iron absorption. An abnormality of this protein in hemochromatosis could result in loss of this negative feedback effect, allowing excess iron absorption. A further possible function is signal transduction of body iron status to the mucosal cell, which may then signal various gene products that control iron absorption. Another mechanism may involve interaction with various components of the immune system that may influence iron metabolism, including various cytokines[66].

Ceruloplasmin

After iron is released from the mucosal cell, it travels into the interstitial space and then passes through the endothelial cells of the portal capillary system. It is likely that small molecular weight chelators or ligands are involved in this step[70]. Ceruloplasmin may be involved in this transfer, because iron absorption is rapidly enhanced after intravenous injection of ceruloplasmin in copper deficient rats. Ceruloplasmin may play a role in oxidizing ferrous iron that diffuses into the portal capillary system, thus producing a ferric iron moiety for binding to transferrin in the portal venous circulation[71].

Nuclear factor-erythroid 2 protein

Nuclear factor-erythroid 2 (NF-E2) is a nuclear DNA-binding polypeptide protein of 45 kD, that plays a role in iron metabolism. NF-E2 is present in increased amounts in placenta, liver, lung, proximal small bowel and fetal liver. NF-E2 recognition sites are found in the H-ferritin gene, upstream of the ferritin transcription initiation site[72]. NF-E2 recognition sites are also present in regulatory regions of erythroid genes including alpha and beta globin gene locus control regions, delta-aminolevulinic acid synthase promotor regions and porphobilinogen deaminase promotor regions. Absence of NF-E2 synthesis and its corresponding mRNA in homozygous Mk mice has been associated with the development of hypochromic microcytic anemia and defective iron uptake by both red blood cells and intestinal musocal cells[73]. The exact localization of NF-E2 in duodenal mucosal cells is unknown[74]. An inverse relationship between duodenal mRNA levels and iron absorption exists in a mouse model; however, increased absorption in conjunction with raised NF-E2 expression was observed in beta-thalassaemic (hbd/hbd) as well as hypotransferrinaemic (hpx/hpx) mouse models[74]. This disparity between NF-E2 levels and absorption in these mouse models suggests that NF-E2 is unlikely to play a direct role in regulating iron absorption, and the relationship of this protein to absorption in humans

is unknown. Furthermore, there appears to be only a minimal regulatory role of NF-E2 on H-ferritin in duodenal cells in mice. Further evaluation of the potential role of this regulatory factor in other mammals and man will be of interest.

Abnormalities of gastrointestinal proteins in hemochromatosis

Increased transfer of iron from the musocal cell into the portal circulation is the major defect of absorption in genetic hemochromatosis[1, 2, 75, 76]. Mucosal transferrin has been evaluated as a possible cause for this excess iron absorption. Transferrin is unlikely to be involved, however, because mucosal transferrin concentrations are decreased in these patients despite increased iron absorption[45].

Duodenal mucosal cells contain lower than predicted ferritin protein concentrations in relation to body iron in genetic hemochromatosis[45, 55, 77]. Based on the usual relationship between serum ferritin and mucosal cell ferritin in normal subjects, the mean mucosal cell L-rich ferritin level was significantly reduced in genetic hemochromatosis (~180 ng/mg), compared with the predicted mean value of ~1000 ng/mg, with an elevated mean serum ferritin of ~1300 μg/l[45]. Similarly, lower than expected fecal ferritin levels have been reported in genetic hemochromatosis in relation to their iron overload[54]. These findings have suggested that dysregulated ferritin synthesis, reduced stability, or enhanced degradation may be the metabolic defect and cause of the disordered iron absorption in this disorder. It is more likely that the diminished ferritin levels in mucosal cells are secondary to enhanced iron transfer from the mucosal cell into the portal circulation with a consequent decrease in ferritin production. The observation that mucosal cell ferritin concentrations can be induced to increase in these patients after oral iron administration, supports enhanced mucosal iron transfer as the cause for the reduced mucosal ferritin content in these patients.

Increased numbers of transferrin receptors are found on the basal surface of duodenal but not gastric mucosal cells in genetic hemochromatosis, in spite of increased whole body iron[61]. This is in keeping with enhanced iron transfer from the duodenal mucosal cell into the circulation. This transfer renders the cell relatively iron deficient. Increased receptor production occurs in an attempt to sequester iron, or may reflect different responsiveness of the duodenal mucosal cells to signals modulating iron absorption in genetic hemochromatosis[61]. Further evidence that the upregulation of transferrin receptor levels is not the cause of the increased iron accumulation is the fact that the gene encoding transferrin receptor is found on chromosome 3[78], but the abnormal gene responsible for genetic hemochromatosis is located on chromosome 6.

Elevated levels of the trimeric receptor of microvillous membrane vesicles described by Stremmel and Teichmann are found in hemochromatosis[79]. Although little is known regarding control of this putative protein, it is possible that the reduced iron content in the labile intracellular iron pool in these patients leads to upregulation of these receptors, as is seen in iron deficiency.

Further evidence of an abnormal transfer of iron from the mucosal cell to the portal circulation has been provided by studies showing normal[80] to consistently elevated IRP activity in duodenal mucosal cells[55] as well as monocytes and macrophages in patients with hemochromatosis[81]. This aberration of IRP activity was not found in other circulating blood cells such as lymphocytes[81]. In vitro experiments in genetic hemochromatosis using monocytes and macrophages indicate that regulation of IRP activity is normal, while abnormal upregulation of IRP activity exists in vivo. IRP activity is reduced in secondary iron overload disorders[55]. Quantification of spontaneous IRP activity in duodenal biopsy extracts show an increased IRP activity of 1.5 in genetic hemochromatosis patients compared to normals, while patients with secondary iron overload show a reduced activity of 0.85 compared to normals. The heightened IRP activity in genetic hemochromatosis patients results in diminished ferritin mRNA translation and thus diminished ferritin production in the cell. The increased activity of IRP is best explained by the inappropriately heightened iron release from the mucosal cell into the portal circulation, rendering the intracellular labile iron pool relatively iron depleted. Similar changes occur in monocytes/macrophages[81] with increased iron release into the circulation. In the studies of duodenal biopsy samples noted above[55], a 25% reduction of ferritin content was found compared to normal subjects, despite markedly elevated serum ferritin levels in this group. In the case of the monocyte/macrophage system, abnormally increased iron release has been shown from macrophages in vitro and from the spleen in ferrokinetic studies in vivo in genetic hemochromatosis[82, 83]. These findings suggest that modulation of IRP protein function is defective in hemochromatosis, indicating a defect involving the labile iron pool in specific cells such as the monocyte/macrophages of the RE system, and duodenal cells in the gastrointestinal tract.

Recently an abnormal gene marker present in 85% of hemochromatosis patients and 3% of controls has been identified in the HLA-H gene region. The abnormal gene

consists of a guanine to adenine transition at nucleotide 845 of the open reading frame, resulting in a cysteine to tyrosine substitution at amino acid 282[66, 84]. The result of this mutation would be an effect on MHC proteins at a highly conserved portion of the molecule involved in intramolecular disulphide bridging. An abnormality involving this portion of the protein could disrupt its normal structure and function[66], and since this portion of the protein is involved in non-covalent interaction with beta$_2$-microglobulin, the correct cell-surface presentation of the HLA-H -beta$_2$-microglobulin complex is disrupted[85]. How and whether this abnormality results in the excess iron absorption occurring in genetic hemochromatosis is unknown at this time. A second mutation of the HLA-H protein, his 63 asp, has been described, but its role in causing iron loading is less likely[66, 84].

The manifestations of iron overload in the recently described β_2-microglobin knockout mouse model closely resemble the metabolic iron abnormalities seen in genetic hemochromatosis. Progressive hepatic iron overload occurs in this model in a manner similar to genetic hemochromatosis, thus implicating β_2 -microglobulin or MHC class 1 proteins as playing a role in the iron overload of this disease[67, 68]. These mutant mice display many of the clinical features seen in genetic hemochromatosis, including the development of hepatocellular carcinoma. The animals fail to downregulate dietary iron absorption, even in the iron overloaded state. The increased iron absorption is due to increased iron transfer from the mucosal cell to the circulation, as is seen in hemochromatosis. Iron absorption levels are 16% to 34% greater than those in control animals at ages ranging from 2 to 12 months[86]. Similar failure to downregulate iron transfer occurs in these mice when experimental iron overload has been induced. These findings all indicate the HLA-H gene product as the likely cause for the clinical manifestations of genetic hemochromatosis, since its normal function is dependent on its close association with the β_2-microglobulin molecule. It has been proposed that defective signaling may occur regarding body iron status between endocytosed transferrin–transferrin receptor complexes and HLA-H proteins within clathrin-coated endocytotic vesicles. Thus, appropriate information regarding iron stores is not conveyed to the appropriate intracellular mechanisms controlling iron absorption from the mucosal cell[87].

Conclusions and future directions

Significant progress has been made in the past decade in understanding the proteins involved in iron absorption and their functions. Our understanding of the precise mechanisms and pathways is still considerably limited, however, and significantly more information is required about each phase of iron absorption . The precise mechanisms of iron uptake into the cell are poorly understood. Whether iron merely diffuses into the mucosal cell via iron channels or is adsorbed by specific receptors and then internalized via specific mechanisms such as pinocytosis or endocytosis is unknown. It appears that transferrin and transferrin receptors do not play a role in this phase, and lactoferrin may only be important for iron procurement in the neonatal period. The integrins, fatty acids and the 54 kD membrane protein[27] may be important, and further study is needed of these, and the exact mechanisms of internalization.

The mechanisms of transcellular iron transport are unknown. Mobilferrin may play a role in transferring iron in the apical segment from membrane receptors such as integrins further into the cell. Better insight has been gained into the prominent role played by ferritin, the control mechanisms regulating its production, and its function as an iron storage protein. Significant strides have been made in understanding the mechanisms by which IRP functions to control ferritin production for iron storage. Not all the mechanisms controlling and influencing IRP activity are clearly understood, however. For example, increased nitric oxide synthase activity is found in duodenal mucosal cells in iron deficiency[88], but how this may influence IRP activity is unknown. Could the localization of the HLA-H protein in the cytoplasm of the enterocyte allow it to interact with IRP in the cytoplasm? A defect in this putative interaction may be an important factor in the abnormal iron regulation of genetic hemochromatosis. The mechanisms of iron acquisition by the cell for its function, via control of transferritin receptor production by IRP, are understood. Although unlikely, a possible role for transferrin in transporting iron out of the cell cannot be entirely excluded. It has been shown that transferrin receptors on the basolateral surface of the cells are capable of binding apotransferrin[64], and the internalization of apotransferrin may permit it to bind and transport iron out of the cell. A significant argument against this possible pathway is the development of iron overload in atransferrinemic models.

Very little is known about iron transfer from the mucosal cell to the portal circulation. The development of iron overload in β_2-microglobulin knockout mice has provided further insight into the possible defect causing increased absorption in genetic hemochromatosis. The discovery of the presumed protein product of the HLA-H gene in the duodenal mucosal cell and its distinct location has thrown

some light on possible mechanisms of regulation of absorption within the cell . Although the HLA-H defect and its presumed protein product have been located, how this affects the dysregulated iron metabolism in genetic hemochromatosis is unclear.The presumed functions of this protein as an iron binding ligand or a possible role in signal transduction are merely speculative at this time[89]. The perinuclear localization of the protein material suggests a signaling function in intranuclear genetic control mechanisms. Considerably more information is required in order to fully comprehend the entire mechanism and regulation of iron absorption.

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11

Intestinal iron absorption and hemochromatosis

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Introduction

Idiopathic hemochromatosis is a familial genetic disorder in which iron absorption consistently exceeds losses of body iron[1]. This leads to accumulation of iron in multiple body organs. When iron concentrations become sufficiently excessive, cellular damage and destruction occur with organ dysfunction and failure. This may become manifest as either cirrhosis of the liver, hepatocellular carcinoma, diabetes mellitus, cardiomyopathy, hypogonadism, or arthritis, or a combination of these disorders. Because hemochromatosis usually becomes clinically manifest during the fifth and sixth decades of life, it usually takes many years of iron accumulation before a homozygote seeks medical attention. Presumably, this long delay occurs because body organs possess a capability to store iron far in excess of body needs. Once the iron-binding capacity of tissues are exceeded, ionic iron becomes available to produce 'free radical' formation and cellular damage[2, 3]. An enigma that remains unexplained is that studies of the prevalence of homozygous hemochromatosis in Caucasians (~0.6%) indicate that genotypic hemochromatosis is at least ten times more commonplace than clinically manifested phenotypic hemochromatosis (1 in 20 000 hospitalizations)[1, 4]. This suggests that factors in addition to a single genetic mutation are necessary to produce massive iron overloading with organ damage.

Although the genetic defect for about 85% of Caucasian hemochromatotics was recently identified (HLA-H, C282Y), the method by which it enhances iron accumulation by the body remains unknown[4]. One possibility is that the genetic abnormality acts directly upon the intestinal absorptive cell to enhance the absorption of iron. The alternative is that the genetic abnormality affects a non-intestinal cell (such as a reticuloendothelial cell) that subsequently informs the absorptive cells to enhance iron absorption regardless of the repletion of body iron stores. The virtual absence of stainable iron in the intestinal absorptive cells of hemochromatotics, similar to observations in the gut of iron-deficient patients, is consistent with both hypotheses[5]. Regardless of the answer to this question, a basic understanding of iron absorption and balance is relevant to the pathophysiology of hemochromatosis and other iron overloading disorders.

Iron balance

A fetus accumulates iron from maternal stores during pregnancy and has a total body iron content of approximately 250 mg at birth (80 ppm). Iron is transferred to the fetus via the placenta at the expense of maternal iron stores; this occurs mostly during the third trimester of pregnancy[6].

The newborn infant is fed an iron-deficient milk diet and acquires little iron during the first few months of life, unless the diet is supplemented with iron[7]. In normal infants the total body iron concentration diminishes to 60 ppm which, in the presence of adequate dietary iron, is maintained throughout life. The growing child must absorb an average of 0.5 mg of iron daily in excess of body losses to maintain this concentration and attain a body iron content of about 4 grams as an adult (Fig. 11.1)[8, 9]. The adult preserves a constant level of body iron by efficient conservation, maintaining rigid control over absorption to balance losses. An adult man loses about 1 mg of iron daily, mostly in defoliated epithelium and secretions from the gut and skin[10–13]. During the childbearing years, the normal female loses an average of an additional milligram of iron daily from menstrual bleeding (40 ml blood loss) and about 500 mg with each pregnancy[14–16]. This results in iron deficiency unless each of these loses is balanced by the absorption of an equivalent amount of iron. Conversely, consistent absorption of more iron than is lost from the body causes siderosis and possible tissue damage.

Previously, it was believed that the quantity of iron in the body was controlled solely by regulation of absorption and that excretion played a totally passive role[17]. However, most cells contain iron somewhat in proportion to the quantity of iron in body stores. Thus the daily obligatory loss of cells from the skin and gut and from secretions such as bile and sweat provides a limited but selective loss of body iron. In addition, there is a normal daily fecal loss of approximately 0.7 ml of blood (0.3 mg iron)[10, 12–14]. Little iron is excreted in urine (<0.1 mg/day) in the absence of significant intravascular hemolysis[18, 19]. In normal iron-replete adults, losses are balanced by absorption of sufficient dietary iron (1–2 mg/day) to maintain a relatively constant amount of body iron throughout life. If consistent alterations in this balance occur, either iron deficiency anemia or siderosis ensues. In dietary or transfusional siderosis, daily excretion of iron can exceed absorption by as much as 4 mg/day in an attempt to re-establish the normal body iron content[12, 20, 21]. This is probably maximal excretion of iron in the absence of hemorrhage or blood-letting. This rate of iron excretion does not occur in the development of phenotypic hemochromatosis[22]. Although excretion is quantitatively as important as absorption in the maintenance of iron balance, absorption usually plays the more active regulatory role[17, 21].

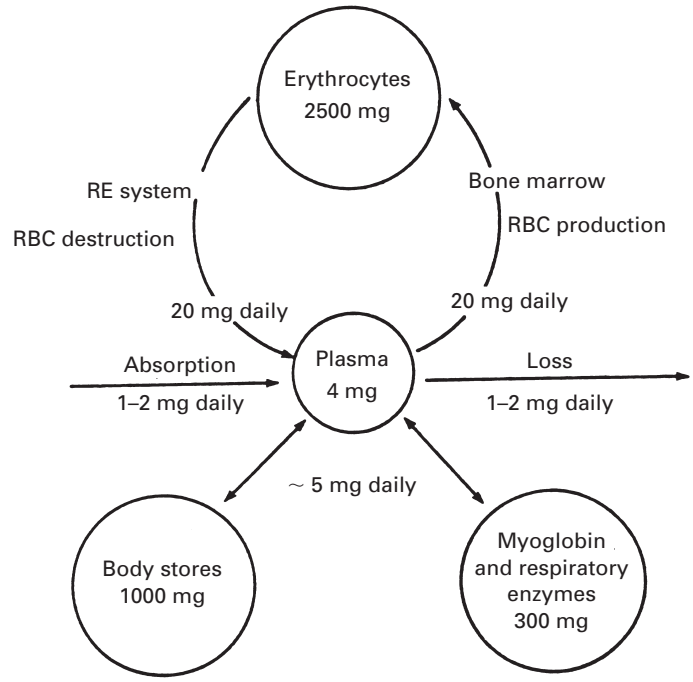


Fig. 11.1 The total body iron in a 70 kg man is about 4 g. This quantity is normally maintained by establishing a balance between absorption and body losses. Although the body absorbs 1–2 mg of iron daily to maintain equilibrium, the internal requirement for available iron is greater (20–25 mg). A red blood cell (RBC) has a normal life span of 120 days; 0.8% of circulating erythrocytes are destroyed daily and must be replaced. A 70 kg man with a 5-liter blood volume has 2.5 g of iron incorporated into circulating hemoglobin with a turnover of 20 mg of iron from hemoglobin degradation and formation and another 5 mg for other requirements. Most of this iron passes through the plasma for reutilization. During childbearing years females have a smaller quantity of iron in body stores (~300 mg) than men (~1000 mg). Persons with hemochromatosis who have clinical manifestations of iron overloading may have 25 g of iron in body stores.

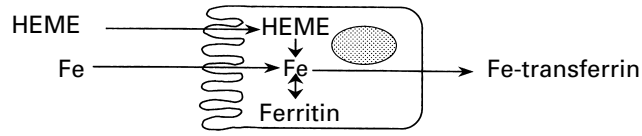
Iron absorption

Iron absorption occurs mostly in the duodenum and jejunum where the mucosal absorptive cells remain attuned to current body requirements for iron[21, 23–25]. Although the exact mechanisms regulating iron absorption across intestinal mucosa are unknown, recent information provides greater insight into factors involved with the absorptive process. The simplest model of iron absorption must consider the mucosal uptake of iron from the lumen of the gut and the transfer of this iron from the intestinal cells into the body (Fig. 11.2). It provides three anatomic locations for studying regulatory factors. These are: (i) the intestinal lumen, (ii) intestinal absorptive cells, and (iiii) corporeal (somatic) factors[21, 24].

Intraluminal factors

Ample dietary iron must be exposed to the intestinal absorptive cells for a sufficient interval and in a physicochemical form that permits absorption of enough iron to meet body requirements.

The dietary iron content can be an important factor affecting iron repletion. A low incidence of iron deficiency is found in North American and European men whose diet contains 10–20 mg of iron. In contrast, iron deficiency is more prevalent in females because they eat less food than men and require, on average, twice as much iron due to blood loss from menstruation and childbirth. Certain Asiatic vegetarian diets are iron deficient and their ingestion is often associated with iron deficiency. However, these diets often contain adequate iron but it is not in a physicochemical form that is readily available for absorption. Populations consuming excessive amounts of iron can develop iron overloading, particularly when this occurs in association with intake of alcohol[26, 27]. This was demonstrated best in Bantus in South Africa who consumed beer brewed in iron kettles. Generally, increased absorption of iron occurs with



Intraluminal factors	Mucosal factors	Corporeal factors
Dietary iron	Anatomic factors	Body iron stores
Quantity	Diminished absorptive surface	Iron turnover
Chemical form	Detective epithelial cells	Erythroporesis
pH	Mucosal lifespan	Anabolism/catabolism
Chelation and precipitation	Intestinal motility	Reticuloendothelial blockade
	Mucosal iron	Sideroblastic disorders
	Quantity	Thalassemia
	Chemical form	Sideroblastic anemia
	Certain metals	Congenital dyserythropoeitic anemia
	Drugs that effect protein synthesis	Hypoxia
		Idiopathic hemochromatosis
		Neonatal hemochromatosis
		Atransferrinemia
		Ferrochelatase deficiency
		Porphyria cutanea tarda
		Ceruloplasmin deficiency
		Transferrin immune complex disease
		Nonalcoholic steatohepatitis
		Cirrhosis
		Portacaval shunt
		Familial hypochromic anemia with siderosis

Fig. 11.2. Dietary iron contains both inorganic iron and heme-iron. Both chemical forms are absorbed by absorptive cells of the proximal small intestine. Many intraluminal factors that enhance or diminish the absorption of inorganic iron have no effect upon the absorption of heme-iron. Within the intestinal absorptive cell, iron is released from heme by heme oxygenase and the iron enters plasma as inorganic iron. Factors that affect various stages of iron absorption are depicted.

increases in the quantity of dietary iron[28, 29]. However, a ten-fold increase in a test dose of inorganic iron results in only a four-fold increase in the quantity absorbed[28] (Fig. 11.3). This indicates that intraluminal iron molecules compete with each other for absorption. This barrier provides a limited 'mucosal block' that can be overcome with massive doses of iron, such as those seen in acute iron poisoning.

The physicochemical form of inorganic iron affects iron absorption[30, 31]. Dietary constituents that solubilize iron enhance absorption, whereas compounds that either precipitate or polymerize iron decrease absorption[31–34]. Iron is absorbed poorly from test doses of ferric salts unless it is either reduced or chelated. This occurs because ferric iron is insoluble in aqueous solutions more alkaline than pH 3, whereas most ferrous iron remains soluble at neutral pH[35–37]. Certain amines, amino acids, and sugars decrease the polymerization and precipitation of iron in aqueous solutions by interfering with the formation of hydroxyl bridges between iron molecules. These iron chelates must be formed at acid pH utilizing ferric iron in a soluble state. Therein lies the importance of hydrochloric acid in facilitation of iron absorption[33]. Long-standing achlorhydria and chronic use of histamine receptor antagonists produce iron-deficiency anemia. Other iron chelators found in the diet such as carbonates, oxalates, phosphates, phytates, and tannates combine with iron to form insoluble precipitates and macromolecules that are poorly absorbed[30, 38, 39]. Similarly, clay and laundry starch render iron less absorbable and their habitual ingestion leads to iron deficiency[40, 41]. Thus when radioiron is either incorporated into foods or added using the extrinsic tag methods[42, 43], the absorption of inorganic iron from bread and vegetables is low compared with that from meat that contains an abundance of amino acids released by proteolytic digestion.

Gastrointestinal secretions play a role in altering iron absorption. Gastric hydrochloric acid solubilizes ferric iron to make it available for chelation with substances that enhance absorption in the less acid milieu of the small intestine[31]. Mucins appear to play an important role in this pH-dependent reaction, and even accept iron complexed to dietary facilitators of iron absorption such as ascorbate, fructose and histidine[44]. Bile enhances iron absorption because it contains significant quantities of ascorbic acid and other substances that reduce and chelate iron[33]. Pancreatic bicarbonate diminishes iron absorption, and its decreased secretion in cystic fibrosis and chronic pancrea-

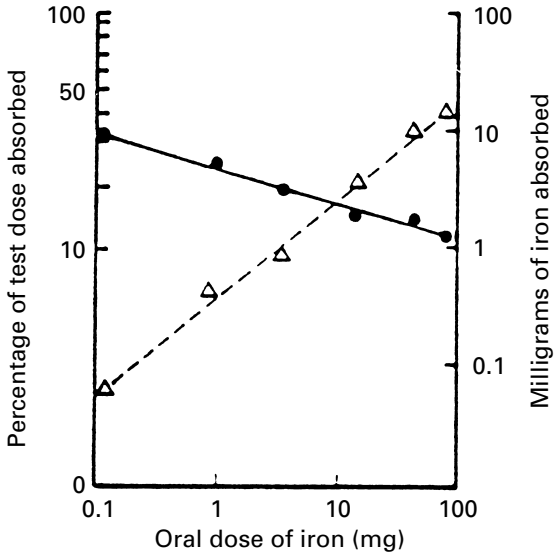


Fig. 11.3. Although more iron is absorbed from larger doses of iron, the percentage absorbed decreases. A 100-fold increase in the quantity of iron in a test dose is associated with only a 40-fold increase in the quantity absorbed. Thus there is a limited 'mucosal block.' In hemochromatosis, more iron is absorbed at each dosage level until the patient becomes iron overloaded. The results of these iron absorption studies are within the expected range for normal subjects.

titis may explain the increased iron absorption reported in these conditions[31, 45–47]. Intestinal enzymes that release sugars and amino acids from food are believed to enhance iron absorption by forming iron chelates that remain soluble in the small intestinal lumen[48].

Absorption of heme iron

The absorption of myoglobin and hemoglobin-iron from meat differs from that of inorganic iron (Table 11.1). People from geographic areas where meat is an important constituent of the diet are usually iron replete, even though they may consume less dietary iron than persons in many populations consuming grain diets. Hemoglobin-iron is absorbed from food more efficiently than inorganic iron and in a different manner[49–53]. Most hemoglobin-iron enters the small intestinal absorptive cell as an intact metalloporphyrin. Current evidence suggests this is facilitated by a heme receptor, and heme enters the cell via a vesicle, similar to observations in bacteria that can catabolize heme from tissue culture media[54, 55]. Once heme is within the cell, iron is released from porphyrin by mucosal heme oxygenase so that it can enter the circulation as inorganic iron[56]. Unlike inorganic iron, heme is soluble in alkaline solutions and is precipitated as hematin in an acid milieu[34, 57]. This makes

Table 11.1. Postulated absorption of inorganic and heme iron

	Fe^{3+} (pH2)	Fe^{2+}	Hemoglobin or myoglobin
Gut Lumen	Mucin; Ascorbate, Histidine, Fructose	Mucin; Ascorbate, Histidine, Fructose	Proteolysis
	Soluble Fe^{3+} chelate	Fe^{2+} or Soluble chelate	Heme; Bile salts, Amino acids
			Soluble heme chelate
Absorptive Membrane	β3 Integrin	?	Heme vesicle
Cytosol	Fe^{3+} Mobilferrin	Fe^{2+}	Heme oxygenase
	Paraferritin	DCT-1	Paraferritin
	NADPH		NADPH
	Fe^{2+}		Fe^{2+}

chelation less important to maintain solubility in the duodenum. However, purified heme is poorly absorbed because it polymerizes. Hemoglobin degradation products and certain amino acids, amines, and amides increase the absorption of heme-iron by diminishing its polymerization within the intestinal lumen[34, 58]. That there is a common absorptive pathway for hemoglobin and inorganic iron was shown by demonstrating competitive inhibition between simultaneously and sequentially administered hemoglobin and inorganic iron[59]. Chase studies demonstrated that the iron released from heme is donated to mobilferrin/paraferritin so that it can be either utilized for production of iron-containing proteins, or be transferred into the body as inorganic iron[60].

Mucosal factors

The quantity of iron in the body is maintained primarily by controlled absorption from the duodenum and jejunum and, to a lesser extent, by control of excretion. To maintain iron balance, iron must be exposed to a sufficient number of absorptive cells that remain attuned to body requirements for iron. Enough iron must be absorbed to compensate for body losses of iron while available but unneeded dietary iron is rejected.

Anatomic and histological abnormalities that decrease the absorptive surface of the proximal small intestine diminish iron absorption[61]. Intestinal resection or surgical procedures that divert ingested food from the duodenum such as gastrojejunostomy are often associated with iron deficiency. A diminished intestinal absorptive surface occurs in normal subjects in the development of populations and may contribute to the occurrence of iron

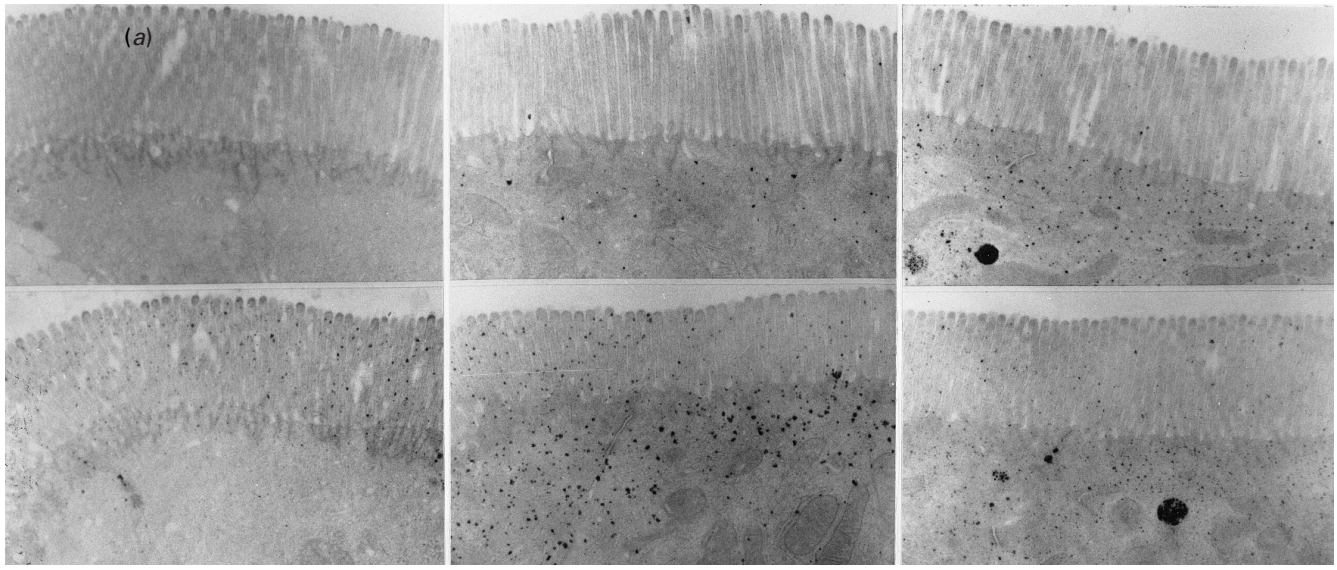


Fig. 11.4(*a*). Ultrastructural photomicrographs of microvilli and apical cytoplasm of duodenal epithelial cells from fasting iron-deficient rat (left), normal control rat (middle), and an iron-overloaded rat (right). Tissues in the top row were stained with acid ferrocyanide. Dense particles represent stainable iron. Virtually no iron was observed in the iron-deficient specimen; increasing amounts of iron are visualized in the more iron-replete rats. Specimens in the bottom row were incubated with Fe-NTA to saturate iron-binding sites before being stained with acid ferrocyanide (presumably mobilferrin). There was no significant difference in the amount of stainable iron in specimens from either iron-deficient (left), normal control (middle), or iron-overloaded (right) rats, suggesting that the quantity of iron binders did not vary with the state of iron repletion. Quantitative measurements of mobilferrin and mRNA for mobilferrin provided similar results. The large densities in the iron-loaded animals are siderosomes containing ferritin.

deficiency in these people[62]. Sprue flattens the intestinal villi and affected patients have an increased incidence of iron-deficiency anemia. Increased intestinal motility decreases iron absorption and drugs that slow intestinal transit increase iron absorption[63]. Thus, the length of time iron is exposed to the absorptive surface can alter iron absorption. In animal studies, the effective intestinal absorptive surface is increased in iron deficiency so that more iron may be absorbed from the distal small intestine. Although alterations in the actual or relative size of the effective absorptive surface are significant in certain circumstances, the effectiveness of the mucosal cells in regulating iron absorption seems more important.

In 1943, Hahn postulated that iron absorption was regulated by a mucosal receptor that blocked iron absorption when it became satiated with iron[64]. Much evidence exists from both human and animal studies to support a limited 'mucosal block' of iron[28, 65]. Iron in the epithelial cells may be derived either from iron within the body or from the lumen of the intestine. Further, the quantity of non-heme iron in intestinal absorptive cells varies directly with the state of iron repletion and inversely with the quantity of iron that will be absorbed from test doses of iron in most experimental circumstances that affect iron absorption[21, 66] (Fig. 11.4). Thus in iron deficiency, enhanced erythropoiesis and hypoxia are associated with a decreased concentration of non-heme-iron in intestinal specimens, and with an increase in absorption of iron[21, 67]. For many years it was believed that ferritin was the receptor that regulated iron absorption, and that apoferritin increased iron uptake into the cell and holoferritin diminished iron absorption. This hypothesis was disproven because immunologic studies showed there was little or no apoferritin in the absorptive cells of iron-deficient animals[68–70]. Presently, it is believed that ferritin serves predominately as a storage protein that incorporates iron to protect cells from oxidative damage from ionic iron, and as a reserve supply of iron.

Unlike other nucleated cells, the luminal surface of absorptive cells contain no transferrin receptors[71, 72]. Thus, iron must enter the cell via a mechanism different than the classical transferrin–transferrin receptor pathway. This led to a search for proteins that mediated uptake of iron by intestinal mucosal cells[73, 74, *].

Unlike other nucleated cells in the body, the luminal surface of absorptive cells contain no transferrin receptors[71, 72]. Thus iron must ingress the absorptive cells via a mechanism different from the classical transferrin–trans-

* Teichman and Stremmel reported a '160 kDa protein with three identical 54 kDa monomers.' It is speculated this is mobilferrin (56 kDa) and the α-subunit of an integrin (160 kDa) that binds mobilferrin[75].

ferrin receptor pathway. This led to a search for proteins which mediated the uptake of iron by the small intestinal mucosal absorptive cells[73, 74]. Two seemingly distinct pathways were identified for uptake of inorganic iron into cells. The mobilferin–integrin pathway was identified by biochemical isolation of proteins which were radiolabeled following injection of radioiron into the duodenal lumen. This pathway is specific for the absorption of ferric iron[75]. The other pathway facilitates the uptake of ferrous iron and was discovered using expression cloning in frog oocytes and a positional cloning strategy in mice[76, 77]. It involves a protein which was identified as Nramp-2 (Natural Resistance Associated Macrophage Protein) but is now named DCT-1 (Divalent Metal Transporter). These pathways are briefly described below because they are probably involved in the iron overloading which occurs in hemochromatosis and other iron-overloading disorders.

Iron-binding complexes were identified from duodenal homogenates after installation of radioiron in isolated gut loops of rats. Four previously unidentified iron-binding complexes were found, in addition to transferrin and ferritin[78](Fig. 11.5). These were mucin[44], a 56 kDa protein named mobilferrin that is a homologue of calreticulin[79, 80] (cytosolic mobilferrin may be identical to calreticulin except for the KDEL carboxy-terminal sequence binding calreticulin to endoplasmic reticulin), a 240 kDa β_3 integrin[81], and a 520 kDa protein complex named paraferritin[82, 83] because it contains β_3 integrin, mobilferrin, β_2 microglobulin, and flavin monooxygenase. Paraferritin behaves as a NADPH-dependent ferrireductase that is probably important in delivering iron in the appropriate redox state for synthesis of iron-containing proteins. The role of β_2 microglobulin in the complex may be important, because β_2 microglobulin knockout mice become iron overloaded, and β_2 microglobulin and mobilferrin/calreticulin are postulated to be important in the etiology of hemochromatosis[84, 85]. The potential role of each of the newly identified iron-binding proteins and complex is shown in Fig. 11.5. This was facilitated by independent observations showing binding of calreticulin to the carboxy-terminal cytosolic end of integrin, and an association of these complexes with flavin monooxygenase[86, 87]. The mobilferrin–integrin pathway appears to exist in most nucleated mammalian cells. In non-intestinal cells, it accepts iron from transferrin, probably to deliver iron in the appropriate redox state for use in the synthesis of end-products such as heme-proteins[88]. Other divalent cations can utilize this pathway for transport. However, certain metals, e.g. zinc, use a β_1 rather than a β_3 integrin. This difference may prevent zinc deficiency in people on an iron-replete diet[89].

While the mobilferrin–integrin pathway was found using classical biochemical methods, DMT-1 was discovered using

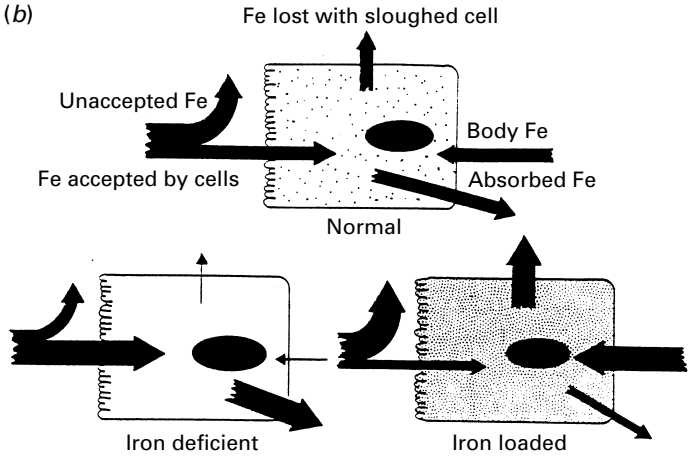


Fig. 11.4(*b*). Iron is absorbed from the lumen of the gut into the small intestinal mucosa and then transferred into the body. The quantity of iron absorbed usually depends on the current body requirements for iron. This figure illustrates a model for the control of iron absorption at the level of the intestinal mucosa. In normal subjects, the mucosal cells contain variable amounts of iron derived from the diet and body stores. This deposit regulates, within limits, the quantity of available intraluminal iron that enters the cells. This probably occurs by saturating iron-binding receptors on iron-binding protein(s); free ionic iron would be toxic. Cellular iron may enter the body to meet current body requirements or may remain within the cell to limit mucosal uptake of iron and be lost when the cells are sloughed from the villus at the end of their 2–3 day life span. In iron-deficient subjects, little iron is incorporated into these cells from body stores, so that absorption is enhanced and excretion is diminished. In iron overloaded subjects, increased amounts of body iron are incorporated into cells to limit absorption and enhance excretion.

molecular biological methodology. DMT-1 was upregulated in iron deficiency and a missense mutation was identified in mk mice and Belgrade rats[76, 77]. The homozygous mutant rodents were born with iron deficiency anemia and a defect in intestinal iron absorption and hemoglobin production. It is postulated that DMT-1 acts as an iron transporter in the intestinal microvilli and in transferrin containing endosomes[90]. In normal iron replete humans (unpublished, observation) and mice, DMT-1 was not identifiable immunologically in the microvilli of intestinal absorptive cells[91]. In contrast, the protein was easily perceived in the mucosa of the proximal duodenum of severely iron deficient mice but not in other portions of the small intestine. Whether this is a technical problem related to antibody sensitivity or evidence that DMT-1 serves an emergent role in iron deficiency to enhance ingress of iron into the mucosal absorptive cell. There is little doubt that DMT-1 serves other roles in cells. The phenotypic manifestations in mk mice and Belgrade rats are most severe in utero (fetal wastage) and shortly after birth at

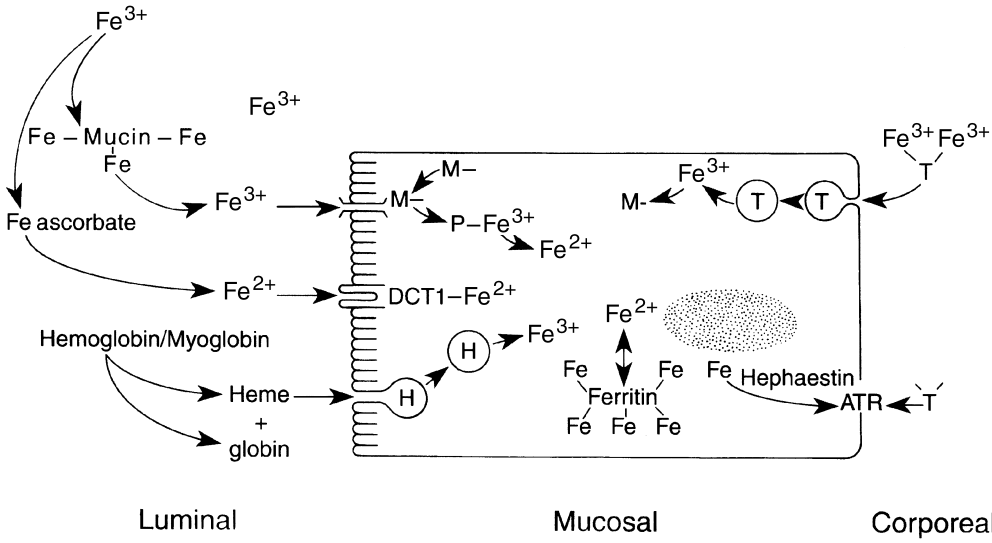


Fig. 11.5. Three pathways exist in the small intestinal absorptive cell for the uptake of food iron. Most dietary iron is in the ferric state. It is mobilized from food in the acid stomach for chelation with mucin, ascorbate, histidine, fructose, etc. Chelation keeps the iron soluble when it enters the less acidic duodenum. Ferric iron enters the absorptive cell via a β_3 integrin in combination with mobilferrin(M). A large complex named paraferritin(P), which contains integrin, mobilferrin and flavin monooxygenase; this permits ferrireduction so the ferrous iron is available for incorporation into compounds such as ferritin and heme. In the lumen of the gut variable quantities of ferrous iron are formed due to reduction of ferric food iron by ascorbic acid and other reducing agents. Entry of ferrous iron into cells is probably facilitated by DCT-1. Heme iron is absorbed via a different mechanism. Heme is enzymatically digested free of hemoglobin in the intestinal lumen and enters the absorbtive cell as an intact metalloporhyrin. This is probably mediated by an endosome. Within the cell the heme is degraded by heme oxygenase with release of inorganic iron. The cell protects itself from free radical formation by incorporation of excess iron into ferritin. On the basolateral membranes there are transferrin receptors. The holotransferrin receptor permit entry of the transferrin–iron complex into the cell via a clathrin coated endosome. Iron is released from the vesicle to enter the cell and inform the cell of the iron status of the body. This probably acts by downregulating DCT-1 and by saturating mobilferrin so that it does not bind the integrin on the microvillus surface to facilitate entry of iron into the cell. The egress of iron from the absorptive cell is facilitated by hephaestin and may involve an apotransferrin receptor on the basolateral membranes.

a time when most body iron was delivered via the transferrin–transferrin receptor pathway in the placenta rather than through the fetal gut. Similarly, fluorescent antibody studies using anti-DMT-1 antibodies show the vast majority of reactivity within cells is intracellular rather than on surface membranes.

The concept that the mobilferrin–integrin pathway facilitates uptake of ferric iron whereas the DMT-1 pathway subserves uptake of ferrous iron is supported by studies of competitive inhibition and separate blocking antibodies against both anti-β integrin and DMT-1[75]. The relative physiological roles of the two inorganic iron pathways are not known. However, available information shows: (i) DMT-1 missense mutations are associated with iron deficiency in rodents, the mobilferrin knockout mouse is not viable; (ii) DMT-1 is drammatically increased in severe iron deficiency in the proximal duodenum of mice[91], the activity of the mobilferrin–integrin pathway is increased in iron deficient rats[92], (iii) the DMT-1 protein is immunologically undetectable in microvilli of freshly obtained human duodenum (Whipple surgery) and in the duodenum of normal mice[91], the mobilferrin–integrin pathway is active in normal human duodenum[93] and (iv) the DMT-1 pathway facilitates uptake of some other non-ferrous metals of nutritional importance (Mn but not Zn and Cu) whereas the mobilferrin–integrin pathway is specific for uptake of ferric iron into cells.

Two additional proteins were identified which appear to be associated with iron transport. Both are located intracellularly rather than on surface membranes. SFT (Stimulator of Iron Transport) was located in close association with endosomes and influences cellular iron uptake[94–95]). Recently, a ceruloplasim homologue, named hephaestin, was described which may explain the absorptive defect in sla mice; it is a ferro-oxidase which appears to regulate the egress of iron from absorptive cells into the plasma[96]. An understanding of the interrelationship of these pathways and proteins is needed to provide a more meaningful knowledge of cellular iron transport.

The mucosal transfer of iron from intestinal absorptive cells into the plasma has been an enigma. Unlike the micro-

villi on the luminal surface, absorptive cells possess transferrin receptors on their basolateral surfaces. The transferrin–transferrin receptors probably function in absorptive cells in the same manner they operate in non-intestinal cells: by facilitating the entry of plasma iron into cells. Thus in absorptive cells, the classical transferrin–transferrin receptor mechanisms would deliver body iron into absorptive cells. This would serve as a mechanism to inform the absorptive cell of the state of iron repletion of the body, and as a means of increasing iron loss from an iron-replete or overloaded host[67]. This does not occur in hemochromatosis despite a markedly elevated serum iron concentration and iron overload body stores of iron. Recently, a 100 kDa complex was identified in human duodenal mucosal membrane that bound apotransferrin. Because apotransferrin does not bind to the classical transferrin receptor effectively at physiological pH[86] and the newly identified apotransferrin receptor has a different molecular mass, it may serve as the gateway for iron to enter the body from the absorptive cell. It is doubtful that the 'docking' of transferrin on the apotransferrin receptor is essential for the transit of iron into the plasma, because patients with hereditary atransferrinemia become iron overloaded[86]. However, the proximity of apotransferrin to iron entering the body would provide a safeguard against 'free radical' formation and iron toxicity[87].

Corporeal factors

The most important known stimuli to iron absorption are the rate of erythropoiesis and status of tissue iron stores[29]. Accelerated red blood cell production caused by bleeding, hemolysis or hypoxia seems related to enhanced iron absorption. Conversely, diminished erythropoiesis, such as that which occurs with starvation, blood transfusion or return to sea level from a hypobaric environment, decreases the absorption of iron[29, 33, 64, 66, 98]. It is tempting to postulate that an erythrocyte stimulating factor acts as a messenger to the gut to increase iron absorption. However, enhanced iron absorption continues in iron-deficient subjects long after the hemoglobin mass is restored to normal levels, and persists until the body stores become normally replete with iron[33, 99, 100]. In addition, certain patients with Laennec's cirrhosis and sideroblastic anemias have increased iron absorption without accelerated erythropoiesis or diminished iron stores[101–103]. In these patients, increased absorption seems related to increased iron turnover. An experiment by Finch et al. showed increased iron absorption after the transfusion of reticulocytes; this increases the number of circulating transferrin receptors and iron turnover in the recipient[104]. Because accelerated hemoglobin production depletes iron stores, the level of labile iron in tissue stores may be a more basic regulatory factor[105]. If one postulates the existence of tissue iron receptors, increased iron absorption would be expected to continue until these receptors were satiated; then surplus iron would become available for incorporation into intestinal cells both to inhibit absorption and to enhance excretion. Thus hemochromatosis could be either a disorder of such iron receptors or an inability to use them properly.

Many investigators searched for a blood factor that signaled the gut to enhance or diminish iron absorption[104, 106]. Clinical observations indicated that the hemoglobin concentration and plasma concentrations of iron, transferrin, and ferritin did not perform those functions acutely[106, 107]. Most corporeal factors that alter iron absorption did not exert an effect for several days[28, 64, 108]. That this lag was not caused by the two- to three-day lifespan of absorptive cells was suggested by the rapid diminution of iron absorption after an injection of endotoxin or removal from a hypoxic environment and the rapid increase in iron absorption that occurs after transfusion of reticulocytes[98, 104, 109, 110]. Thus factors that have a delayed effect on absorption such as phlebotomy, or the administration of erythropoietin, thyroid and pituitary extracts probably influence iron absorption by increasing body iron requirements and not by a direct effect on the gut[105, 110, 111]. Additional evidence that erythropoietin hormone is not a direct regulator of iron absorption was provided by the lack of effect of erythropoietin hormone on iron absorption when the bone marrow was depressed by irradiation[112]. This seems to indicate that hypoxia can act as a factor independent of the body iron stores and iron turnover, because it causes increased iron absorption in animals following marked bone marrow suppressive by radioactive strontium administration[112].

Several days after phlebotomy or pathologic hemorrhage, humans have a transient decrease in the serum iron concentration and a sustained increase in the rate of plasma iron turnover. These changes occur simultaneously with the onset of enhanced iron absorption. The rapid iron turnover persists as long as iron absorption is abnormal[113]. Animal studies show a similar relationship between the increased absorption of iron and the rapid plasma clearance of radioiron in many physiologic circumstances[114, 115]. Moreover, the injection of endotoxin causes a reverse relationship; a decreased absorption of iron with rapid plasma iron clearance, but a diminished total plasma iron turnover[109]. Whether this indicates that plasma iron turnover has no etiologic relationship to iron absorption, or that endotoxin alters iron absorption by a different mechanism of action than more physiological factors, is unknown. Studies in which the transfusion of reticulocytes was associated with

increased iron absorption support the hypothesis that the number of available iron receptors influences iron absorption; this would also affect plasma iron clearance[104]. However, the manner in which either plasma iron clearance or the number of available iron receptors could affect absorption by the intestinal mucosal cells independent of changes in the serum iron concentration or the quantity of apotransferrin in the circulation has not been delineated. Further, the subject is complicated by clinical studies showing that aged individuals who should have a less active erythroid bone marrow are as capable of absorbing inorganic iron as effectively as young adults[116].

Although corporeal factors must be the most important regulators of iron absorption, the way they inform the duodenum to transfer appropriate amounts of iron into the plasma is unknown. It is unlikely that hormones such as erythropoietin serve as the usual messengers to the gut because their concentration in plasma is often unrelated to changes in iron absorption[106, 110–112]. Humoral factors that enhance or inhibit iron absorption have been sought by many investigators. Fischer[117] showed the increased iron absorption in rats transfused with iron-deficient plasma, but failed to exclude erythropoietin as a stimulatory factor. Brittin reported increased iron absorption in the parabiotic partner of a hypoxic rat[118]. These experiments could reveal a dilute factor undetected by transfusion experiments. However, they do not exclude plasma iron turnover as an etiology; it should be considered because parabionts share the same circulation. The results of these experiments seem different from the outcomes of many other studies in which plasma transfusions neither increased nor decreased iron absorption[30, 104, 119].

Idiopathic hemochromatosis

Hemochromatosis is a clinical example of an exception to many of the observations described above. Affected patients absorb approximately three milligrams of iron daily in excess of body losses, and become markedly iron overloaded without stimuli such as diminished tissue iron stores, accelerated erythropoiesis, or hypoxia[22, 111, 112]. The net daily gain of iron in hemochromatotic patients seems to be within the normal humans capability to excrete excess iron (~4 mg/day)[12, 22]. In hemochromatotics, absorption is increased after phlebotomy to 8–10 mg of iron daily from an American diet that contains15–20 mg of iron. This is significantly greater than that observed following phlebotomy of normal individuals (~5 mg/day). Stimuli of iron loss and hemochromatosis are additive and may occur via different mechanisms. Patients with certain hereditary hemolytic anemias, e.g., sickle cell anemia or hereditary spherocytosis, absorb increased amounts of iron throughout their lives without becoming markedly iron overloaded unless they are multiply transfused[106]. Thus the defect of hemochromatosis appears to produce a relative diminution in iron excretion in addition to excessive absorption of iron. Patients with transfusional siderosis can excrete 4 mg of body iron daily in excess of iron absorption, whereas persons with hemochromatosis sustain positive iron balance despite excessive body stores of iron[22]. This makes it difficult to attribute the iron overloading to intestinal intraluminal factors, even though many hemochromatotics have diminished production of pancreatic bicarbonate and may have abnormal secretion of iron-binding substitutes in their gastric juice (mucin?)[115, 120–122]. A partial explanation for increased absorption and decreased secretion of iron is found in ultrastructural studies of the duodenal mucosa from patients with hemochromatosis[123]. Their intestinal mucosal cells are devoid of both ferritin and stainable iron[123–126]. Hemochromatotic intestinal epithelial cells resemble those from iron-deficient individuals, except that the lamina propria of the hemochromatotics is iron replete and contains excess ferritin, whereas this is not found in iron-deficient persons[5] (Fig. 11.6). Incubation of specimens from hemochromatotic patients or iron-deficient persons with iron-nitrilotriacetic acid shows that non-ferritin iron-binding complexes are present in both specimens, and this is indistinguishable from intestinal specimens from normal subjects similarly treated[5]. These observations support the hypothesis that these non-ferritin iron-binding complexes (presumably mobilferrin and/or DMT-1) affect iron absorption by either stimulating uptake or producing a relative mucosal block, or both. Likewise, they provide an explanation for the increased mucosal uptake of iron by the hemochromatotic intestine and diminished loss of iron from the body in defoliated intestinal epithelial cells at the end of their two–three-day lifespan. Additional support for the hypothesis that the basic defect of hemochromatosis resides in intestinal absorptive cells is the identification of mobilferrin/calreticulin and β_2 microglobulin within the cells closely associated with paraferritin[79, 80]. The β_2 microglobulin knockout mouse becomes iron overloaded, and both complexes have been associated with class I major histocompatibility (MHC-1) complexes and the genetic defect of the majority of persons with hemochromatosis[81, 82]. Proponents of DCT-1 postulate a relationship of this protein to hemochromatosis. However, persons with hemochromatosis have relative increases in the absorption of both heme-iron and inorganic iron[127, 128]. Inorganic iron absorption is decreased in hemochromatotics as iron overloading progresses[1]. In contrast, increased absorption of heme-iron was unaffected by increasing body stores of iron. Because hemochromatosis is rarely seen in vegetarian populations,

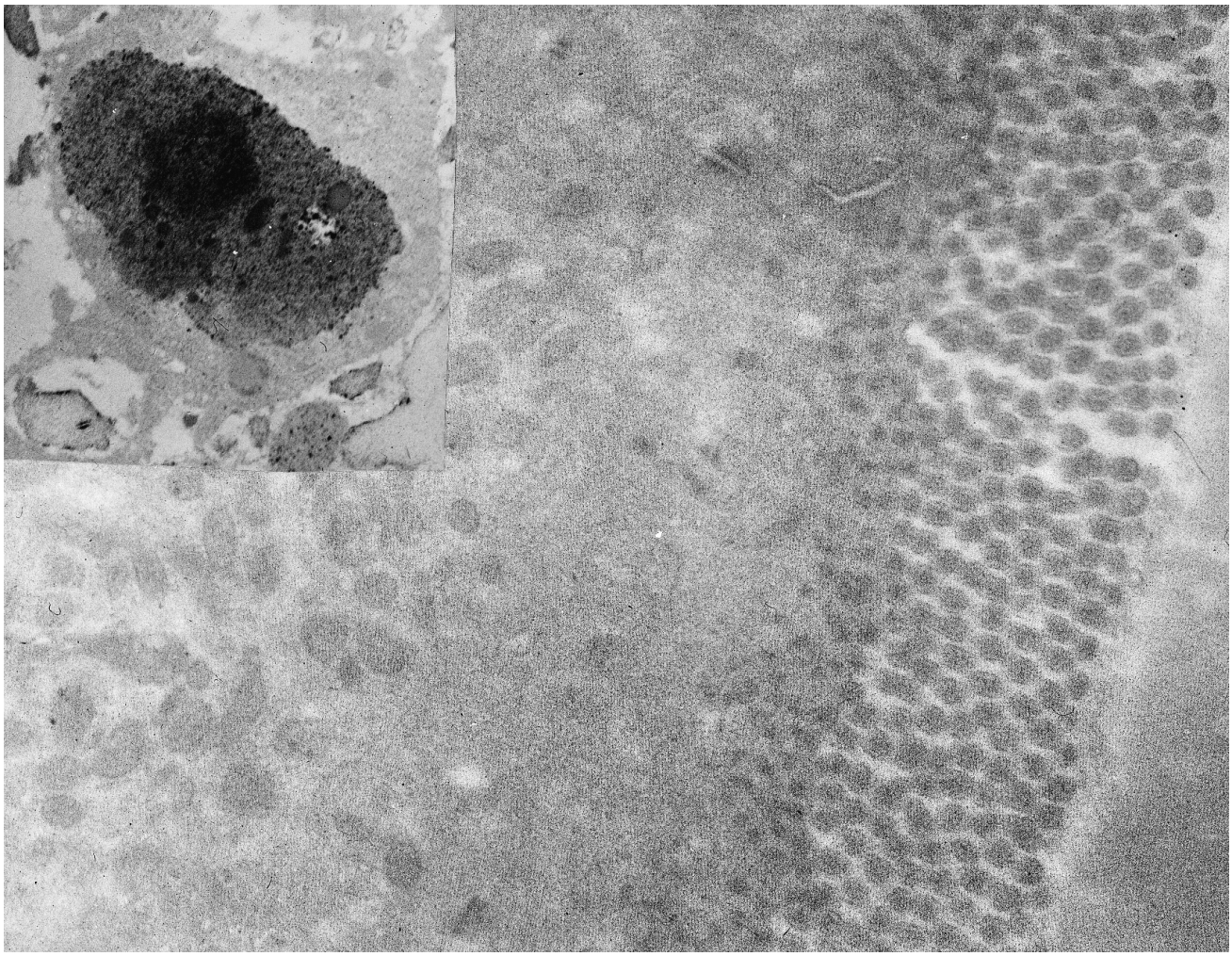


Fig. 11.6. Duodenal biopsy specimen from a patient with symptomatic hemochraomatosis. The microvilli and apical cytoplasm of the intestinal epithelial cell contain little or no iron (by acid ferrocyanide staining). This resembles the findings in a specimen from an iron-deficient individual. In contrast, the submucosa contains excess stainable iron. The insert shows a phagocyte in the submucosa containing a large quantity of ferritin. Preincubating this specimen with Fe-NTA showed that there were multiple iron-binding sites in the apical cytoplasm, similar to the findings in iron-deficient subjects and rats (Fig. 11.4(*a*)).

and about two-thirds of body iron in meat eaters is derived from absorption of heme iron, one could speculate that the iron overloading in hemochromatosis is primarily an abnormality of heme absorption, rather than absorption of inorganic iron. However, increased absorption of both forms of iron make this unlikely and the HFE mutation is found predominantly in Caucasian populations of Celtic derivation. Similarly, unless the difference in the absorption of heme-iron and inorganic iron is an artifact, the major functional defect of hemochromatosis is not located in the microvilli, because heme-iron and inorganic iron uptake are non-competitive until iron is released from heme within the cell. The stimulus to increase iron absorption could exist in a more proximal location within the intestinal absorptive cell (hephaestin?), or in a distant organ that misleads the absorptive epithelial cell to believe the host is iron deficient. The preferential deposition of iron in parenchymal cells[1] rather than reticuloendothelial cells in patients during the development of iron overload due to hemochromatosis may provide support of this later hypothesis.

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12

The absorption and metabolism of non-ferrous metals in hemochromatosis

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Introduction

Many non-ferrous metals are encountered as normal dietary constituents, environmental contaminants, or occupational hazards. However, the absorption and metabolism of non-ferrous metals and their relationships to that of iron are incompletely understood. Early investigators discovered that there is increased retention of certain non-ferrous metals in the liver, blood, or other tissues of persons with hemochromatosis[1–3]. These observations indicate that increased absorption, altered transport and tissue and organ deposition, or abnormal excretion of the metals occur in hemochromatosis. Further, many Caucasians of western European descent have inherited hemochromatosis allele(s), and may have increased susceptibility to the deleterious (or beneficial) effects of increased non-ferrous metal retention.

Non-ferrous metal absorption and retention in hemochromatosis

Cobalt (in an inorganic form) is the first metal the absorption of which was shown to be increased in hemochromatosis subjects[4, 5]. Increased absorption of lead also occurs in hemochromatosis homozygotes, based on studies using measurements of blood lead concentrations and a mathematical model of human lead absorption[6]. To a lesser extent, the absorption of lead (and iron) is also increased in hemochromatosis heterozygotes[6]. The degree of augmentation of intestinal absorption of inorganic iron, cobalt, and lead in hemochromatosis homozygotes is approximately 1.5–3 times that observed in normal control subjects; this is consistent with increased absorption of each element by the same transport pathway (Table 12.1)[6]. In hemochromatosis, increased absorption of cobalt and lead occurs in men and women, and is not affected by the presence or absence of iron overload, hepatic cirrhosis, or diabetes mellitus[4–6]. No other measurements of non-ferrous metal absorption appear to have been reported in hemochromatosis, or in genetic knockout mice with features similar to those of hemochromatosis[7].

In hemochromatosis homozygotes, hepatic concentrations of manganese, copper, zinc, and lead are increased several-fold, apparently unassociated with the presence or absence of hepatic cirrhosis[1, 2, 8–11]. Early measurements of hepatic manganese and zinc in the livers of hemochromatosis patients did not reveal increased concentrations, probably due to insensitivity of the methods used[1, 2, 8, 11]. Increased concentrations of copper also occur in many other tissues in hemochromatosis, especially in the choroid plexus and thyroid[1, 2, 10]. In contrast, aluminum concentrations in the livers of hemochromatosis patients are subnormal[2], and cobalt was almost undetectable in multiple tissues in hemochromatosis[2]. Blood zinc concentrations are often normal in hemochromatosis homozygotes[12], and thus do not reflect increased hepatic zinc retention[11]. However, mean blood concentrations of lead are increased several-fold in hemochromatosis homozygotes and are slightly increased in heterozygotes[6].

Increased absorption of non-ferrous metals in hemochromatosis resembles that in iron deficiency

In rats, iron deficiency increases the absorption of inorganic forms of many non-ferrous metals[13]. Subsequent investigations confirmed and extended these initial observations to include other species and metals (Table 12.1)[14–24]. Although published studies of this phenomenon in iron-deficient humans are limited, they have confirmed

Table 12.1. Increased absorption and organ retention of iron and non-ferrous metals[a]

Disorder [refs]	Subjects	Metals
Hemochromatosis[1–12]	humans	Mn[b], Fe, Co, Cu[b], Zn[b], Pb
Iron deficiency[13–24]	many mammalian species	Al, Mn, Fe, Co, Ni, Cu, Zn, Cd, Pb, Pu
Iron deficiency[25–28]	humans	Fe, Co, Pb
Aceruloplasminemia[29, 30]	humans	Fe, Cu
Co-inheritance of hemochromatosis and Wilson disease[31 c]	human[c]	Fe, Cu
African iron overload[32]	humans	Fe, Pb[d]
Wilson disease[33, 34]	humans	Cu
Bedlington terriers[35]	dogs	Cu, Fe[e]
Congenital hypotransferrinemia; atransferrinemia[36, 37]	humans; mice	Fe
Hereditary, acquired anemias[f]	humans; mice	Fe
Knockouts of *HFE*, β_2-microglobulin genes[7, 38]	mice	Fe

Note:

[a] This table provides information on disorders in which the distribution of excess absorbed iron is widespread; metals are listed in order of their appearance in the periodic table. Possible excess deposition of non-ferrous metals in localized forms of iron overload with a genetic basis such as Friedrich's ataxia, pulmonary hemosiderosis, Zellweger's syndrome, and Hallervorden–Spatz disease seem unreported.

[b] These entries are based on demonstrations of increased non-ferrous metal concentrations in livers and other tissues[1–11], and the likelihood that non-ferrous metal absorption is increased in hemochromatosis as it is in animals with iron deficiency.

[c] This case appears to be unique[31].

[d] In African iron overload patients from South Africa, significantly increased concentrations of lead were detected in liver, pancreas, jejunum, heart, and spleen. This suggests that lead absorption is increased in such persons, but the authors did not provide data on dietary lead intake or other lead exposure[2].

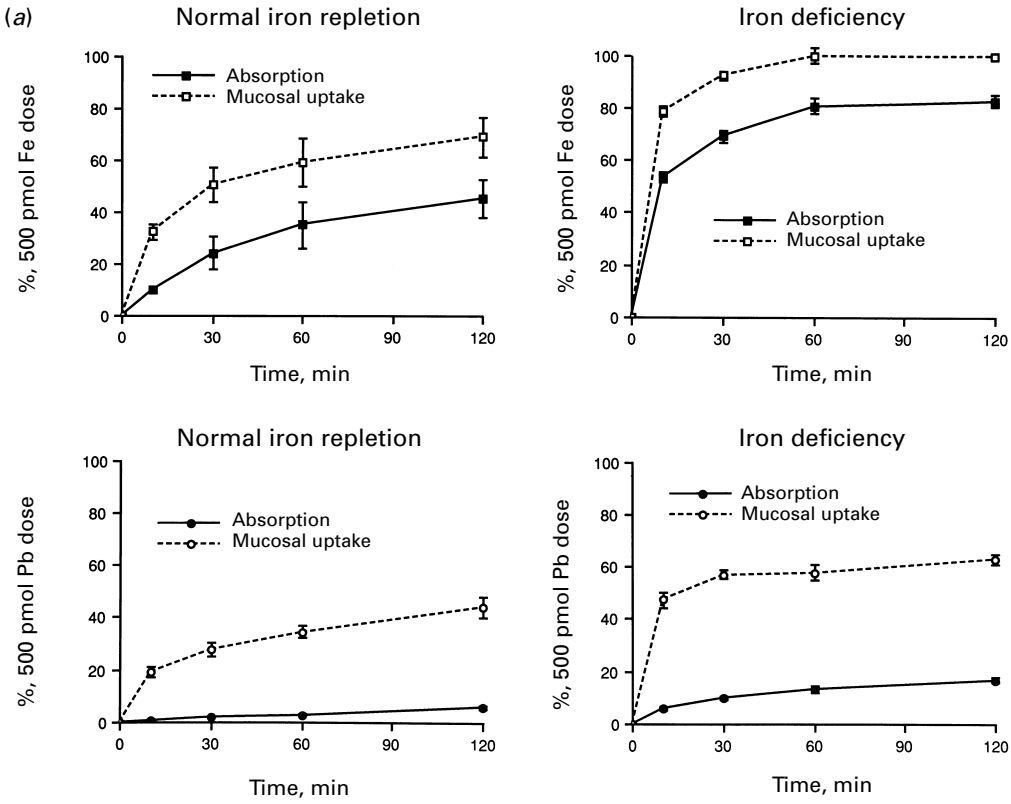
[e] Bedlington terriers with copper toxicosis have increased hemosiderin in the liver, bone marrow, spleen, and lymph nodes. This may be due to increased iron absorption or increased erythrocyte turnover related to copper toxicity[34].

[f] Iron loading due to increased absorption is most commonly reported in thalassemias, hereditary and acquired forms of sideroblastic anemia, congenital dyserythropoietic anemias, and pyruvate kinase deficiency.

observations regarding cobalt and lead originally made in experimental animals (Table 12.1)[25–28]. Heritable disorders other than hemochromatosis are also associated with increased iron absorption, and non-ferrous metal absorption may also be perturbed in these conditions (Table 12.1)[2, 7, 29–38]. Taken together, these results suggest that inorganic forms of metals share and compete for absorptive pathways with iron in the proximal small intestine[17, 20, 39–45]. In vivo, iron deficiency increases the uptake of non-ferrous metals into enterocytes from the intestinal lumen and metal transfer into the circulation[17, 46]; a similar effect is observed in duodenal enterocytes derived by endoscopic biopsy[45]. When iron deficiency occurs, however, specificity of the transport mechanism(s) for iron may be increased (Figure 12.1)[17, 46]. Non-hemochromatosis iron overload in experimental animals and in humans is often associated with subnormal absorption of inorganic iron, but a corresponding decrease in the absorption of non-ferrous metals does not always occur[4, 17, 20, 44].

General principles of metal absorption

Fundamental physiologic properties by which inorganic forms of iron and many non-ferrous metals are absorbed by the small intestine are similar. There are multiple pathways by which various chemical forms of iron and non-ferrous metals are absorbed. Many are not metal-specific, and multiple pathways can function simultaneously. However, all involve entry of metal into the mucosal cell from the intestinal lumen and its transfer through the basolateral membrane of the absorptive cell into the blood, usually by active, energy-dependent mechanisms[47, 48]. The absorption of common organic forms of cobalt and iron, i.e., cobalamin and heme, are well understood. Vitamin B_{12} absorption is probably normal in hemochromatosis, but heme iron absorption is increased (but to a lesser extent than is that of inorganic iron)[49]. Heme iron is reduced to an inorganic form by heme oxygenase within enterocytes, and is subsequently transferred into the



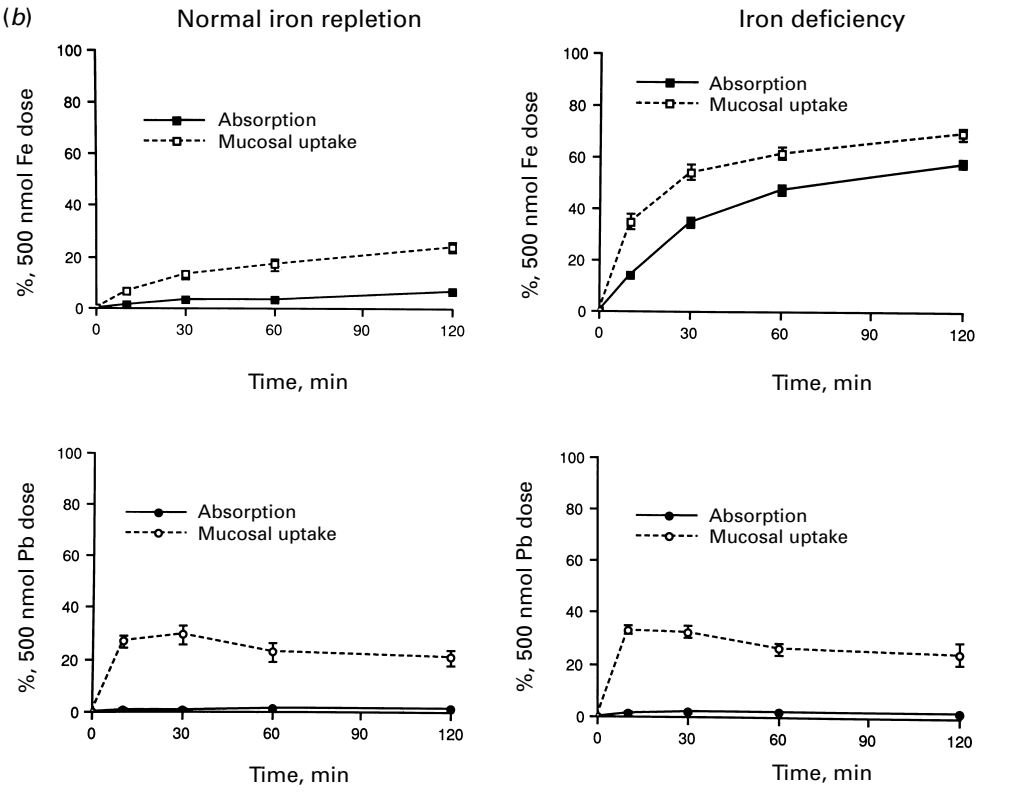


Fig. 12.1. Mucosal uptake and absorption of ^{59}Fe and ^{210}Pb (as divalent chlorides) from closed duodenal loops in mature male Wistar rats; each datum represents the mean ± S.D. for eight animals[17]. (*a*) Data representing rats tested with 'physiologic' doses of 500 picomoles of either metal are displayed. Mucosal uptake and absorption of iron were greater than those of lead, but iron deficiency induced by phlebotomy and feeding an iron-deficient diet significantly enhanced the mucosal uptake and absorption of both metals. (*b*) Data representing rats tested with 500 nanomoles of either metal are displayed. At this higher dose, only the mucosal uptake and absorption of iron were increased by iron deficiency.

circulation by the same mechanisms that effect inorganic iron absorption[50, 51]. Relatively little is known about the absorption of many non-ferrous metals bioincorporated into organic molecules in normal or hemochromatosis subjects[52].

Intraluminal factors that affect metal absorption

The dose and chemical form of metals influence their absorbability. Physicochemical effects within the gastric or intestinal lumen that affect valence, solubility, and chemical reactivity of metals also affect metal absorption. The acid milieu of the stomach and proximal duodenum promote metal solubility and absorption, whereas alkaline succus entericus and bicarbonate-rich pancreatic juice can decrease metal solubility and absorption. Many common ligands present in gastric, biliary, or intestinal secretions, foods, or products of digestion (e.g., hydrochloric acid, sugars, amino acids, and ascorbate) bind iron and non-ferrous metals and maintain or increase their solubility, thereby enhancing their absorption. Anions (e.g., phosphates, phytates, and tannates) bind metals and often inhibit their absorption. Fasting typically increases the absorption of test doses of inorganic metals, probably by decreasing intraluminal precipitation of metal and decreasing competition for absorptive sites. Factors that retard intestinal motility often augment metal absorption; those that shorten gastrointestinal transit time usually diminish metal absorption. Many combinations of these factors can occur, and this partly explains the variability of metal absorption measurements observed in human and animal subjects[53, 54]. However, none of these factors is peculiar to hemochromatosis.

Uptake of iron and non-ferrous metals by enterocytes

In general, there is an inverse relationship of metal absorption to age. The capacity of enterocytes in immature animals or humans to absorb most nutrients is great, and maturation or 'closure' of the absorptive cells to create a more adult-like pattern of nutrient absorption occurs at, or before, weaning[55]. In mature small intestine, inorganic forms of iron and many non-ferrous metals are absorbed in a gradient duodenum > jejunum > ileum[44, 53]. Similarly, absorption of iron and many non-ferrous metals occurs in a gradient along the intestinal villus: villus tip > lateral villus > crypt[44, 53, 56]. Approximately one-half of rat and human enterocytes contain unsaturated iron-binding substances (siderophilia) in a cellular distribution that corresponds to metal-absorbing gradients along the small intestine and intestinal villi[56]. Although the biochemical identity of this substance(s) has not been established, its distribution corresponds to the enterocyte uptake of intraluminal radioiron in vivo[56].

In microcytic anemia (*mk*) mice and in Belgrade (*b*) rats, homologous mutations in the *Nramp2*/DCT1 (natural resistance-associated macrophage protein; divalent cation transporter) gene cause defective entry of iron through the apical portion of absorptive enterocytes due to the production of an abnormal iron chelator. This results in iron deficiency; these animals also have manganese deficiency[57–59]. In addition to transporting iron, the *Nramp2*/DCT1 gene product also binds and transports manganese, cobalt, copper, cadmium, nickel, zinc, and lead[46, 57, 58]. The expression of *Nramp2*/DCT1 is upregulated in response to dietary iron deficiency[58].

The absorption of iron and non-ferrous metals is inhibitable by physiologic concentrations (5–10 mM) of inorganic calcium in the intestinal lumen[60]. In the case of iron, this effect appears to occur at the surface of the microvillus membrane[60–62]. This cannot be explained by altered metal solubility, ultrastructural lesions in enterocytes, or competition for binding sites on transferrin or vitamin D-dependent calcium-binding protein[63–65]. Inhibition of *Nramp2*/DCT1 by physiologic concentrations of calcium suggests a molecular site of action of calcium ions in decreasing the absorption of iron and non-ferrous metals, and is consistent with impaired mucosal uptake of cobalt, iron, zinc, lead, and plutonium in the presence of calcium (Fig. 12.2)[22, 60, 61, 66, 67]. Whether the *Nramp2*/DCT1 gene product is the metal-binding moiety that interacts with the hemochromatosis-associated *HFE* gene product in the small intestine is not presently known. However, *Nramp2*/DCT1 is an excellent candidate chelator moiety that explains many aspects of increased absorption of inorganic forms of iron and non-ferrous metals in hemochromatosis. Further, mutations in the human *Nramp2*/DCT1 gene could explain the occurrence of a hemochromatosis phenotype in persons who lack the C282Y and H63D mutations.

Mobilferrin, a component of an intestinal iron transport pathway, is probably identical to calreticulin[68–70]. Mobilferrin (calreticulin) binds both iron and zinc with high affinity[71], and its calcium-binding properties could also explain the calcium inhibition of the absorption of iron[60], cobalt (Fig. 12.2), and lead[72]. Although calreticulin is involved with the transport of major histocompatibility (MHC) class I molecules, the calreticulin gene is not mutated in persons with hemochromatosis who lack the

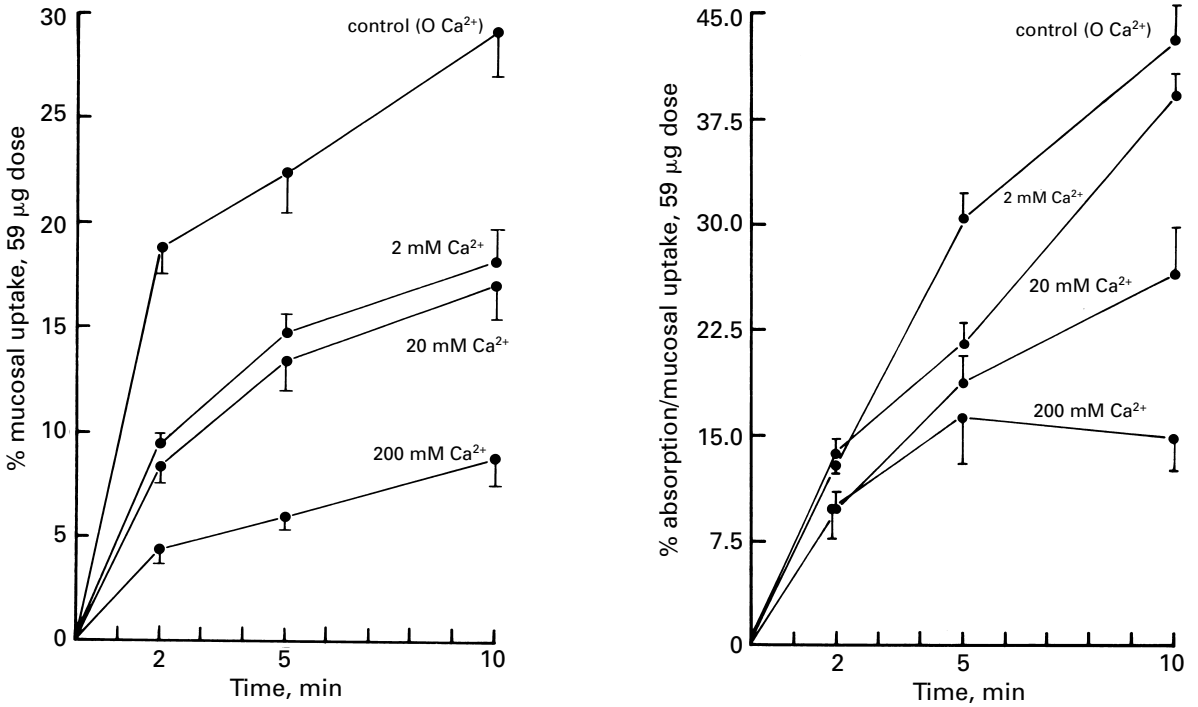


Fig. 12.2. Mucosal uptake and rate of transfer into the body (expressed as the percentage [absorption/mucosal uptake]) of a standard dose of ^{60}Co-labeled cobaltous chloride from closed duodenal loops in mature male Wistar rats; each datum represents the mean ± S.D. for eight animals[20]. Test doses of radiolabelled cobalt were administered without $CaCl_2$ (control), and with 2 mM $CaCl_2$, 20 mM $CaCl_2$, and 200 mM $CaCl_2$ (left). Mucosal uptake was significantly inhibited by $CaCl_2$ (right). The rate of radiocobalt absorption was inhibited by $CaCl_2$ in a dose-related manner.

C282Y *HFE* mutation[73]. A 54–kDa protein isolated from human intestinal microvillus membranes and important for iron uptake by microvillus membrane vesicles in vitro[74] is highly expressed in the duodena and livers of patients with hemochromatosis[75]. The subcellular localization of this protein and its ability to bind non-ferrous metals indicates that it is also a candidate for participation in iron and non-ferrous metal transport.

Lactoferrin coats the luminal surface of the small intestine and binds to specific receptors on the apical cytoplasm of enterocytes. In addition to iron, lactoferrin can bind manganese, aluminum, copper, and other divalent and trivalent cations[76–78]. In adults, the process probably does not occur by pinocytosis[79]. Therefore, lactoferrin may function in the mucosal uptake and absorption of iron, manganese, and other metals in normal infants and adults[77, 79, 80]. In adults with hemochromatosis, concentrations of lactoferrin in duodenal fluid are lower than in normal subjects, and exogenous human apolactoferrin inhibits radioiron absorption in normal subjects, but not in those with hemochromatosis[81]. However, the pertinence of these observations to iron and non-ferrous metal absorption in hemochromatosis is unknown. Transferrin, a normal constituent of bile[82], binds iron and many non-ferrous metals. However, transferrin receptors are located on the basolateral, but not apical, membranes of enterocytes[83, 84]; there are other persuasive data that transferrin does not participate in the uptake of metals from the intestinal lumen by enterocytes[85]. Therefore, it seems unlikely that the abnormal interaction of mutant *HFE* protein with transferrin receptor could account entirely for the regulation of entry of iron and non-ferrous metals at the level of the microvillus membrane. Metallothionein transports zinc and cadmium across the small intestine. Although iron, zinc, and cadmium share absorptive relationships in subjects with iron deficiency and hemochromatosis, iron is probably not transported by metallothionein.

Metal movement and binding within enterocytes

Once within the microvillus membrane, metal atoms or particles move proximally through microvilli and appear rapidly in multiple intracellular loci, particularly along the lateral or basolateral membranes. Ultrastructural studies reveal metal- and compound-specific similarities and

differences in these processes[86–88]. Numerous enterocyte cytosolic iron-binding moieties that bind or transport iron and other metals have been tentatively identified[89]. Iron derived from the intestinal lumen (or from the circulation via the abluminal aspect of enterocytes) is sometimes detectable in intracytoplasmic ferritin of enterocytes. This may permit temporary retention of iron that is transferred to the circulation later, or represent a mechanism for the elimination of excess iron via exfoliation of enterocytes. Non-ferrous metals are also incorporated into ferritin in vitro[90], but no role of enterocyte ferritin in their absorption or excretion has been defined.

Metal transfer across enterocyte basolateral membranes

In normal subjects and in hemochromatosis, the rate of mucosal transfer of iron to the plasma is the major determinant of increased iron absorption[47, 91]. Similarly, mucosal transfer of lead in rats occurs at the basolateral membrane by active transport, and is rate-limiting for absorption[48]. Transferrin receptors in enterocytes occur only at the basolateral membranes[83, 84]. Normal (wild-type; wt) *HFE* gene product expressed in enterocytes is important in regulating iron absorption in enterocytes[38, 92–95]. It has been proposed that this protein, like other MHC class I gene products, is posited in a transmembrane location, binds β_2-microglobulin and, presumably, an iron- or metal-binding moiety[92, 94, 95]. The gene that codes for the attached β_2-microglobulin is normal in hemochromatosis[73]. However, transferrin receptor in cultured cells forms stable complexes with wt and H63D *HFE* protein. The binding of wt *HFE* protein to transferrin receptor decreases the receptor's affinity for transferrin, whereas the H63D protein binding to the receptor does not alter transferrin binding. The C282Y *HFE* protein, associated with most 'classical' cases of hemochromatosis, binds transferrin receptor poorly[96]. These abnormal interactions of mutant *HFE* proteins with enterocyte transferrin receptors are almost certainly important in the pathogenesis of increased iron absorption characteristic of hemochromatosis, perhaps by causing more rapid transmembrane movement of the iron-binding moiety[92]. Further, mRNA for transferrin receptor is inappropriately increased in the duodenum in hemochromatosis[97]. Persons with hereditary atransferrinemia and hypotransferrinemic (*hpx)* mice absorb excess iron[36, 37], but data about the absorption of non-ferrous metals in persons and mice that lack transferrin have not been reported. Mice with sex-linked anemia (*sla*) have a defect of cobalt, iron, and copper transport at the basolateral enterocyte membrane[43, 46, 98, 99]. Identification of the *sla* abnormality could explain normal transfer of iron and non-ferrous metals from enterocytes to the blood, or unusual cases of hemochromatosis or other iron overload disorders.

Transport of non-ferrous metals in blood

The affinity of unsaturated transferrin appears to be greatest for iron, but transferrin binds many other metals in a competitive (or non-competitive) manner, including Al, V, Cr, Mn, Co, Ni, Cu, Zn, Ga, Ru, Cd, In, Hf, Pb, Bi, Ac, Gd, Th, Pa, U, Np, Pu, Am, Cm, and Cf[100–122]. Erythropoiesis and biochemical, electrophoretic, and physiologic characteristics of transferrin in hemochromatosis are normal[123–127]. Like iron, non-ferrous metals can alter transferrin synthesis by modulating regulatory sequences or altering post-translational production[128]. Although the transferrin gene has been cloned[129], there are no reports of mutations in the gene or its regulatory elements that could account for unusual cases of hemochromatosis. In animals or humans, many non-ferrous metals in inorganic forms are bound to transferrin after injection or absorption[101, 108, 117–120, 130], and thus transferrin may be a physiologic transporter for these metals. However, many non-ferrous metals, even those that can also bind to transferrin, are largely transported by albumin (e.g., zinc) or more specific binders (e.g., ceruloplasmin). Transferrin saturation is usually elevated in untreated persons with hemochromatosis[131, 132]; after phlebotomy therapy, saturation values decrease but remain supranormal[131]. Accordingly, non-transferrin-bound iron (NTBI) is often detectable before or after iron depletion therapy in hemochromatosis patients[133, 134], and this implies that few or no binding sites may be available for the transport of non-ferrous metals.

Uptake of non-ferrous metals by non-intestinal cells

Lead uptake in liver is increased when transferrin is iron-saturated[112], and the decreased availability of transferrin binding sites for non-ferrous metals may promote their entry into cells via an alternate receptor(s)[135]. Transferrin increased the uptake of gallium into human lymphocytes in vitro, but decreased Pu uptake[136]. Consistent with this observation, the binding sites for transferrin-bound iron and plutonium are different in cultured hepatocytes[137]. In several non-intestinal, non-erythroid cell types in hemochromatosis, transferrin receptor function and regulation

appear to be normal[138–142]. This includes decreased expression of transferrin receptors on the surfaces of hepatocytes[143] that return to normal levels when iron overload is relieved. Taken together, these observations suggest the fractions of non-ferrous metals that enter cells via transferrin and transferrin receptor is small, especially in untreated patients.

In reticuloendothelial cells, the movement of iron from intracellular vesicles formed during receptor-mediated endocytosis of the transferrin–iron complex may require the function of the iron transporter *Nramp1*, a homologue of *Nramp2/DCT1* expressed in the intestine. In yeast, the *Nramp1* homologue *Smf1* is necessary for manganese transport[144, 145], and therefore *Nramp1* may function as a regulator of intraphagosomal manganese, iron, and other divalent cations[146, 147]. Normal or mutant *Nramp1* proteins could be involved in the intracellular traffic of iron and non-ferrous metals in hemochromatosis. Investigation of the coding and regulatory sequences for this gene have not been reported in hemochromatosis or African iron overload (in which macrophage iron deposition is predominant).

Hepatocyte uptake of NTBI is much more efficient than that of transferrin-bound iron, and this probably accounts for the predominantly hepatocellular iron loading with a portal to central gradient observed in hemochromatosis[148–150]. This suggests that non-transferrin mechanisms could account for the relatively selective hepatic deposition of manganese and zinc in hemochromatosis[9, 11]. In hypotransferrinemic mice, the uptake of injected gallium is increased in all tissues, and aluminum uptake in bone is increased[151]. In cultured fibroblasts and brain cells, aluminum uptake occurs by a transferrin-independent mechanism[152]. Using the isolated perfused liver model, Wright and colleagues[150] provided evidence that the hepatic uptake of NTBI is mediated by a membrane carrier and occurs by an electrogenic mechanism in which there is net movement of positive charge into the cell. These authors concluded that because there is evidence that manganese, copper, and zinc share a common carrier with iron[149], hepatic uptake and accumulation of these metal ions may be driven by similar transmembrane gradients. This also implies that other transition metal ions may compete with NTBI for incorporation into cytosolic binding sites in many cell types[149, 150].

Pre-incubation of a wide variety of cell types with ferric ammonium citrate results in marked stimulation of ^{59}Fe incorporation from ^{59}Fe-transferrin at concentrations greater than those required for saturation of the transferrin receptor[153]. There is evidence that the stimulation of iron uptake from transferrin was caused by ·OH produced via the iron-catalyzed Haber–Weiss reaction. This implies that NTBI present in the plasma of iron-overloaded patients may further promote the loading of cells with iron derived from diferric transferrin[153, 154]. A similar phenomenon could explain the cellular entry of non-ferrous metals.

Organ distribution and storage of non-ferrous metals

The sites of increased deposition of non-ferrous metals in hemochromatosis generally correspond to the physiologic sites of non-ferrous metal deposition in normal animal and human subjects. Likewise, these sites are similar to those of deposition of non-ferrous metals given to experimental animals or normal human subjects in normal or supranormal amounts. However, this does not exclude the possibility that the rates of transport of non-ferrous metals into or out of targeted storage cells or their preferential organ deposition differ from normal in hemochromatosis.

Ferritin, the primary iron storage protein, can also bind non-ferrous metals in vivo and in vitro, including Be, Al, Cu, Zn, Ga, Cd, Pb, and Bi[90, 155, 156]. Absorbed cobalt is not incorporated into ferritin, consistent with its rapid excretion after absorption in rats[157], and its undetectability in multiple tissues of hemochromatosis patients[2]. Body retention of intravenously administered radiochromium is depressed in hemochromatosis[158]. In rats, manganese is also rapidly excreted[40]. Therefore, the incorporation into or non-binding of non-ferrous metals by ferritin in hemochromatosis may affect their organ deposition and excretion significantly. Similarly, induction of hepatic metallothionein synthesis could explain zinc storage in hemochromatosis livers, although this is unstudied. Mutations in ferritin genes are not detectable in most cases of hemochromatosis[159]. The cytoplasmic mRNA-binding iron-regulatory proteins IRP1 and IRP2 appear to coordinate the expression of transferrin and ferritin by binding of the proteins to genes whose transcripts contain RNA stem-loop structures known as iron-responsive elements (IRE)[160]. Heavy metals can also bind to IRE[128]. Mutations in IRP1 cause hereditary, autosomal dominant hyperferritinemia and premature cataracts, but affected persons do not appear to develop iron overload. Further, cell lines lacking IRP1 and IRP2 appear to grow readily, and genetic knockout mice lacking the proteins also appear to be unaffected by major abnormalities of metal absorption[161]. It seems unlikely that an abnormality of IRP1 or IRP2 can explain the increased absorption of non-ferrous metals in hemochromatosis.

Excretion of non-ferrous metals

The renal excretion of absorbed inorganic cobalt is rapid and quantitative in normal persons and in those with hemochromatosis[4, 26]. As a corollary, the increased gastrointestinal absorption of radiocobalt and its subsequent excretion in the urine were once the basis of a presumptive diagnostic test for hemochromatosis and iron deficiency[5, 162]. In metabolic balance studies, whole-body zinc excretion in hemochromatosis homozygotes is normal or increased[163]. Similarly, the urinary excretion of intravenously administered chromium is normal or increased in hemochromatosis[158], and studies of lead metabolism suggest that urinary excretion of lead in affected persons is also probably normal[6]. Taken together, these observations suggest that the overall function of excretory mechanisms for non-ferrous metals (and iron) in hemochromatosis via the kidneys, gastrointestinal tract, or other pathways is normal or increased. This is consistent with the generally normal function of these organs in hemochromatosis (with the exception of increased small intestinal absorption of iron and non-ferrous metals). However, studies of urinary or gastrointestinal excretion of non-ferrous metals in hemochromatosis are few, and there is little information about transport mechanisms of non-ferrous metals to excretory cells and concentrations of non-ferrous metals in sweat, milk, and other external secretions.

Implications of increased non-ferrous metal absorption and retention in hemochromatosis

Diagnostic testing

In persons with hemochromatosis, their family members, and other persons with hemochromatosis alleles, it seems prudent to demonstrate deficiencies of non-ferrous metals using appropriate blood, urine, or other clinical measurements before recommending corresponding dietary supplementation. However, zinc absorption and retention are increased in hemochromatosis, yet serum zinc concentrations in affected persons are usually normal[12] and thus are unreliable indicators of zinc nutriture. Persons with avocational or occupational exposure to toxic metals, especially cadmium, lead, and the actinides need appropriate testing on a regular basis.

Therapeutic phlebotomy and diet management

The excess retention of zinc, manganese, and lead in persons with hemochromatosis is not decreased significantly by therapeutic phlebotomy, because concentrations of these metals in the blood are low[6, 12]. Many nonprescription nutritional supplements contain chromium, manganese, copper, or zinc, in addition to iron. It is prudent for known hemochromatosis homozygotes or heterozygotes to limit their use of non-ferrous metal supplements to the treatment of specific nutritional deficiencies[164]. Oral calcium supplementation is commonly used as part of prevention or treatment regimens for osteoporosis or as an antacid. Although calcium supplements can decrease the absorption of non-ferrous metals, their use (or that of other diet changes) to reduce metal absorption is not recommended.

Effects on iron overload disease

There is no documented synergy of the excess retention of zinc, manganese, or lead in the livers or other tissues of persons with hemochromatosis. In an unusual patient with hemochromatosis and Wilson disease, there was no evidence of severe hepatic injury despite significant accumulations of iron and copper in the liver[31]. Hemochromatosis patients who undergo iron depletion therapy before the development of diabetes mellitus or hepatic cirrhosis have normal longevity by actuarial criteria, despite the fact that therapeutic phlebotomy probably alters overall body retention of certain non-ferrous metals relatively little[6]. This at first suggests that metals absorbed and retained in excess in hemochromatosis are not harmful. However, a role for any non-ferrous metals known or suspected to be absorbed in excess in the causation (or amelioration) of hemochromatosis-associated hepatocellular injury or carcinogenesis, arthropathy, endocrinopathy, or cardiomyopathy remains largely unstudied.

Toxicology

Co-incidental diagnosis of non-ferrous metal toxicity in persons with hemochromatosis appears to be rare[165]. In hemochromatosis patients whose mean blood lead concentrations were greater than normal (including some subjects with occupational or avocational lead exposure), no evidence of plumbism was observed[6]. However, ~12% of western Caucasian populations are C282Y heterozygotes and ~25% are H63D heterozygotes. Thus, undue exposure to and absorption and retention of iron and non-ferrous metals is of concern on a potentially large scale[166]. Common conditions that increase iron absorption (even in persons with hemochromatosis) such as young age, iron deficiency, pregnancy, blood donation, and therapeutic phlebotomy could also increase the absorption of non-

ferrous metals. Lead is excreted slowly, and the total body lead burden increases progressively, even among normal persons. Because lead absorption is increased in hemochromatosis, affected persons could have increased susceptibility to lead intoxication, especially those with occupational exposure to lead. In many children, 'physiologic' iron deficiency and low-iron-content diets suggest that those who inherited hemochromatosis alleles may also be particularly susceptible to the development of plumbism. However, normal iron repletion in experimental animals may diminish some toxic manifestations of lead poisoning[167]. Environmental or occupational exposure to inorganic cobalt consisting of stable isotopes (in relatively small quantities) may pose little hazard due to its rapid renal excretion, whereas increased absorption of ^{60}Co is a significant radiologic risk. Copper depletion is the only known consequence of excess zinc absorption in humans, but other ill-effects may await discovery. Increased gastrointestinal absorption of aluminum and actinides is be predicted to occur in persons with hemochromatosis alleles. Aluminum is a ubiquitous contaminant of food and drink due largely to the its use in water purification and in the manufacture of food containers and cooking vessels. The toxicity of aluminum is cumulative, and increased aluminum absorption could precipitate or aggravate complications in persons who have renal or neurologic disorders. Actinides occur as general environmental contaminants due to their accidental release in association with nuclear power generation and weapons testing; workers in nuclear industries can sustain greater exposures. In additional to chemical toxicity, these substances pose enduring threats to personal health and future generations due to their radiologic properties. Chelation therapy for non-ferrous metal retention in persons with hemochromatosis is not recommended unless toxicity clearly exists.

Conclusions

Persons who inherited hemochromatosis alleles are very common, and therefore certain issues related to non-ferrous metals are potentially important for individuals and populations. Abnormal absorption and metabolism of many non-ferrous metals probably occur in many hemochromatosis homozygotes and heterozygotes, but are incompletely understood and inadequately studied. Increased absorption of non-ferrous metals probably occurs largely by several normal and abnormal pathways that are also important for increased iron absorption and tissue deposition. Absorbed non-ferrous metals are probably transported and enter non-intestinal cells predominantly via non-transferrin-associated mechanisms. More comprehensive analyses of blood and tissues from persons with hemochromatosis for non-ferrous metals are needed; histologic studies can be used to demonstrate metals in situ and understand their possible effects at the subcellular level. Renal and gastrointestinal mechanisms for metal excretion appear to be normal or enhanced in hemochromatosis, although there are few data on mechanisms of transport of non-ferrous metals to excretory sites or the concentrations of non-ferrous metals in external secretions. Because normal excretion of many non-ferrous metals is limited, progressive retention of certain metals occurs and may cause toxic (or beneficial) effects that are presently undescribed. Despite the paucity of available information pertinent to hemochromatosis, current knowledge of non-ferrous metal absorption and metabolism suggests implications for performing diagnostic testing for mineral deficiencies, recommending diet changes to hemochromatosis patients, understanding the pathogenesis of iron overload disease, and evaluating preventive and therapeutic toxicology issues.

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Ferritin metabolism in hemochromatosis

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Introduction

In all mammalian cells, iron in excess of current metabolic requirements is incorporated into ferritin. Effective iron storage is an essential component of cellular iron homeostasis, because iron not sequestered within the cell can catalyze potentially cytotoxic free radical-generating reactions. Although all cells can store iron in ferritin, macrophages and hepatocytes are particularly adapted for this function and retain excess iron as a reserve for times of increased body iron needs. The hepatocyte can take up iron in a variety of different forms and act as a major site of available iron stores, and thus has a central 'buffering' role in internal iron exchange.

Because hemochromatosis is an iron storage disorder, ferritin, the principal iron storage protein, plays an important role in the disease. Ferritin sequesters the iron distributed throughout the body as a consequence of elevated intestinal iron absorption. The serum ferritin concentration accurately reflects the body iron load and provides a valuable diagnostic tool. The iron in ferritin is not biologically inert but can be utilized readily for various cellular functions. The ability of ferritin to release iron in times of demand is essential physiologically but also underlies the treatment of hemochromatosis by phlebotomy therapy.

Aspects of ferritin metabolism relevant to hemochromatosis will be discussed in this chapter. The areas covered include a brief overview of ferritin biochemistry, a discussion of ferritin synthesis and its regulation in the intestinal mucosa, the liver and the reticuloendothelial (RE) system, and the role played by the serum ferritin concentration in the diagnosis of hemochromatosis. Iron loading and ferritin synthesis can be demonstrated in many tissues in hemochromatosis, but there is no convincing evidence that the metabolism of ferritin in any of these tissues differs significantly from that in the liver, and they are not discussed here. The pathology of iron overload and iron-related toxicity in advanced iron storage disease are discussed briefly; these topics are covered in greater depth in other chapters in this volume and elsewhere[1, 2].

Ferritin biochemistry and cellular homeostasis

Structure

Tissue ferritins

Ferritin is a large protein consisting of a spherical protein shell (M_r of ~480000) surrounding a hollow core that can hold as many as 4500 atoms of iron[3, 4]. The protein shell consists of 24 polypeptide chains of two structurally distinct types: H- (heavy) and L- (light) chains with molecular weights of 21000 and 19000, respectively. The H-subunit has a more acidic isoelectric point, while the L-subunit is more basic[3]. Different proportions of each subunit give rise to isoferritins of characteristic isoelectric points and specific tissue distributions[5, 6]. Isoferritins with a higher proportion of L-subunits are found predominantly in the liver, spleen, and placenta[7]. Isoferritins with an increased percentage of H-subunits occur in tissues not primarily involved in iron storage, such as the heart[8, 9], erythrocytes[10], and lymphocytes and monocytes[11]. Although homopolymers of either the H-subunit or L-subunit of ferritin have been useful in in vitro investigations of ferritin structure and assembly, heteropolymers are the norm in mammalian ferritins in vivo. The heteropolymers appear to have an advantage as iron storage proteins by combining the ability of H-chains to facilitate rapid ferrous iron uptake and oxidation and the ability of L-subunits to act as efficient nucleation centers for the formation of the internal ferrihydrite iron core[3]. Consistent with this proposal is

the demonstration that the administration of iron to rats results in the preferential synthesis of L-subunits, thereby favoring the assembly of L-rich ferritins in iron overload conditions[12]. This observation has been supported by in vitro studies showing that ferritins with a high L:H subunit ratio are more efficient in incorporating iron when the external iron concentration is high[13]. The isoferritin profile of various tissues in hemochromatosis has been investigated by a number of groups[8, 14, 15]. In treated hemochromatosis patients, the observed profile is very similar to that found in normal individuals. In iron-loaded patients, most tissues contain isoferritins similar to hepatic isoferritins. This suggests that there is an increase in the proportion of L-subunits in these ferritins and that ferritin with a higher proportion of L-subunits is found in iron-loaded tissues[1, 2].

The molecular structure of each of the ferritin subunits and their assembly to form the intact protein shell have been studied by a combination of physico-chemical and molecular biological approaches[3, 4]. The 24 subunits are assembled into a molecule with 4:3:2 symmetry. There are two types of channels in the protein shell (along the 3- and 4-fold axes of symmetry) through which small molecules or iron can enter the internal cavity of the protein. The process of iron incorporation into ferritin is complex and incompletely understood, but it involves the entry of Fe(II) into the channels after which its oxidation, hydrolysis, and polymerization occur[3, 16]. The mechanisms by which iron is released from ferritin, particularly in vivo, are less well understood[17].

Hemosiderin

When excessive iron accumulates in tissues, e.g., in hemochromatosis or in iron-loading anemias such as thalassemia, most iron is found in hemosiderin rather than in ferritin. In contrast to ferritin, a well-defined biochemical entity, hemosiderin is an amorphous deposit of iron and peptide that appears to consist of degraded ferritin protein and ferric hydroxide polymers or cores of varying size[18–20]. Histologic and immunologic studies have demonstrated a close relationship between ferritin and hemosiderin[18, 21, 22], and confirm that the major peptide in hemosiderin is derived from ferritin. The structures of the iron cores of hemosiderin appear to be disease-specific[19, 20, 23]. For example, there are significant differences between the hemosiderins from the livers of patients with thalassemia and hemochromatosis[7]. Such differences could play a role in the relative rates of iron mobilization in the two disorders, and in iron-related toxicity. Storage iron can be mobilized readily when the body's metabolic requirements are increased, and this is the basis of iron depletion through phlebotomy therapy in hemochromatosis. However, the precise mechanisms by which iron leaves ferritin and hemosiderin in vivo have not been elucidated[3].

Serum ferritin

In addition to its major role in intracellular iron storage, small amounts of ferritin are found in the serum[5, 24]. This ferritin could be released into the circulation from damaged cells (i.e., 'tissue' ferritin), or arise through the specific export of ferritin from its source cells via the secretory pathway. Both processes probably operate in vivo. In certain disease states, the most likely source of the extracellular ferritin is tissue damage; in normal subjects and in hemochromatosis patients who do not have tissue damage, secreted ferritin probably predominates[25]. The demonstration that serum ferritin is glycosylated and that ~15% of hepatocyte ferritin is synthesized on membrane-bound polysomes[26–29] supports the hypothesis that secreted ferritin makes a major contribution to the serum ferritin pool. Further, serum ferritin from both normal and iron-loaded subjects has a low iron content[30], unlike tissue ferritin. The cellular origin of serum ferritin is not clear, although the two most likely sources are the tissues most closely associated with iron storage: the liver and the RE system[24]. Linder et al.[31, 32] have suggested that contributions to the serum ferritin pool may be made by a wide range of tissues.

The immunoreactivity of serum ferritin with antibodies raised against tissue ferritins and preliminary biochemical analyses suggest that serum ferritin is very similar to its tissue counterparts[3, 25, 33, 34]. However, Linder and colleagues[31, 32] have shown that purified horse serum ferritin consists of four polypeptide subunits with M_rs of 26 k, 50 k, 59 k and 65 k. The 26 k polypeptide appears to be present in greatest amounts and may be the glycosylated ferritin G-subunit identified by Cragg et al.[35]. Each of the four subunits was immunoreactive to antibodies raised against horse and human tissue ferritins. Partial cDNA clones from rat liver for the 26 k and 59 k polypeptides show ~50% homology to each of the rat L- and H-subunits of tissue ferritin at the nucleotide and amino acid levels. The use of probes for both cloned serum ferritin subunits shows that the genes are expressed in a variety of tissues, with the exception of skeletal muscle. Further, the level of mRNA for both subunits could be regulated inversely by iron. Because there is a direct, not inverse, correlation between tissue iron stores and the serum ferritin concentration[5], the amount of ferritin destined for secretion may be determined by regulation at the post-transcriptional level (see below).

Synthesis and regulation

Ferritin is synthesized primarily on free polysomes[3, 36], as expected for a cytoplasmic protein. However, some synthesis also occurs on membrane-bound polysomes (~15% in hepatocytes)[28, 29] and this may be relevant to the secretion of serum ferritin. Assembly of the nascent ferritin chains and incorporation of iron into the protein are discussed in detail elsewhere[3].

The biosynthesis of ferritin is under the control of multiple factors and the regulatory mechanisms involved (transcriptional and translational) vary according to the metabolic state, the degree of differentiation, and the iron status of the cell[3, 17]. In normal healthy humans, iron exerts the strongest regulatory influence on ferritin synthesis. Because the major function of ferritin is to sequester potentially toxic free iron within the cell, the synthesis of ferritin can respond rapidly to an increase in the intracellular iron concentration. Most of the increase is mediated at the translational level via an RNA-binding protein known as the iron regulatory protein (IRP) that can respond to the intracellular iron level by undergoing an iron-dependent conformational change[37, 38]. When the intracellular iron concentration is low, IRP is iron-depleted and its binding to RNA is increased. The IRP binds to its cognate iron-responsive element (IRE), a stem–loop structure in the 5′-untranslated region of the ferritin mRNA, and blocks translation. When the intracellular iron concentration is increased, however, the affinity of the IRP for the IRE is decreased, and consequently the block to ferritin translation is removed. Although iron exerts most of its effects on ferritin synthesis at the translational level, iron-dependent stimulation of L-ferritin, but not H-ferritin, transcription has been demonstrated in the liver in vivo[39, 40]. Both H- and L-subunit mRNA levels can be induced in response to iron in various types of cells in culture[3].

Ferritin synthesis is also stimulated at the transcriptional and translational levels by cytokines, the cellular differentiation state, and some hormones[3, 41–43]. Cytokine-dependent control is particularly relevant to inflammation, because ferritin is an acute phase reactant[44] and several pro-inflammatory cytokines stimulate ferritin synthesis. Among these are IL-1 and IL-6 that enhance H-ferritin synthesis at the translational level[45–48], and tumor necrosis factor-α that stimulates H-ferritin transcription[43, 49].

The regulation of ferritin synthesis in specific tissues in hemochromatosis will be considered in detail in following sections, but there do not appear to be major abnormalities in ferritin expression in this disorder. Apparent disturbances in intestinal ferritin synthesis in hemochromatosis are probably secondary to a defect in signaling between the intestine and other body tissues. In addition, no abnormalities in the regulation of the IRP-IRE system in hemochromatosis have been found[50], nor in the regulation of the transferrin receptor[40, 51], the expression of which, like that of ferritin, is IRP dependent[37]. Similarly, in neonatal hemochromatosis, ferritin and transferrin receptor regulation are normal[52].

Genetics

The H- and L-subunits of ferritin are encoded by separate genes on chromosomes 11 and 19[53, 54] respectively. In addition, L-ferritin sequences are found on chromosomes 20 and X, and H-subunit sequences have been localized to at least thirteen chromosomes, including chromosome 6[55–57]. These additional ferritin sequences represent pseudogenes and none appears to be actively transcribed. A possible role for the chromosome 6 ferritin H gene in the etiology of hemochromatosis was investigated using ferritin H-chain cDNA probes to search for linkage of restriction fragment length polymorphism alleles to the hemochromatosis gene. No correlations were found[58, 59]. Subsequent studies have shown that this chromosome 6 ferritin pseudogene does not map close to the site of the hemochromatosis locus[60, 61]. The recent identification of the hemochromatosis gene as one that encodes a HLA class I-like molecule[59] confirms that a primary disturbance in ferritin gene expression is not responsible for the hemochromatosis phenotype.

Ferritin receptors

The existence of a specific tissue receptor for ferritin was proposed after studies demonstrated that tissue ferritin, when injected intravenously into rats, is rapidly cleared from the circulation by the liver[62, 63]. Subsequent studies culminated in the isolation of a specific ferritin-binding protein from rat liver[64]. Ferritin receptors have since been identified on the surfaces of a wide range of cell types[65–68], and have been isolated and partially characterized from human and pig liver[69, 70].

The role of the ferritin receptor in cellular and whole-body iron homeostasis is not fully defined. Possible functions include the clearance of tissue ferritin released into the circulation after cell damage[7] and the transfer of iron bound to ferritin from the hepatic Kupffer cells to hepatocytes[71]. H- but not L-ferritin acts as a growth-regulatory cytokine and inhibits the proliferation of certain hematopoietic progenitor cells, and this activity probably requires a specific cell surface ferritin-binding protein[72–74]. Although no specific role for the ferritin receptor in the

pathogenesis of hemochromatosis has been described, it could contribute to the regulation of iron absorption, given the close correspondence between body iron stores, the serum ferritin concentration, and the rate of iron absorption. A receptor for ferritin has recently been identified on hepatic stellate cells, the cells which are the major source of extracellular matrix in the liver[75]. Ferritin may play a role in maintaining the state of differentiation of these cells, and disturbances in this state may contribute significantly to the hepatic fibrosis that accompanies advanced hemochromatosis.

Intestinal ferritin in hemochromatosis

Although the ferritin gene itself does not appear to be abnormal in hemochromatosis, many studies have investigated ferritin synthesis and its regulation in this disease, because an analysis of ferritin expression could provide valuable insights into the basic abnormality of iron metabolism underlying the disorder. Particular attention has been paid to the small intestine, the major site at which the hemochromatosis defect appears to exert its influence.

Ferritin in the regulation of intestinal iron transit

When body iron demands are high, iron rapidly traverses the intestinal epithelial cells and enters the circulation. If the iron is not immediately required, it is sequestered in ferritin within absorptive enterocytes[76, 77]. Investigations in animals and humans have shown that ferritin synthesis in the small intestine is stimulated by intraluminal doses of iron[78–80]. Over a period of hours there may be movement of some of this ferritin-bound iron into the body, but most remains within the enterocytes and is lost via the gastrointestinal tract when enterocytes are shed at the villus tip. Early studies led to the suggestion that ferritin may play an active role in the regulation of intestinal iron absorption and hence the 'mucosal block' theory was proposed[81]. However, ferritin probably plays a passive, rather than active, role in the absorptive process[77, 82]. Thus enterocyte ferritin sequesters iron not required by the body, although it does not appear to withdraw iron actively from the transit pool.

The regulation of intestinal ferritin synthesis

In normal subjects, the amount of iron in intestinal epithelial cells appears to vary in direct proportion to body iron stores and serum ferritin concentrations[80]. Thus when body iron stores are low, intestinal iron levels and consequently ferritin levels are low. When body iron stores are high, intestinal ferritin concentrations are also increased. Halliday et al.[80] showed that in patients with iron overload (due to hemochromatosis and other causes), the ferritin content of duodenal biopsy specimens was much lower than expected based on the serum ferritin concentrations. These data suggest that when the body iron load is high, the relationship between serum ferritin and duodenal stores does not hold to some extent, irrespective of the cause of the iron loading. However, in hemochromatosis patients who had undergone phlebotomy therapy and had much reduced body iron loads, the duodenal ferritin level remained inappropriately low in relation to the serum ferritin concentration, suggesting that there is a relative defect in ferritin synthesis in the small intestine in this disorder. To investigate this further, Halliday et al.[80] gave a small dose of oral iron to a hemochromatosis patient and observed a highly significant increase in the duodenal ferritin concentration. This experiment indicates that duodenal enterocytes in hemochromatosis patients remain capable of ferritin synthesis, and implies that the lower than expected level of ferritin in the mucosal cells reflects an abnormality in the way the intestinal epithelium senses or responds to alterations in body iron stores, rather than an abnormality in ferritin synthesis per se.

Other investigations have added detail to our understanding of duodenal ferritin synthesis in iron overload. Whittaker et al.[83], using immunoassays for individual ferritin subunits, found that the mucosal levels of both H- and L-ferritin were directly related to body iron stores in normal subjects. In seven patients with hemochromatosis, however, neither the H- nor L-ferritin level increased in parallel with the serum ferritin concentration. They found no differences in the ratio of H- and L-ferritin chains between hemochromatosis patients and normal subjects, confirming earlier studies that found no differences in the intestinal isoferritin profiles between normal persons and patients with hemochromatosis[8, 14, 15]. The authors concluded that in both normal and hemochromatosis subjects, the level of mucosal ferritin was appropriate for the level of iron absorption. Francanzani et al.[84] addressed the same issue using immunohistochemical techniques. They reported an absence of ferritin granules in the enterocytes of the apical villi in 87% of patients with hemochromatosis, whereas ferritin could be detected readily in intestinal biopsy specimens from normal individuals. They also were unable to demonstrate differences between H- and L-subunit expression. Pietrangelo and co-workers[85] examined H- and L-ferritin gene expression in the duodena of normal persons, patients with anemia, and others with siderosis, and found that the levels of the mRNAs for both

ferritin subunits were abnormally low in hemochromatosis patients in comparison to patients with secondary iron overload. These data are consistent with the studies on ferritin protein levels described above, but also suggest that there is a significant degree of iron-dependent transcriptional regulation of the ferritin genes in the duodenum. This is unusual, because iron regulates ferritin expression at the translational level in most tissues.

When assessing the regulation of ferritin synthesis in tissues, it is also informative to examine the regulation of the transferrin receptor, because the two proteins are coordinately regulated by the IRP-IRE system. Unlike ferritin, the synthesis of the transferrin receptor is depressed in iron overload and enhanced in iron deficiency. In hemochromatosis patients, the observed levels of transferrin receptor in the small intestine are either similar to or greater than those observed in normal subjects, whereas in secondary iron overload transferrin receptor expression is depressed (as expected)[85–87]. Thus there is a failure to appropriately down-regulate transferrin receptor expression in the duodenal mucosa in hemochromatosis. This is consistent with the ferritin studies described above and suggests that the coordinate regulation of ferritin and transferrin receptor synthesis in hemochromatosis is normal and appropriate for the observed level of iron absorption. A recent study confirms that the duodenal IRP activity in hemochromatosis patients is normal and appropriate for the intracellular iron concentration of the intestinal epithelial cells[50].

These studies appear to suggest that an abnormality exists in ferritin (or transferrin receptor) synthesis or its regulation in hemochromatosis. However, because the level of mucosal ferritin is always appropriate for the observed or expected level of iron absorption, the defect in hemochromatosis probably lies not in the passage of iron across the absorptive enterocytes of the small intestine, but in the way the body signals the gut to alter iron absorption, or the way in which the gut responds to this signal. The demonstration that the protein affected in hemochromatosis (*HFE*) is expressed in the cells of the intestinal crypts[88] is consistent with the hypothesis that the defect occurs within these cells, at least in part. *HFE* may be part of the mechanism by which the crypt cell senses body iron status, and a mutation in the *HFE* gene may reduce the capacity of these cells to respond to some as yet undefined ligand that conveys information on body iron stores, although this is unproven.

Hepatic ferritin metabolism in hemochromatosis

Iron deposition and ferritin synthesis in the liver

The liver is the most important iron storage organ in the body, and the organ in which many of the most severe clinical effects of hemochromatosis are manifest. Iron that enters the body due to increased intestinal absorption is first directed to the liver via the portal vein where it is deposited in hepatic parenchymal cells[21, 89]. Within hepatocytes, iron deposition is most prominent within lysosomes in the pericanalicular cytoplasm. Initially, iron deposits are found around the portal tracts, but the area of deposition becomes progressively larger as iron accumulation proceeds over time[21] and can ultimately occupy the entire lobule. In the early stages of iron deposition, iron is largely restricted to hepatocytes and the absence of iron in cells of the RE system is a prominent feature of the pathology of hemochromatosis[21, 90]. The liver has an enormous capacity to store iron, and hence to synthesize ferritin and hemosiderin, such that large quantities of iron can be present in the liver before overt disease symptoms are apparent. It is only when the iron storage capacity of the tissues is exceeded that non-sequestered iron accumulates and can participate in chemical reactions that lead to the generation of toxic oxygen radicals and cell and tissue damage[89, 91]. Iron stored in the liver is not biologically inert but can be mobilized whenever the body's metabolic requirement for iron is increased. This is clearly demonstrated in hemochromatosis when hepatic iron is mobilized for new hemoglobin synthesis during phlebotomy therapy.

At the subcellular level, excessive iron storage in hemochromatosis occurs from the earliest stages of the disorder[21, 92]. Iron can first be detected by electron microscopy as electron-opaque ferritin iron cores dispersed throughout the cytoplasm. As cellular iron loading progresses, the concentration of ferritin molecules increases and they ultimately coalesce to form hemosiderin (see above). Ferritin and hemosiderin are found within siderosomes, membrane-bound bodies derived from secondary lysosomes of various types. Within siderosomes, there are various structured arrays of ferritin iron cores[92, 93]. As the quantity of iron within the cell increases, the level of ferritin in the cytoplasm appears to reach a plateau, but the amount of siderosomal iron continues to increase indefinitely[21].

Hepatic ferritin contains both L- and H-subunits, but it is particularly rich in L-subunits, a feature consistent with its pre-eminent role in iron storage. Many studies have investigated the expression of both ferritin subunits in the liver in hemochromatosis, but have identified no

abnormalities in ferritin synthesis[40, 86]. Hepatic ferritin concentrations appear to be modulated normally, in contrast to those of the duodenum (described above). The hepatic ferritin concentration in hemochromatosis patients is much higher than that of normal subjects, and comparisons with secondary iron overload patients indicate that the amount of ferritin in hemochromatosis livers is appropriate for the body iron load. The IRP–IRE system[50] and the transferrin receptor[94] are regulated appropriately in the livers of hemochromatosis patients, consistent with the apparently normal regulation of hepatic ferritin synthesis.

Intrahepatic pathways of ferritin metabolism

Although the human liver can release stored iron to the circulation readily, it has a very limited capacity to excrete iron in the bile. In some laboratory animals, however, the biliary excretion pathway is quantitatively far more significant[95]. To study the role of biliary excretion of iron in hemochromatosis, Hultcrantz et al.[96] measured hepatic and biliary iron and ferritin concentrations in hemochromatosis and control subjects. The former had an eight-fold greater hepatic iron concentration than normal individuals, but their biliary iron and ferritin concentrations were only two-fold and five-fold greater, respectively. There was a significant positive correlation between serum ferritin concentrations and biliary ferritin. These results show that the liver can excrete iron and ferritin in the bile, and that the level of excretion is increased in iron loading. However, the increase is not proportional to the increase in hepatic iron concentration. The authors suggest that the apparent limited capacity of the liver to excrete iron in hemochromatosis may contribute to iron loading.

Aisen and colleagues[71, 97] observed that hepatic Kupffer cells in rats can release iron derived from phagocytosed erythrocytes in the form of ferritin, and that this ferritin can be taken up by hepatocytes. This pathway could play a significant role in intrahepatic iron transfer, although it probably does not contribute significantly to the iron loading seen in hemochromatosis. However, Kupffer cell-derived ferritin could play a role in the development of hepatic fibrosis by acting locally on hepatic stellate cells.

Detailed studies of iron and ferritin release from the livers of rodents have been performed. Ramm and colleagues have used a number of inhibitors of intracellular trafficking to elucidate the pathways of intrahepatic ferritin metabolism in rats[98–100]. They demonstrated that tissue ferritin is cleared from the hepatic circulation of both normal and iron dextran-loaded rats by a receptor-mediated process that is dependent on microtubules[98, 99] and requires chloroquine-sensitive vesicles[100]. These studies also suggested that both intracellular trafficking and the release of ferritin into serum and bile of iron-loaded rats require a network of intact microtubules, microfilaments, and chloroquine-sensitive vesicles[98–100]. Similar pathways may be involved in ferritin release from the liver in hemochromatosis, albeit at a somewhat reduced level.

Potential role for ferritin in the development of hepatic fibrosis

A non-parenchymal cell called the hepatic stellate cell has been identified as the cellular source of increased collagen production leading to hepatic fibrosis in a variety of animal models of iron overload[101–104]. Activated hepatic stellate cells have also been demonstrated in the livers of patients with hemochromatosis[105], and are presumed to play a similar role. However, the precise mechanisms involved in iron-induced hepatic stellate cell activation are not fully understood. A specific receptor for liver ferritin has recently been characterized on rat hepatic stellate cells[75]. The binding of ferritin to the hepatic stellate cell ferritin receptor requires the presence of H-ferritin subunits within the ferritin holopolymer. Ferritin receptor expression is detectable on activated, but not quiescent, hepatic stellate cells[106], and these cells can internalize bound ferritin. Ferritin receptor expression appears to be more than a marker of the activated phenotype, because the addition of ferritin to hepatic stellate cell cultures inhibits the expression of both the intracellular microfilament protein α-smooth muscle actin, a marker of the activated phenotype[107], and the M_r 80 000 isoform of protein kinase C-ζ[108]. Thus the interaction of ferritin and the ferritin receptor may be important in regulating hepatic stellate cell biology and the development of hepatic fibrosis, although further studies are necessary to elucidate the role of ferritin and iron in these processes.

Reticuloendothelial cell ferritin metabolism in hemochromatosis

RE cells play an important role in iron homeostasis because they are involved in recovering iron from senescent erythrocytes. To facilitate this function, RE cells have the capacity to store iron and to release it for re-utilization. A prominent histologic feature of hemochromatosis is the relative paucity of iron in cells of the RE system until late in the course of the disorder[109, 110]. This is particularly apparent in the liver. Hepatic biopsy specimens are stained for iron as part of the diagnosis of hemochromatosis, and

Kupffer cells are deficient in iron in relation to the surrounding parenchymal cells[21]. Bone marrow iron stores are also very limited in the early stages of hemochromatosis[110]. In contrast, RE cells from individuals with secondary iron overload contain large amounts of iron[111]. Part of this dichotomy between primary and secondary iron overload can be explained by the route of iron entry into the body, i.e., enteral vs. parenteral, but the differences are so striking that many believe that the basic defect in hemochromatosis may be expressed in RE cells[90].

The decreased amount of storage iron in macrophages in hemochromatosis could be explained by a defect in the incorporation of iron into ferritin or an increased release of ferritin from these cells. Ferritin synthesis by peripheral blood monocytes is similar in both hemochromatosis and normal subjects[112, 113], and no abnormalities in the regulation of H- or L-ferritin synthesis have been detected in macrophages from hemochromatosis patients[114]. The release of iron by hemochromatosis macrophages has been investigated by several workers. Fillet et al.[115] used ferrokinetic studies to demonstrate increased release of iron from the RE system in hemochromatosis, and Flanagan et al.[116] found that ferritin release from isolated peripheral blood monocytes was increased in patients with hemochromatosis. In the latter study, the same result was found in both iron-loaded hemochromatosis patients and those who had been treated by phlebotomy. Baynes et al.[117] compared iron chelator-mediated iron release and transferrin and transferrin-bound iron uptake in peripheral blood monocytes from normal subjects and hemochromatosis patients, but did not detect any significant differences.

Further evidence for a defect in the RE system in hemochromatosis comes from studies on β_2-microglobulin knockout mice. These mice are unable to process and express MHC class I molecules correctly, including, presumably, the murine homologue of the hemochromatosis protein. As a result, the mice develop iron overload histologically very similar to that seen in hemochromatosis, including a relative paucity of iron in macrophages[118]. However, if the bone marrow of these mice is ablated and they are then transfused with normal bone marrow, redistribution of iron from hepatocytes to Kupffer cells occurs[119]. These studies suggest that at least part of the basic defect in hemochromatosis involves the RE system and its ability to handle iron.

Ferritin metabolism and the basic physiological defect in hemochromatosis

The identification of *HFE* as the gene mutated in most persons with hemochromatosis[59] has not yet led to the elucidation of the basic biochemical or physiological defect in iron metabolism that underlies the disorder. Nevertheless, data about the hemochromatosis phenotype suggest that the basic abnormality is in the regulation of intestinal iron absorption and possibly in iron trafficking by RE and other cell types. Although no direct links have been demonstrated between *HFE* and ferritin metabolism, this has not been investigated in detail. It is possible that the abnormal protein synthesized as a consequence of a mutated *HFE* gene directly or indirectly leads to disturbances in ferritin synthesis or iron loading. The gradual and progressive nature of the disorder suggests that a subtle defect in some aspect of iron metabolism could produce the observed phenotype. Regardless, an important aspect of understanding the function of the *HFE* protein is to determine how the activity of other proteins of iron metabolism varies with modulation in *HFE* expression. The analysis of ferritin in hemochromatosis has already led to important findings with respect to the regulation of iron absorption in this disorder. The analysis of ferritin synthesis and that of newly discovered proteins of iron metabolism such as the metal transporter *Nramp2*[120, 121] will be important in analyzing *HFE* protein function now that more directed experimental studies are possible.

Serum ferritin concentration as an indicator of body iron stores and its role in the diagnosis of hemochromatosis

Serum ferritin concentration and body iron stores

The concentration of ferritin in the plasma of normal, healthy persons remains remarkably constant, and there is little or no diurnal variation. The concentration is high at birth (~300 μg/l) and gradually falls over the first six months of life to concentrations approximating those found in the adult (10–200 μg/l). The concentration in normal men rises slowly and progressively with age, whereas in women the level remains relatively constant in the absence of excessive menstrual blood loss until menopause, after which it rises to similar concentration to those in men[5]. The plasma ferritin concentrations is positively correlated with body iron stores in normal subjects and in patients with iron deficiency or uncomplicated iron storage disease[122–125]. Consequently, in persons with iron

overload, the concentration of serum ferritin is greatly elevated. There is a progressive rise in the plasma ferritin concentration with increases in body iron stores, such that an increase in the ferritin concentration of 1 μg/l is equivalent to an increase in storage iron of ~8 mg[123, 124]. In hemochromatosis, elevated serum ferritin concentrations occur in the absence of architectural damage to the liver, suggesting that the elevated plasma ferritin results from enhanced secretion of ferritin, rather than tissue damage[126].

The plasma ferritin concentration is also increased in certain other disorders. These include hepatocellular necrosis, malignancy, leukemia and related disorders of the RE system, and inflammation[5, 24, 127]. In some situations, e.g., inflammation, ferritin may act as an acute phase reactant and its serum concentration may be elevated irrespective of body iron stores. Accordingly, the transcription of genes for tissue ferritin can be stimulated in cells treated with inflammatory cytokines[43–49].

Role of serum ferritin concentration in the diagnosis of hemochromatosis

Serum transferrin saturation, serum ferritin concentration, hepatic biopsy, and HLA typing are the tests currently available for diagnosing this disorder. A DNA-based test may soon become widely available, but will not determine the biochemical expression or clinical stage of the disorder. The first three tests mentioned above are therefore likely to remain important in diagnosing and managing patients with hemochromatosis in the near future. Elevated serum ferritin concentrations and transferrin saturation values in a patient strongly suggest the diagnosis of hemochromatosis. An elevated serum ferritin concentration and a normal fasting transferrin saturation value suggest an alternative diagnosis, such as alcoholic disease of the liver or non-alcoholic steatohepatitis, particularly if hepatic function tests are abnormal. In pre-cirrhotic cases, hepatic function tests are usually normal and do not preclude diagnosis[128]. Serum iron concentrations alone are of little use in diagnosing hemochromatosis due to their wide diurnal variation. However, many patients with hemochromatosis are eventually diagnosed after an elevated serum iron concentration is detected incidentally on an automated panel as part of general physical or insurance examination. HLA typing, although of benefit in screening siblings of an index case, has no role in diagnosing the index case itself. This is because HLA-A3 occurs in ~25% of the normal population and in ~75% of hemochromatosis patients[129].

At present, hepatic biopsy with a histologic assessment of the degree and distribution of iron stores and a biochemical quantification of the hepatic iron concentration is the standard in diagnosing an index case of hemochromatosis[130, 131]. The subsequent diagnosis of iron overload in a sibling who is HLA-identical to the proband, or who is shown by a DNA test to have the C282Y mutation of the *HFE* gene, confirms the diagnosis. However, hepatic biopsy is the only reliable way to determine the presence or absence of cirrhosis, the most important prognostic factor in hemochromatosis[128]. Increasing age[132] and serum ferritin concentrations[133] are also associated with cirrhosis. A threshold level of hepatic iron concentration above which cirrhosis occurs has been found in some studies[130, 134] but not others[132, 133]. In any case, hepatic biopsy is required to determine this. Obtaining hepatic biopsies should therefore continue to be necessary in the management of hemochromatosis until a reliable serum marker or other non-invasive test of cirrhosis is identified.

Conclusions

Ferritin is one of the most studied proteins of iron metabolism and it fulfils two important functions in hemochromatosis. First, it is the major intracellular iron storage protein. Ferritin sequesters iron within the cell and prevents it from catalyzing potentially cytotoxic free radical-generating reactions. Ferritin synthesis is strongly induced when the intracellular iron concentration is elevated, and thus it is present within cells and tissues in large quantities in untreated patients with hemochromatosis. Second, the level of ferritin outside cells also reflects the whole-body iron load. Thus secreted or serum (or plasma) ferritin plays an important role as a diagnostic tool for hemochromatosis and for monitoring hemochromatosis therapy, because it provides an accurate index of body iron stores.

Ferritin synthesis in the liver appears to be normal in hemochromatosis and its levels are regulated according to the tissue iron load via the IRP-IRE system. In the small intestine, however, ferritin levels are inappropriately low relative to body iron stores. This does not appear to represent an abnormality in the synthesis of ferritin per se, but a defect in the signaling system by which the small intestine, and hence the iron absorption mechanism, is informed of body iron requirements. A potential abnormality in ferritin secretion by cells of the RE system has also been postulated to occur in hemochromatosis, and this could explain the paucity of macrophage iron stores in this disorder. The analysis of ferritin synthesis in hemochromatosis helps to understand the basic defect on iron physiology underlying the disorder. The recent cloning of the *HFE* gene that is mutated in many persons with hemochromatosis will

foster further investigations to define the role of this gene in iron homeostasis.

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14

Hepatic iron metabolism in hemochromatosis

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Introduction

As a major site of iron storage under normal circumstances, the liver bears a disproportionate share of the burden of increased body iron in hemochromatosis. Storage iron normally accounts for 20–30% of total body iron, or about 1 g in an average adult man. Of this, approximately 25% is deposited in the liver. Thus, in normal individuals, hepatic iron concentrations range from 500 to1500 μg/g dry weight. In hemochromatosis, hepatic iron concentrations can exceed the upper limit of this range by more than 20-fold, reflecting the dramatic expansion in iron stores.

Excessive absorption of dietary iron on a chronic basis produces a characteristic pattern of hepatic iron deposition. In hemochromatosis, the parenchymal cells of the liver are the primary sites of iron accumulation, whereas in secondary iron overload and in parenteral iron overload, iron deposition is found in Kupffer cells and in hepatocytes[1]. Iron deposition in hemochromatosis is first evident in periportal hepatocytes (acinar zone 1 of Rappaport), but with progressive iron loading involves hepatocytes of zones 2 and 3. As a consequence, a decreasing portal to central gradient of hepatocellular iron is observed. At the cellular level, granules containing iron are localized along the pericanalicular aspect of the siderotic hepatocytes and thus outline bile canaliculi. Morphological and biochemical data indicate that these granules are iron-laden lysosomes[2]. As hepatic iron increases, there is deposition of iron in the mesenchymal structures of the portal tracts (bile ducts, connective tissue, vascular walls, etc.). Although cell necrosis and inflammation are not conspicuous features of hemochromatosis, sinusoidal iron deposits that are presumed to result from phagocytosis of necrotic iron-loaded hepatocytes by Kupffer cells are also seen with heavy iron loading[3]. The appearance of sinusoidal iron deposits is associated with hepatic fibrosis, a lesion that portends the development of cirrhosis.

In this chapter various mechanisms are reviewed by which iron is taken up by the liver and the potential involvement of these mechanisms in hepatic iron overload due to hemochromatosis is considered. The chapter begins with a review of normal hepatic iron metabolism. Dietary iron is absorbed by the intestine and taken up by the liver. Depending on tissue requirements for iron, it may be retained within the liver and used for the synthesis of iron-containing enzymes such as the cytochromes, or exported to extrahepatic sites (e.g., bone marrow, muscle) where it is used in erythropoiesis or in the synthesis of myoglobin respectively. Iron not utilized for the synthesis of iron-containing proteins remains in the liver bound to the storage proteins ferritin and hemosiderin. The sequestration of iron by these binding proteins is an important means of preventing iron from participating in reactions that lead to the generation of free radicals, and thus to cellular injury. There is evidence that this protective mechanism can be overwhelmed in iron overload, leading to the presence of catalytically active iron in hemochromatosis.

In addition to processing iron absorbed through the gastrointestinal tract, the liver participates in the metabolism of iron derived from hemoglobin from senescent erythrocytes. However, unlike the metabolism of dietary iron that primarily involves hepatocytes, Kupffer cells play a key role in this process. Like the reticuloendothelial cells of the spleen and bone marrow, Kupffer cells ingest senescent or damaged erythrocytes, catabolize their hemoglobin, and release the iron. Under physiological conditions, the Kupffer cells in a rat liver ingest about one erythrocyte per macrophage per day. A single erythrocyte contains approximately 10^9 iron atoms, of which about half are released by the Kupffer cell within 24 hours[4], and bound to ferritin. The released ferritin is rapidly taken up by hepatocytes, a

process that may involve ferritin receptors on the hepatocellular plasma membrane[5]. Ferritin has thus been proposed to function as an intrahepatic iron carrier, transferring iron from Kupffer cells to hepatocytes. Moreover, ferritin resulting from erythrophagocytosis by splenic macrophages may also enter the portal circulation to be taken up by hepatocytes. Although this mechanism is probably not involved in the pathophysiology of hemochromatosis, it is of potential importance to hepatic iron loading occurring as a result of accelerated erythrocyte turnover (e.g., hemolysis, repeated blood transfusion, etc.).

Transferrin receptor-mediated uptake of transferrin-bound iron

There are several mechanisms by which hepatocytes take up iron. The predominant pathway under physiological circumstances is the uptake of transferrin-bound iron (TBI) by its interaction with the transferrin receptor on the hepatocyte cell membrane. The transferrin receptor is a transmembrane protein with an extracellular ligand-binding domain. Upon binding of TBI to the receptor, the entire complex is internalized via receptor-mediated endocytosis. The interior of the endocytic vesicle is relatively acidic, promoting dissociation of iron from transferrin. The transferrin receptor and its bound apotransferrin is then rapidly recycled to the membrane. The iron exits the endosome by an unknown mechanism and enters the low-molecular-weight chelate pool from which it is either utilized or bound to ferritin for storage.

Under normal circumstances, most iron in serum is bound to transferrin; thus transferrin receptor-dependent uptake of TBI constitutes the major route of iron acquisition by the liver. Iron uptake by this pathway is a function of the density of transferrin receptors on the cell surface. The regulation of transferrin receptor synthesis involves a complex feedback loop by which iron uptake and storage are linked to intracellular iron status. This loop is orchestrated by the iron-responsive element binding protein (IRE-BP), also known as the iron regulatory factor[6]. The IRE-BP is an iron–sulfur protein with a 4Fe–4S cluster that regulates the synthesis of transferrin receptor and ferritin through interactions with specific stem–loop structures known as iron-responsive elements (IREs) in the mRNAs coding for those proteins. The formation of the iron–sulfur cluster of the IRE-BP is dependent upon cellular iron status. Under conditions of iron deficiency, the cluster is disassembled, yielding the apo-IRE-BP that is capable of interacting with the IREs. In the case of transferrin receptor, the binding of IRE-BP to IREs in the 3′ untranslated region of its mRNA increases the stability of the message and leads to increased transferrin receptor synthesis. Conversely, binding of the IRE-BP to the IRE in the 5′ regions of ferritin heavy and light chains acts as a translational repressor. The binding of the IRE-BP to the IREs thus stimulates synthesis of transferrin receptor, leading to increased iron uptake, while suppressing synthesis of the iron-storage protein ferritin. When iron stores are replete, the iron–sulfur cluster remains assembled and the IRE-BP is unable to bind to the IREs. In the absence of the IRE-BP–IRE interaction, transferrin receptor mRNA is rapidly degraded while translation of ferritin is unimpeded, resulting in decreased uptake of TBI and enhanced iron storage.

How is it possible for hepatocytes to accumulate excessive stores of iron as occurs in hemochromatosis? One obvious possibility is that of defective signaling. For example, a mutant IRE-BP capable of binding IREs, but not iron–sulfur clusters, could lead to constitutive production of transferrin receptor regardless of cellular iron stores. Results obtained with a mutant IRE-BP overexpressed in a human cell line confirm that such a mutation abrogates iron-dependent regulation of transferrin receptor expression[7]. However, the relevance of this observation to hepatocellular iron loading in hemochromatosis is doubtful. Not only are the genes for IRE-BP and hemochromatosis located on different chromosomes (chromosome 9 for IRE-BP and chromosome 6 for hemochromatosis), but a variety of data indicate that this system responds appropriately to iron overload. For example, Sciot et al. demonstrated the absence of transferrin receptor immunoreactivity in biopsy specimens of heavily iron-loaded livers[8]. In this study, occasional hepatocytes were positive for transferrin receptor in a biopsy from a patient with treated hemochromatosis, who had no stainable iron, and in a biopsy from a patient with a mild degree of iron loading. Lombard et al. were also unable to detect transferrin receptor in iron-loaded hepatic biopsy specimens from a group of untreated hemochromatosis patients, but observed immunoreactivity for the receptor in specimens from a second group of phlebotomized hemochromatosis patients[9]. The reciprocal nature of hepatic iron stores and transferrin receptor expression was illustrated by a patient in whom transferrin receptor was absent on a pretreatment biopsy, but in whom the receptor became detectable following phlebotomy. Conversely, a previously treated hemochromatosis patient who had not been phlebotomized for several years and who had re-accumulated substantial iron stores had no detectable transferrin receptor present in his biopsy specimen. In a moderately iron-loaded liver, a reciprocal relationship was also observed

with respect to the lobular distribution of iron stores and transferrin receptor expression, such that the receptor was absent from the heavily iron-loaded periportal areas and present in iron-poor pericentral hepatocytes. These observations indicate that hepatocellular iron loading due to hemochromatosis results in a physiologically appropriate down-regulation of transferrin receptor expression.

Studies examining the regulation of transferrin receptor expression have likewise concluded that this mechanism is intact in hemochromatosis. Pietrangelo et al. found that steady-state levels of transferrin receptor mRNA were significantly decreased in hepatic biopsy specimens from patients with hemochromatosis compared to controls, as would be expected if message turnover were increased in the absence of an alteration in gene transcription[10]. An increase in ferritin light chain mRNA was also observed in heavily iron-loaded patients, suggesting that iron induces ferritin expression at the transcriptional level, in addition to its effects on translation. More recently, IRE-BP was assessed in samples of liver from patients with hemochromatosis and other forms of liver disease. Both the binding activity of IRE-BP (as assessed by gel retardation assay) and IRE-BP immunoreactive protein were decreased in iron-loaded livers, demonstrating that this regulatory factor responds normally to increased hepatic iron content[11].

Transferrin receptor-independent uptake of transferrin-bound iron

Thus, it appears that excessive accumulation of hepatocellular iron in hemochromatosis may not be attributed to aberrrant regulation of transferrin receptor-mediated uptake of TBI. An alternative pathway exists for uptake of TBI into hepatocytes. Evidence for the existence of this pathway comes from studies with isolated hepatocytes demonstrating that iron uptake is not saturable with increasing concentrations of TBI, and that protonophores have only a minor effect on iron uptake. These observations suggest a route of TBI uptake that is not receptor-mediated and that does not involve acidification of the endosome. Although non-saturable TBI uptake has been ascribed to fluid phase endocytosis, recent data indicate the involvement of a plasma membrane ferric reductase. Specifically, it has been proposed that a membrane-bound NADH:ferricyanide oxidoreductase acts as an electron/proton symport, providing reducing equivalents and generating a local increase in proton concentration at the plasma membrane[12]. At transferrin concentrations in excess of that needed to saturate transferrin receptors, ferric iron is labilized from transferrin by the local decrease in pH. This destabilizes the iron-transferrin bond, allowing the ferric iron to be reduced to the more soluble ferrous form by the action of the ferric reductase. Ferrous iron then binds to plasma membrane ferrous iron ligands and is transported across the membrane where it is transferred to intracellular iron ligands. Although it seems likely that this mechanism, operating independently of transferrrin receptor at high transferrin concentrations, could account for continued iron uptake by iron-replete hepatocytes, the involvement of this pathway in hepatocellular iron loading in hemochromatosis has not been investigated.

Transferrin-independent iron uptake

Like non-transferrin receptor-dependent uptake of TBI discussed above, a third route of cellular iron acquisition involving uptake of non-transferrin-bound iron (NTBI) may be particularly important under conditions of iron overload. NTBI refers to low-molecular weight iron complexes, primarily iron-citrate, that form as a consequence of saturation of the binding capacity of transferrin[13]. In normal individuals, $>$99% of circulating iron is bound to transferrin; plasma concentrations of NTBI are therefore extremely low. Much higher concentrations of NTBI are observed in iron overload[14]. Thus, the contribution of NTBI to hepatocellular iron uptake is presumably much lower in normal individuals than in those who are iron loaded. Clearance of NTBI by isolated perfused rat livers has been characterized[15, 16]. These studies have demonstrated that the removal of NTBI by the liver is highly efficient; as much as 75% of NTBI is removed on a single pass through the liver. In contrast, the corresponding hepatic uptake of iron from TBI is less than 2%. Furthermore, removal of NTBI by the liver is unaffected by hepatic iron stores, indicating that this process is not regulated by iron status[16]. Continued delivery of NTBI to the liver could therefore result in progressive accumulation of iron within that organ.

Other observations suggest that removal of NTBI by the liver plays an important role in hepatic iron loading due to hemochromatosis. In experiments using autoradiography and radioiron NTBI perfused via the portal vein was taken up primarily by hepatocytes[15]. Because NTBI is rapidly removed as it passes through the hepatic sinusoids, it is probable that a gradient is produced, such that central areas are exposed to lower concentrations of iron than periportal areas These features are consistent with the anatomical distribution of hepatic iron seen in hemochromatosis, i.e., predominantly hepatocellular iron loading with a portal to central gradient. In addition, the avidity with which the liver removes NTBI from the circulation

Table 14.1. Characteristics of non-transferrin-bound iron uptake by whole liver or liver-derived cells

Model	K_m	Calcium dependence	Competition by other metals	Effect of iron loading	Inhibition by ferrozine
Isolated perfused rat liver[15, 16]	14–22 μM	(+)	(+)	(−)	ND
Hep G2 cells[19]	3.6–4.3 μM	ND	(+/−)	increased uptake	(+)
Primary rat hepatocytes[21]	1.25 μM	(+)	(−)	ND	(−)

Note:
ND – not determined.

could account for the fact that hepatic iron loading precedes iron accumulation in other organs in hemochromatosis.

The importance of NTBI in hepatic iron overload is further supported by data from humans and mice with hypotransferrinemia. This inherited disorder results in a near-total absence of circulating transferrin, microcytic anemia unresponsive to iron supplementation, and iron loading of the liver and pancreas[17, 18]. Because this pattern of iron deposition is similar to that seen in hemochromatosis, it has been suggested that reduced concentrations of apotransferrin (or conversely, increased concentrations of NTBI) could account for the tissue distribution of iron in hypotransferrinemia and in hemochromatosis. The disposition of an intragastric dose of radiolabeled iron has been compared in hypotransferrinemic (HP) mice and normal mice made anemic by phlebotomy[18]. HP mice and anemic mice absorb a greater percentage of ingested iron than iron-replete normal mice. In HP mice, however, most of the labeled iron is found in the liver and less than 1% is found in erythrocytes; in anemic mice a large proportion of the iron is found in erythrocytes and a minor fraction is found in the liver. A similar shift of iron from the bone marrow and spleen to the liver and pancreas is observed in normal mice in which transferrin has been saturated with non-radioactive iron prior to administration of a dose of radiolabeled iron[18]. Kinetic studies have demonstrated that the clearance of a tracer dose of iron from the circulation is much more rapid in transferrin-saturated animals than in controls ($T_{1/2} < 30$ seconds vs. 50 minutes, respectively), consistent with removal of NTBI in the former case and transferrin-receptor mediated uptake of TBI in the latter. These data emphasize that when the binding capacity of transferrin is exceeded, a pool of NTBI results that is cleared very rapidly from the circulation and is deposited predominantly in the liver.

In both isolated perfused rat livers and cultured hepatic cells, uptake of NTBI is saturable and temperature dependent, suggesting the involvement of an enzyme or membrane carrier protein[15, 16, 19, 21]. Although there are similarities in NTBI uptake by whole livers and cultured cells, some important differences have been observed. The features of NTBI uptake in these models are summarized in Table 14.1. It is unclear whether the differences are due to experimental conditions or represent multiple mechanisms of NTBI uptake. For example, the affinity for NTBI differs between isolated livers and Hep G2 cells, although uptake is calcium dependent and inhibitable by divalent transition metal ions in both[15, 16, 19]. Calcium dependence and competition by other transition metals are also characteristic of non-transferrin receptor-mediated uptake of TBI[12]. The possibility that the latter shares a common mechanism with NTBI is suggested by the observation that ferrozine, a membrane-impermeable specific chelator of Fe^{2+}, inhibits NTBI uptake by Hep G2 cells[19].

The requirement for reduction to the ferrous state prior to transport into cells implies the involvement of a cell surface ferric reductase. Although ferric reductases play an important role in iron transport in plants and yeast, little is known about their mammalian counterparts. However, a variety of cell types, including Hep G2 cells, upregulate NTBI uptake in response to iron-loading[19, 20]. Kinetic measurements indicate that the increased rate of uptake of NTBI by iron-loaded cells is attributable to an increased number of transporters. Excess quantities of NTBI thereby induce a cellular response that is the converse of that elicited by TBI. This apparent paradox may be explicable in terms of the reactivity of the two forms of iron, because NTBI, unlike TBI, may participate in reactions that generate free radicals. In the presence of high concentrations of circulating NTBI, hepatocellular sequestration of iron and the resultant iron overload is possibly less injurious to the liver. Nonetheless, the relevance of the up-regulation of

NTBI uptake to iron overload in vivo is unclear, because studies with iron-loaded isolated livers demonstrated no increase in rates of uptake of NTBI compared to control livers[16]. Because these livers were very heavily iron-loaded (their iron concentrations were increased nearly 40-fold), the possibility that clearance of NTBI is accelerated at modest levels of iron loading and plateaus as iron concentrations increase cannot be excluded.

Another pathway for uptake of NTBI has been described in freshly isolated rat hepatocytes[21]. This mechanism, saturable and calcium-dependent, has a lower K_m for NTBI than those previously described, suggesting that it would be fully saturated at concentrations of NTBI observed in iron overload states. Furthermore, this pathway is distinct from others in that uptake of NTBI is unaffected by other transition metals and by ferrozine. Whether rat hepatocytes lack plasma membrane ferric reductase activity such as that described in Hep G2 cells is unknown. Because the reductase activity of the latter is significantly decreased by mild proteolytic treatment[19], such activity could be lost from hepatocytes by perfusion of the liver with proteolytic enzymes during the isolation process. In any case, it appears that hepatocytes have the capacity, by one or more mechanisms, to remove NTBI from the circulation and that this may play an important role in hepatocellular iron overload due to hemochromatosis.

Conclusions

The liver plays a central role in the uptake, distribution and storage of iron. Under normal circumstances, hepatocellular uptake of transferrin-bound iron via the transferrin receptor is the major pathway by which iron enters the liver. The regulation of this pathway appears to be intact in hemochromatosis, suggesting that other routes of iron uptake are involved in hepatocellular iron loading in that condition. These include clearance of non-transferrin-bound iron and transferrin receptor-independent uptake of transferrin-bound iron. Whether hepatic iron uptake by these mechanisms is solely a consequence of elevated levels of circulating iron resulting from dysregulated intestinal iron absorption, or whether the regulation of these pathways is defective due to a generalized alteration in iron metabolism, remains to be established. Further characterization of the biological effects of the candidate mutation associated with hemochromatosis will help to distinguish between these possibilities.

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15

Extra-hepatic iron metabolism in hemochromatosis

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Introduction

Body iron balance normally remains relatively constant over time. Because humans have no mechanism for regulating the rate of iron excretion, this balance must be maintained by control of intestinal iron absorption. Excessive iron absorption in patients with hemochromatosis leads to body iron overload, the hallmark of the disease. The pattern of increased tissue iron deposition is quite characteristic, with progressive iron accretion in parenchymal cells of the liver and many other organs, but relatively little iron accumulation in reticuloendothelial cells such as hepatic Kupffer cells or bone marrow macrophages until late in the course of the disease[1]. This distribution pattern represents an enigma, given the normal role of the reticuloendothelial system (RES) in iron storage[2], and raises the possibility of a combined defect in hemochromatosis that involves not only the absorptive epithelium of the gastrointestinal tract but also the RES.

The discovery of a strong candidate gene for hemochromatosis (*HFE*) promises to accelerate progress toward elucidation of the biochemical basis of the disease[3]. Most hemochromatosis patients are homozygous for a missense mutation in *HFE* that produces a Cys-282 → Tyr substitution (C282Y). A second mutation, His-63 → Asp (H63D), is found in some hemochromatosis patients who are heterozygous for the C282Y mutation. The function of the normal gene product is unknown, however, nor is it understood how these mutations alter iron metabolism in affected individuals. This chapter will focus on studies of iron kinetics in hemochromatosis patients and the significance of these studies for understanding the mechanism of the increased iron absorption in this disorder. The development of a novel physiologically-based mathematical model of intestinal iron absorption and internal (systemic) iron exchange, coupled with relatively recent advances in computational methods, has made it possible to examine intestinal mucosal and systemic iron transport kinetics in vivo non-invasively[4]. This model has been used to characterize the changes in mucosal iron kinetics associated with increased intestinal iron absorption in hemochromatosis.

Iron absorption and ferrokinetics in hemochromatosis

Transport of non-heme iron across the mucosal epithelial cells of the upper small intestine can be divided into three steps: (i) uptake of iron from the intestinal lumen across the brush border of the enterocyte; (ii) transfer of this mucosal iron across the basolateral (serosal) membrane of the enterocyte to the systemic circulation; or (iii) incorporation of mucosal iron into an intracellular storage pool thought to represent mucosal ferritin, followed by removal of this sequestered iron from the body as the mucosal epithelial cells are sloughed into the intestinal lumen[1]. Although there is general agreement on the importance of these steps, controversy exists about how each step is regulated under various conditions to determine the rate of iron absorption. Retention of orally administered radioactive iron measured by whole-body counting is often used to assess overall iron absorption. This technique has been combined with simultaneous oral administration of non-absorbable markers to determine the amount of iron initially taken up by the mucosal epithelium, the amount retained by the mucosa (and subsequently removed from the body during exfoliation), and the amount finally absorbed into the body[5–7]. These studies indicated that in hemochromatosis and in iron deficiency anemia, there is increased mucosal iron uptake in addition to increased transfer of mucosal iron to the systemic circulation. However, this approach does not distinguish between the

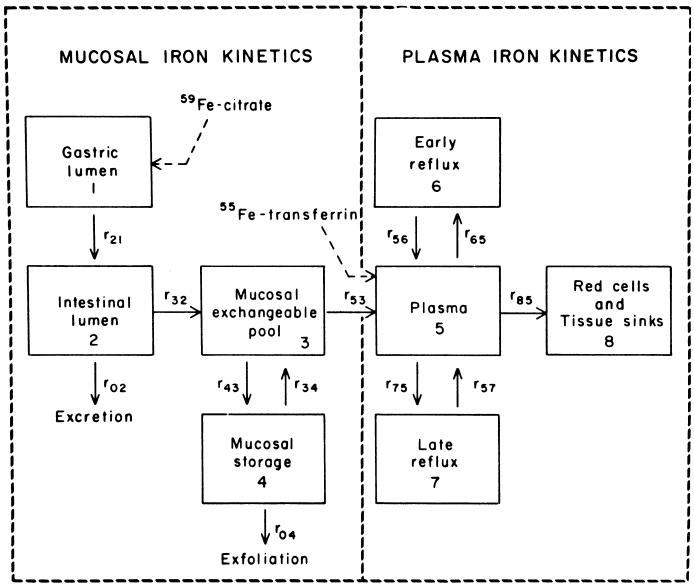

Fig. 15.1. A combined model of iron absorption and plasma iron kinetics. Rate constants of plasma iron kinetics (r_{65}, r_{75}, r_{85}, r_{56}, and r_{57}, right panel) are determined by least-squares fit of the computer- simulated plasma clearance curve to measured plasma ^{55}Fe levels. Rate constants of mucosal iron transport (r_{32}, r_{02}, r_{43}, r_{53}, r_{34}, and r_{04}, left panel) are estimated by least-squares fit of simulated plasma and whole-body clearance curves to measured plasma and whole-body ^{59}Fe levels, respectively[12].

net amounts of iron transported between compartments and the rate constants for each of those transport steps. Classical ferrokinetic studies in hemochromatosis patients have not revealed any striking abnormality of internal (systemic) iron exchange. Plasma iron turnover (PIT) is increased, reflecting a markedly elevated plasma iron concentration, but erythroid iron turnover is normal[8].

Combined model of mucosal and systemic iron kinetics

A mathematical compartment model has been developed of intestinal iron absorption (Fig. 15.1, left panel) that permits analysis of mucosal iron kinetics in vivo[9]. The model represented in Fig. 15.1 corresponds to the set of differential equations that describes the transport of iron between each of the compartments. To fit the model to experimental data, it is also necessary to consider another compartment: the plasma. It has been shown that a previously described stochastic model that represents plasma ferrokinetics as the sum of three decaying exponentials[10] is mathematically equivalent to a specific deterministic (compartmental) model[11] (Fig. 15.1, right panel). The combined model is uniquely identifiable, i.e., only a single set of estimates of the rate constants corresponds to a given set of data. With the combined model (Fig. 15.1) and the appropriate tracer studies, it is possible to estimate simultaneously all the rate constants of mucosal iron transport and systemic iron kinetics.

Animal studies

The model was initially applied to the study of mucosal iron kinetics in beagle dogs under normal conditions[9]. An oral dose of ^{59}Fe was administered in the form of ferric citrate, and an intravenous bolus of ^{55}Fe bound to autologous plasma transferrin was injected simultaneously. Plasma ^{59}Fe- and ^{55}Fe-activities and whole body ^{59}Fe-activity then were monitored at frequent intervals according to a predetermined schedule. The rate constants of mucosal iron transport were estimated by determining the best nonlinear least-squares fit of the model to the data. These studies showed that the rate constant for mucosal iron uptake was at least two orders of magnitude less than the rate constants for either incorporation of iron into the mucosal storage pool, or transfer of mucosal iron to the plasma. This indicated that mucosal iron uptake is the rate-limiting step in intestinal iron absorption under normal conditions.

Studies of beagles with iron-deficiency anemia induced by serial phlebotomy were then conducted. Iron absorption under these conditions was approximately five times greater than under normal conditions. This was accompanied by a marked increase in the mucosal iron uptake rate constant, and a comparable decrease in the rate constant for incorporation of iron into the mucosal storage pool. In contrast, the rate constant for transfer of mucosal iron to the plasma was unchanged. These data suggest that the combination of an increase in the mucosal iron uptake rate constant and a decrease in the mucosal iron storage rate constant leads to expansion of the mucosal exchangeable iron pool, resulting in greater transfer of mucosal iron to the plasma despite an unchanged transfer rate constant[12].

In subsequent studies, we combined our model of mucosal iron kinetics with an expanded model of plasma iron exchange[13] (Fig. 15.2, right panel) to analyze systemic iron kinetics in greater detail[14]. The results confirmed our previous studies of the same animals, and revealed that the changes in mucosal iron kinetics observed in iron-deficiency anemia were paralleled by strikingly similar changes in systemic iron exchange. For example, there was a strong positive relationship between the increase in the rate constant for mucosal iron uptake under these conditions and the increase in the rate constant for uptake of

iron by the erythroid marrow. At the same time, there was a marked decrease in the rate constant for incorporation of iron into tissue storage (represented in this model primarily by the liver), corresponding to the decreased rate constant for iron storage in the intestinal mucosa. These similarities between mucosal and systemic iron kinetics in iron deficiency suggest that each tissue regulates iron exchange independently according to the need for iron within that tissue. Thus, the increased iron absorption in iron-deficiency anemia may represent a response to a contracted intracellular iron pool within the mucosal epithelium, reflecting the state of body iron stores.

More recently, intestinal iron absorption was examined in iron-deficient beagles fed an iron repletion diet containing stearic acid, a fatty acid in beef tallow that enhances non-heme iron absorption. Both iron absorption and the rate of rise of the blood hemoglobin level were increased by the stearic acid-supplemented diet compared with a control diet containing safflower oil. Analysis of mucosal iron kinetics indicated that this effect was not mediated by further alterations in the uptake or storage rate constants, but rather by a marked increase in the rate constant for transfer of mucosal iron to the plasma[15]. Enhancement of non-heme iron absorption by beef tallow may have important implications for the rate of iron accumulation in hemochromatosis patients consuming a typical Western diet.

Studies of normal humans and patients with hemochromatosis

The non-invasive nature of these techniques for compartmental analysis of intestinal mucosal iron kinetics makes it possible to apply this approach to the study of iron absorption in humans, including patients with hemochromatosis. To examine the mechanism of the increased iron absorption in this disorder, we studied six hemochromatosis patients who previously had undergone serial phlebotomy therapy, and were in various stages of iron depletion[16]. Five hematologically normal persons served as controls. Iron absorption in hemochromatosis patients was inversely related to the serum ferritin concentration but was inappropriately high for any given ferritin concentration, consistent with previous studies[17, 18]. Analysis of mucosal iron kinetics showed a similar inverse relationship between the rate constant for mucosal iron uptake and serum ferritin concentration in both normal persons and hemochromatosis patients, indicating that the uptake step is regulated according to body iron stores in humans. However, there was no significant difference in the parameters (slope and intercept) of the regression lines for the two groups.

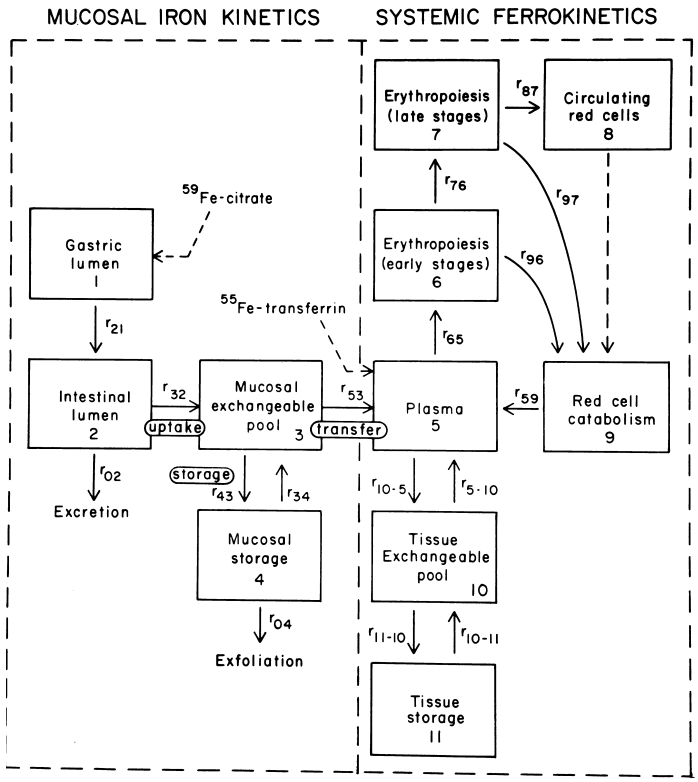

Fig. 15.2. Combined model of iron absorption (left) and internal iron exchange (right). Experimental inputs to the system are a bolus of ^{59}Fe-citrate to the gastric lumen and a bolus of autologous ^{55}Fe-transferrin to the plasma. Outputs are serial measurements of plasma ^{59}Fe and ^{55}Fe, red blood cell ^{55}Fe and whole-body ^{59}Fe. The system parameters (intercompartment transport coefficients) are uniquely identifiable with this input–output arrangement and are estimated by fitting the outputs of model simulations to the tracer measurements. Parameters of particular importance for the modulation of iron absorption are those corresponding to uptake of iron across the brush border of the mucosal epithelium, transfer across the serosa to the plasma, and retention within the mucosal storage pool[13, 14].

Unlike the inverse relationship between the mucosal iron uptake rate and iron stores, there was no consistent relationship between the rate constant for incorporation of iron into the mucosal storage pool and the serum ferritin concentration, nor was there a significant difference between the mean mucosal iron storage rate constant in normal subjects and hemochromatosis patients. In contrast, there was a strong inverse relationship between the rate constant for transfer of mucosal iron to the plasma and serum ferritin concentration in both groups, indicating that the transfer step is regulated according to body iron stores in a manner similar to the uptake step. In

hemochromatosis patients, however, the transfer rate constant was inappropriately high for any given serum ferritin level ($p = 0.0001$), a pattern similar to the relationship in this group of patients between serum ferritin concentration and overall iron absorption. These data indicate that the rate constant for transfer of mucosal iron to the plasma is the major determinant of increased intestinal iron absorption in hemochromatosis. Multiple regression analysis showed that the transfer rate constant was the best predictor of the variability in iron absorption among normal persons and hemochromatosis patients ($R^2 = 0.87$); the relationship between the transfer rate constant and absorption was highly significant ($r = 0.93$; $p = 1.65 \times 10^{-5}$). This increase could be mediated either by an intrinsic abnormality within the intestinal mucosa or by a response of the absorptive epithelium to increased systemic demand. Although the PIT in patients with hemochromatosis was increased, a finding consistent with previous studies[8], this was attributable to an elevated plasma level. The fractional iron clearance rate in hemochromatosis patients was normal, however, suggesting that the increased transfer of mucosal iron to the plasma in hemochromatosis represents an abnormality intrinsic to the intestinal mucosa rather than a response to accelerated plasma iron clearance.

In more recent studies of iron absorption kinetics in human subjects with iron-deficiency anemia, increased intestinal iron absorption in this condition is found to be associated with increases in the rate constants for both mucosal iron uptake and transfer of mucosal iron to the plasma[19]. This is consistent with previous work in mice and humans showing increased mucosal iron uptake in iron deficiency[20, 21]. Considered together, our studies of normal and iron-deficient human subjects and patients with hemochromatosis indicate that both the uptake and transfer steps are important in the control of iron absorption in humans. However, our data indicate that it is primarily the altered regulation of the transfer step that is responsible for the increased iron absorption in hemochromatosis. These results suggest that different mechanisms are responsible for the increased intestinal iron absorption in hemochromatosis and in iron-deficiency anemia.

Implications for regulation of mucosal iron transport

Our studies of iron absorption kinetics in hemochromatosis point to a defect in the control of mucosal iron transport at a site proximal to the mucosal iron uptake step. According to our analysis, the increased non-heme iron absorption in this disorder is attributable primarily to an increase in the rate constant for serosal transfer of iron across the basolateral membrane of the enterocyte to the systemic circulation. Although we cannot rule out the possibility of an associated increase in mucosal iron uptake from the intestinal lumen as suggested by previous studies using other methodological approaches[5–7, 20], our data indicate that the increased transfer rate is the predominant factor that determines the net increase in iron absorption. The ability to make this type of distinction illustrates the value of compartmental analysis in identifying the steps of mucosal iron transport that are modulated in disorders of iron metabolism, thereby informing further investigations into the mechanism of altered regulation of iron absorption in these conditions.

The importance of the transfer step in mediating increased iron absorption in hemochromatosis may offer an explanation for the fact that both heme and non-heme iron are absorbed at an increased rate in this disorder[17]. Heme iron is taken up by a distinct pathway, and release of iron from the protoporphyrin ring by the action of heme oxygenase is an intracellular event[1]. A single genetic defect in hemochromatosis could increase transport of both forms of iron across the mucosal brush border if the mutation affects a regulatory process within the cell that controls the two pathways in tandem. On the other hand, because both forms of iron eventually are incorporated into the same exchangeable pool within the enterocyte[1], a mutation in hemochromatosis leading to acceleration of iron transport at a single step more proximal than the uptake step could account for increased absorption of both heme and non-heme iron. Although the regulation of heme iron absorption has not been examined in our investigations, this mechanism would be consistent with our finding that hemochromatosis patients have an inappropriately increased rate constant for transfer of mucosal iron to the plasma. Of related interest is the enhancing effect of dietary stearic acid on iron absorption. Based on our results in beagles, this effect also appears to be mediated at the level of mucosal iron transfer. Thus, in addition to providing a rich source of iron in the form of heme, meat ingestion may increase iron absorption further in hemochromatosis through a synergistic effect on the transfer step.

One consequence of an increased mucosal iron transfer rate in hemochromatosis might be a contracted mucosal iron storage pool. The mucosal ferritin level does not increase in parallel with the serum ferritin concentration in hemochromatosis[22]. The intestinal mucosal epithelium of patients with hemochromatosis is essentially devoid of immunohistochemically identifiable storage iron in the form of ferritin[23]. However, the mucosal ferritin content in hemochromatosis patients, although much lower than

would be predicted on the basis of serum ferritin concentration, is appropriate for the level of iron absorption[22]. One explanation for these observations could be that in hemochromatosis the exchangeable iron pool within the enterocyte is depleted as the result of the increased rate constant for transfer of mucosal iron to the plasma, allowing little opportunity for incorporation of iron into ferritin.

It is also possible that the mucosal crypt cells in hemochromatosis patients fail to assimilate iron normally from the systemic circulation, despite increased iron stores elsewhere in the body, as the result of a defect in the ability to take up transferrin-bound iron. By immunohistochemical techniques, it has been demonstrated that *HFE* is associated with both β_2-microglobulin and transferrin receptor in human placenta[24]. *HFE* is a major histocompatibility class I-like protein that requires association with β_2-microglobulin in order to be presented on the cell surface, and this capability is lost as a result of the C282Y mutation in hemochromatosis[25]. If the association between *HFE* and transferrin receptor is involved in the regulation of cellular iron uptake and is also altered by the C282Y mutation, this could affect the ability of cells that express *HFE* (including mucosal epithelial cells) to acquire iron. A relative lack of intracellular iron in the crypt cells could establish conditions that favor increased intestinal iron absorption once the cells reach the tips of the villi. A failure of the crypt cells to accumulate iron in hemochromatosis and rapid transfer of dietary iron from the absorptive epithelium to the plasma would have similar effects in limiting the amount of intracellular iron available for incorporation into ferritin. In contrast, a schema to account for the increased iron absorption in hemochromatosis solely on the basis of an increase in the rate constant for mucosal iron uptake from the intestinal lumen would not explain the lack of storage iron within the mucosal epithelium.

An alternative hypothesis is that in hemochromatosis there could be a defect in regulation of mucosal ferritin synthesis that limits the capacity to sequester iron in the mucosal epithelium, thereby interfering with a normal mechanism for controlling iron absorption. Most absorbed iron reaches the systemic circulation by direct transfer from the mucosa to the plasma, without any contact with the mucosal iron storage pool[26, 27]. Iron that is incorporated into mucosal ferritin is not absorbed, but is removed from the body by exfoliation of mucosal epithelial cells[1, 28, 29]. Ferritin synthesis is controlled by iron regulatory proteins (IRPs) according to the availability of iron in the intracellular low molecular weight (LMW) iron pool[30]. Mucosal IRP activity in hemochromatosis patients is increased, indicating a contracted LMW iron pool and suggesting that the relatively low mucosal ferritin level in this disorder is attributable to a low intracellular iron level, rather than to a defect in ferritin synthesis[31]. Consequently, a contracted intracellular iron pool could be the result of increased transfer of mucosal iron to the plasma. The recent identification of *Nramp2*, a transmembrane divalent metal transporter[39] that is mutated in the microcytic anemia (*mk*) mouse strain[32], raises the question whether this protein interacts with *HFE*. If so, altered regulation of *Nramp2* in hemochromatosis patients could be a factor in restricting intracellular iron accumulation in crypt cells.

Our earlier results in iron-deficient beagles indicating a decreased mucosal iron storage rate offer an interesting contrast with our studies of iron-deficient humans, in whom no evidence was found for regulation of the storage step. Thus, whereas iron absorption in dogs may be controlled in part by regulation of mucosal iron storage, this step does not appear to have a major role in the control of iron absorption in normal humans, in patients with hemochromatosis, or in persons with iron-deficiency anemia. These observations suggest differences among mammalian species in the mechanism of control of iron absorption. In humans, the function of mucosal ferritin may be similar to the role of ferritin in other tissues, i.e., storage of intracellular iron that is in excess of requirements for essential cellular functions (and, in the case of the enterocyte, for storing iron taken up across the brush border but not transferred to the systemic circulation). Further studies may reveal a regulatory role for mucosal ferritin in certain other conditions, such as the anemia of chronic disease, in which there is an inflammatory stimulus to ferritin production.

Regulation of systemic iron exchange

An increased PIT was observed in hemochromatosis patients[16], a finding that is in agreement with the results of previous investigators[8]. However, this increase was attributable entirely to an elevated plasma iron concentration. In contrast, the fractional iron clearance rate was normal, consistent with a normal demand for iron to support the needs of the erythroid marrow and other tissues. Other studies have demonstrated that release of iron to the plasma after infusion of heat-damaged, ^{59}Fe-labeled erythrocytes is accelerated in patients with hemochromatosis, suggesting a defect in processing iron derived from hemoglobin degradation within the RES[33]. Blood monocytes of hemochromatosis patients contain less iron and ferritin than do monocytes of patients with transfusion siderosis. However, the rate of ferritin synthesis in hemochromatosis monocytes is normal[34], a finding that could be interpreted to mean that circulating hemochromatosis mononuclear

phagocytes also may have a defect involving cellular iron release. Although the regulation of ferritin synthesis has been characterized in a variety of cell types, the mechanism of cellular iron release is less well understood[35]. The copper-dependent ferroxidase activity of ceruloplasmin is thought to facilitate iron binding to transferrin by catalyzing oxidation of iron (II) to iron (III), although it is not clear that this is necessary for transfer of iron to the plasma. Enhanced activity of a membrane iron transporter involved in the release process could reduce the intracellular LMW iron pool, resulting in decreased ferritin synthesis. Monocyte IRP activity is inappropriately high in hemochromatosis patients in relation to body iron stores, but is modulated normally in vitro in response to manipulations of iron levels[36]. The high monocyte IRP activity levels and low ferritin levels in hemochromatosis monocytes suggest a paradoxical contraction of the LMW regulatory (or exchangeable) iron pool. One mechanism that could account for this phenomenon is increased transfer/release of iron from mononuclear phagocytes to the circulation, like that observed previously in hemochromatosis patients after injection of heat-damaged erythrocytes[33].

Conclusions

Several lines of evidence suggest that there is a defect in the control of cellular iron transfer/release in hemochromatosis that involves both the intestinal mucosa and internal (systemic) iron exchange. Further support for this concept has emerged from recent studies of β_2-microglobulin knockout mice. This animal has increased iron absorption and develops iron overload with a predominance of iron accumulation in hepatocytes and relatively little in Kupffer cells, a pattern similar to that seen in hemochromatosis[37, 38]. Although the mechanism of these abnormalities is not fully understood, these animals fail to express MHC class I products normally. The increased iron absorption in these mice is associated with a failure to limit transfer of intestinal mucosal iron to the systemic circulation[38], a finding that is similar to our observation of an increased mucosal iron transfer rate constant in hemochromatosis. Reconstitution with normal hematopoietic cells after lethal irradiation of β_2-knockout mice redistributed hepatic iron from parenchymal to Kupffer cells, but did not correct the defect in control of mucosal iron transfer. These observations in β_2-microglobulin knockout mice suggest that they have a defect involving iron transport in both the intestinal mucosa and the RES as the result of impaired *HFE* function. The existence of an abnormality in hemochromatosis that affects iron metabolism in both the intestine and the RES is consistent with the concept of an abnormality in regulation of cellular iron transfer/release, because this takes place in both tissues and could be explained by a single genetic defect. In contrast, different pathways are involved in uptake of dietary iron by the intestinal mucosa and incorporation of hemoglobin iron by phagocytosis of senescent red blood cells, the major iron source of iron input to the RES.

Studies of mucosal iron kinetics and of RES iron metabolism are consistent with the existence of a defect in hemochromatosis that results in accelerated iron transfer/release to the plasma. A single mutation involved in regulation of this pathway could account for the shared defect that appears to exist in both tissues, suggesting that the *HFE* gene may be important in the control of iron metabolism both in mucosal epithelial cells and in the mononuclear-phagocyte system.

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16

Mathematical models of metal metabolism in hemochromatosis

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Introduction

Hemochromatosis is an autosomal recessive disorder that affects approximately 0.5% of Caucasians of Western European descent. Many persons homozygous for hemochromatosis genes absorb increased quantities of dietary iron and eventually develop iron overload. Approximately one in seven persons in general Western Caucasian populations are heterozygous for a hemochromatosis gene, and 1–3% of these also develop iron overload. Many other heterozygous persons absorb slightly increased quantities of dietary iron but do not develop symptomatic iron overload. Because the intestinal pathways for iron absorption are not unique to iron, the absorption of certain nonferrous metals is also increased in hemochromatosis homozygotes and some heterozygotes[1].

Traditional measurements of iron absorption, metabolism, and balance have provided valuable, but incomplete, information on the kinetics of iron in hemochromatosis and its relation to normal iron kinetics. These measurements indicate that elevated absorption of iron may start before adulthood and, after many years, may gradually decline as the iron stores enlarge[2]. There appears to be a defect in the ability of the reticuloendothelial (RE) cells to store iron, with the result that the plasma iron concentration is increased and plasma transferrin becomes saturated with iron[3,4]. Non-transferrin bound iron is deposited mainly in the hepatic parenchyma, although the pancreas, thyroid, heart and other tissues may accumulate many times their normal iron content[2,3,5]. Iron excretion is increased slightly due to accumulation of iron in skin and modest increases in biliary and urinary excretion, but increased losses from the body are far outweighed by increased gains due to the elevated absorption from diet[2,6]. Erythropoiesis remains effective and the rates of red cell production and destruction are essentially normal[2]. The quantity of storage iron in bone marrow is often within the normal range even when massive deposits are present in the parenchymal cells of the liver and other organs[2].

The recent appearance of relatively sophisticated mathematical models of the internal kinetics of iron and physiologically related metals[7,8] has presented the opportunity to use computers to explore the implications of previous findings on hemochromatosis. This chapter provides two illustrations of such applications of computer models. In the first example, a new biokinetic model for iron is used to investigate the levels of absorption of iron from the gastrointestinal tract that are necessary to produce observed concentrations of iron in subjects with hemochromatosis. In the second example, a detailed biokinetic model for lead is used to examine possible causes of increased blood lead concentrations in hemochromatosis.

Accumulation of iron at various levels of absorption

Structure and parameter values of the biokinetic model for iron

A schematic of the biokinetic model for iron is shown in Fig. 16.1. This is a modification of a model recently introduced by the International Commission on Radiological Protection (ICRP[8]). Some compartments and paths of movement of iron have been added to the ICRP model, and some components of the ICRP model that were intended mainly for applications in radiation protection have been removed. Efforts are underway to introduce greater detail and realism into the model indicated in Fig. 16.1, but this preliminary version is sufficiently detailed to illustrate potential applications of computer models to the study of abnormal iron metabolism.

Parameter values for a normal adult male are given in Table 16.1. These values are transfer coefficients, meaning that they represent fractional transfers per unit time between compartments. For example, if Compartment A contains 0.4 g of iron, and the transfer coefficient from Compartment A to Compartment B is 2 d^{-1}, then the rate of mass transfer of iron from A to B is 0.4 g $\times$ 2 d^{-1} = 0.8 g d^{-1}.

Although the transfer coefficients in Table 16.1 were developed for an adult male, they will be applied here to males of age 15–50 years, on the basis that only modest changes in the internal kinetics of iron are expected between age 15 years and adulthood[8]. Although there appear to be substantial changes in the distribution of iron in males after age 15 years due to the accumulation of iron stores over time, this does not imply that there are equally large changes in the fractional rates of transfer per unit time between compartments.

The model for iron metabolism in the normal adult male

Parameter values describing the fate of iron in the first few weeks after entry into blood plasma were based on results of radioiron studies on healthy male subjects[2, 8–10]. After those parameter values had been set, values controlling long-term retention and excretion of iron were set for consistency with: estimated iron contents in various iron pools in a male of age 50 years; estimated daily losses of iron along various excretion pathways; and the assumption that 0.9 mg of iron are absorbed each day from food[2, 8]. The normal 50-year-old male is assumed to have a total-body iron content of about 3.9 g, and this is assumed to be divided roughly as follows among major iron pools[2]: erythrocytes (RBC), 2300 mg; hepatocytes, 400 mg; hepatic reticuloendothelial (RE) cells, 50 mg, RE cells of bone marrow, 320 mg; spleen (mainly RE cells), 80 mg; other RE cells, 300 mg; erythroid marrow, 80 mg; plasma transferrin, 2.9 mg; remaining plasma, 0.4 mg; and remainder of the body (including several of the compartments shown in Fig. 16.1), approximately 400 mg. The precise total-body and compartmental contents calculated for age 50 years depend to some extent on the age at which the calculation is started and the assumed compartmental contents at the starting age. The compartment contents given above for a 50-year-old male are based on a starting age of 15 years, with the initial iron content of a given storage pool being 30% of the value indicated above for age 50 years and the initial iron content of a non-storage pool being 80% of the value indicated above.

Iron absorbed from the gastrointestinal tract or returning to plasma after destruction of red blood cells enters a compartment in blood plasma called other plasma, which represents plasma iron that is not bound to transferrin. Iron in other plasma transfers to plasma transferrin. Iron is removed from plasma transferrin with a half-time of 90 min, with about 85% moving to erythroid marrow (marrow synthesis), 5% to the hepatic parenchyma (liver parenchyma 1), and 10% to a compartment representing relatively rapidly exchanging extravascular spaces (extravascular transferrin).

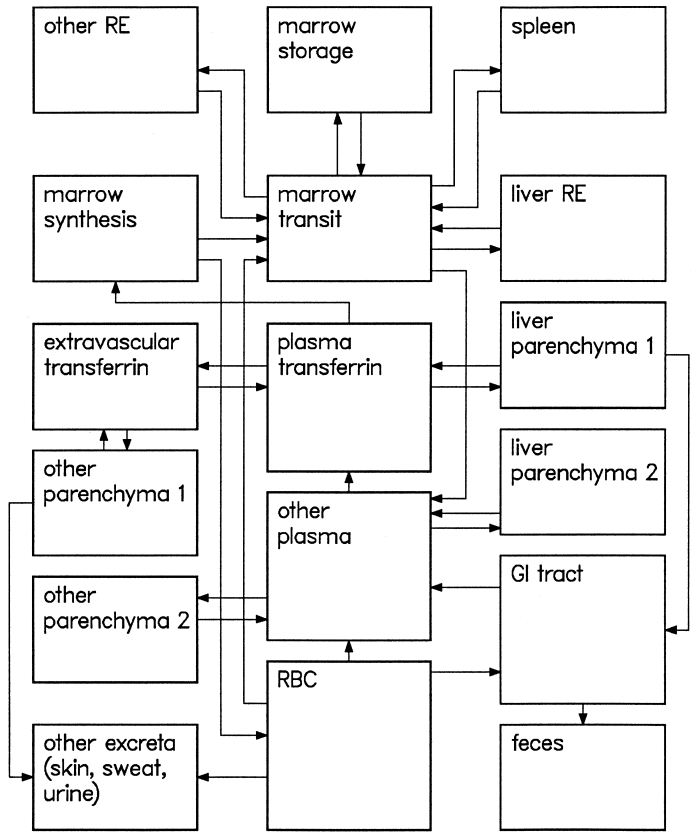

Fig. 16.1. Structure of the biokinetic model for iron.

Iron is removed from marrow synthesis with a half-time of 2 days, with 70% transferring to RBC and the remaining 30%, representing ineffective erythropoiesis, transferring to a marrow RE compartment called marrow transit. The removal of aging erythrocytes from the circulation is depicted as a transfer from RBC to marrow transit, representing phagocytosis by RE cells, plus a smaller transfer (about 10% of the total) from RBC to other plasma, representing intravascular breakage of red cells and release of the hemoglobin into the plasma. Most of the iron entering marrow transit is returned to other plasma with a half-time of 12 h. To account for relatively long- term storage of iron throughout the RE system, a small fraction of iron leaving

Table 16.1. Transfer coefficients for the iron model, for adult males

Path	Transfer coefficient (per day)
Other plasma to plasma transferrin	70 (normal), 0 (saturated plasma transferrin)
Plasma transferrin to marrow synthesis	9.43 (normal), 0 (saturated marrow synthesis)
Plasma transferrin to liver parenchyma 1	0.555
Plasma transferrin to extravascular transferrin	1.11
RBC to other plasma	0.000833
RBC to marrow transit	0.00729
RBC to GI tract	0.0002
RBC to other excreta	0.000015
Marrow synthesis to RBC	0.243
Marrow synthesis to marrow transit	0.104
Marrow transit to other plasma	1.39
Marrow transit to marrow storage	0.0635
Marrow transit to liver RE	0.0106
Marrow transit to spleen	0.017
Marrow transit to other RE	0.0635
Marrow storage to marrow transit	0.0038
Liver RE to marrow transit	0.0038
Spleen to marrow transit	0.0038
Other RE to marrow transit	0.0038
Liver parenchyma 1 to plasma transferrin	0.00364
Liver parenchyma 1 to GI tract	0.00037
Extravascular transferrin to plasma transferrin	0.888
Extravascular transferrin to other parenchyma 1	0.222
Other parenchyma 1 to extravascular transferrin	0.00127
Other parenchyma 1 to other excreta	0.0006
GI tract to feces	0.5
Other plasma to liver parenchyma 2	0.0 (normal), 0.9 (saturated plasma transferrin)
Other plasma to other parenchyma 2	0.0 (normal), 0.1 (saturated plasma transferrin)
Liver parenchyma 2 to other plasma	0.000019
Other parenchyma 2 to other plasma	0.000019

marrow transit is distributed to the RE storage compartments in marrow, liver, spleen, and other tissues called, respectively, marrow storage, liver RE, spleen, and other RE. Iron is removed from these storage sites to marrow transit (which quickly transfers most of the inflowing iron to other plasma) over a period of months. The use of a central compartment within the RE system is a simplification of the real events, in that destruction of red blood cells (including red cell precursors) actually does not occur entirely in the marrow, and iron entering or leaving RE cells in the liver, spleen, and other extra-skeletal sites is not actually channeled through the marrow.

In addition to the RE system, an important storage site for iron is the hepatic parenchyma, represented in this model by the compartment liver parenchyma 1. This compartment receives 5% of the outflow from plasma transferrin. Iron entering liver parenchyma 1 is returned over a period of months to plasma transferrin, except for a small amount, representing biliary secretion, that transfers to small intestine.

It is assumed that most of the iron that transfers from plasma transferrin to extravascular transferrin returns to plasma over the next day or two, but a portion (20%) is taken up by a compartment called other parenchyma 1 representing functional or storage iron not accounted for by explicitly identified tissues and fluids. The compartment other parenchyma 1 also is used to account for losses of iron due to exfoliation of skin, sweating, and losses in urine associated with exfoliation of kidney cells. Iron in other parenchyma 1 that is not lost in excreta returns over a period of months to extravascular transferrin.

In addition to the excretion pathways indicated above, iron is lost from the body in erythrocytes that enter the gut and urinary bladder. According to the model, about two-

thirds of iron losses are in feces and the remainder are in skin, sweat, and urine in normal adult males.

Modifications of the transfer coefficients to address hemochromatosis

The model for normal iron kinetics is used together with a set of 'on/off' switches on certain transfer coefficients to simulate iron kinetics in hemochromatosis. Specifically, inflow into plasma transferrin is turned off if the content of this compartment exceeds an effective iron saturation level, which is assumed to be 9 mg iron; inflow into marrow synthesis is turned off if the content of this compartment rises more than 10% above normal, which is assumed to be about 80 mg iron; and transfer coefficients from other plasma into the 'overflow' compartments, liver parenchyma 2 and other parenchyma 2, are turned on when plasma transferrin approaches its saturation level (specifically, when the iron content of plasma transferrin exceeds 6 mg). The on/off switches are used in lieu of more realistic but more complicated saturation kinetics in which there is a gradual decline in transfer coefficients. The simplistic kinetics applied here suffices for consideration of the long-term behavior of iron.

The switches described above serve to maintain a nearly normal amount of iron in marrow synthesis, in all compartments representing the RE system, and in RBC, despite the elevated iron content of plasma transferrin and the large amounts of iron accumulating in tissues outside the erythroid circuit. Because fractional transfers per unit time from plasma transferrin to liver parenchyma 1 and to other parenchyma 1 are assumed to remain unchanged, the contents of the latter two compartments – in addition to the daily losses from these two compartments in excreta – increase in parallel with the content of plasma transferrin. If plasma transferrin becomes saturated with iron, the masses of iron transferred per unit time into liver parenchyma 1 and other parenchyma 1 have reached their maximum values. Iron in other plasma that would normally enter plasma transferrin is then diverted to the compartments liver parenchyma 2 (90%) and other parenchyma 2 (10%), that have extremely slow losses back to plasma and no direct excretion outlets. Because losses from liver parenchyma 1 and to other parenchyma 1 are limited by the iron- binding capacity of plasma transferrin while losses associated with RBC remain nearly unchanged, total losses of iron from the body lag well behind gains from elevated absorption, and there is a steady net increase in total-body iron, particularly in the liver.

Model predictions of iron accumulation and excretion

The model was used to predict the time-dependent accumulation and excretion of iron for various levels of absorption of iron from the gastrointestinal tract, beginning at age 15 years. This age was used as a starting point because significant iron overload is usually not observed below this age in hemochromatosis homozygotes. Age 50 years was used as the end-point of the analysis because it is the average age at which hemochromatosis is diagnosed in routine medical care. The amount of iron in each storage compartment in a 15-year-old male was assumed to be 30% as much as that in the corresponding compartment of a normal 50-year-old male, and the amount of iron associated with other compartments was assumed to be 80% as much as that in the corresponding compartments of a normal 50-year-old male. Model predictions for times remote from age 15 years were relatively insensitive to assumptions concerning compartment contents at age 15 years.

Calculations were made for five different levels of daily absorption in excess of normal: 1, 2, 3, 4, or 5 mg. Because normal daily absorption is assumed to be 0.9 mg, these values correspond to total daily absorption of 1.9, 2.9, 3.9, 4.9, and 5.9 mg, respectively.

Model predictions of the iron content of the total body, the liver, and daily excreta as a function of age (or time since the start of exposure) are shown in Figs. 16.2, 16.3, and 16.4, respectively. For absorption of 1 mg per day in excess of normal absorption, the model predicts that the total-body content at age 50 years is about 10 g, of which 5.5 mg is in the liver. For absorption of 2, 3, 4, or 5 mg per day in excess of normal absorption, the model predicts a total-body content of about 19, 30, 43, or 55 g, respectively, and a hepatic content of about 13, 23, 34, or 45 g, respectively. As indicated in Fig. 16.4, predicted daily losses of iron do not increase in proportion to absorption at high levels of absorption but converge to a maximum level of about 1.8 mg of iron per day. This is in reasonable agreement with the maximum excretion rate of 1.5 mg per day suggested by Bothwell et al.[2].

Lead absorption and kinetics in hemochromatosis

There is experimental evidence that the absorption of iron and lead are influenced similarly by the state of body iron stores. For example, iron deficiency enhances the absorption of iron and lead[11–13]. Based on observed increases of mean blood lead concentration in a large number of

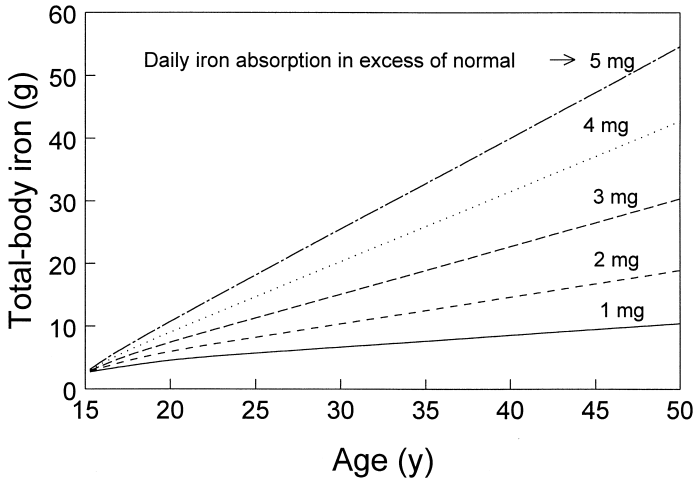

Fig. 16.2. Model predictions of the total-body content of iron in hemochromatosis, for various rates of absorption of iron to blood.

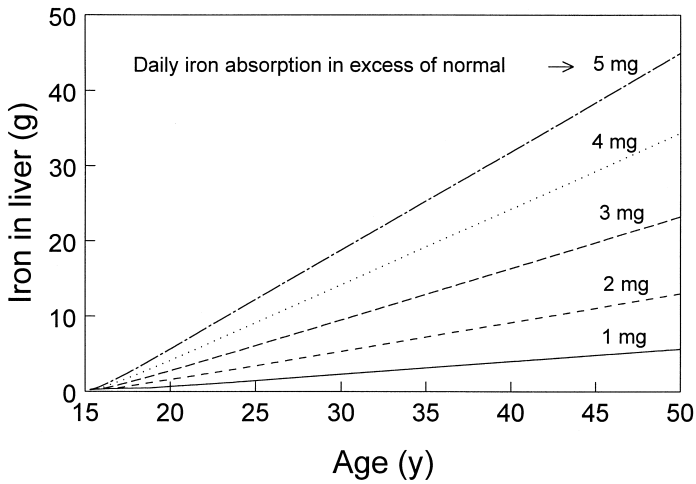

Fig. 16.3. Model predictions of the liver content of iron in hemochromatosis, for various rates of absorption of iron to blood.

subjects, Barton and co-workers[1] postulated that lead absorption is also increased in hemochromatosis. To test this hypothesis, they quantified blood lead levels in homozygotes and obligate heterozygotes, in normal subjects, and in abnormal control patients with transfusion-induced iron overload. These data were analyzed to determine the effects of age, gender, serum ferritin concentration, and extent of phlebotomy and/or blood transfusion on blood lead concentration. Occupational, avocational, and geographic variables that could influence lead exposure were also evaluated. Finally, a detailed, physiologically realistic biokinetic model for lead in humans was used to examine different factors that might contribute to the observed differences in blood lead concentrations[1].

The structure of the biokinetic for lead is shown in Fig. 16.5. The model has been described in detail in previous publications[7, 14]. Because the model has apparently high predictive accuracy and physiologically realistic structure, it can be used to examine changes in the blood lead concentration in response to changes in various aspects of lead metabolism (for example, gastrointestinal absorption, renal clearance, biliary secretion, bone resorption), and thus can be used to test the feasibility of various explanations of the elevated blood lead concentration observed in cases of hemochromatosis.

A chronic exposure scenario was designed to produce the mean blood lead concentration observed in normal controls (3.5 μg dl^{-1}). Under any plausible exposure scenario, the model predicts that childhood exposures have little affect on the blood lead concentration beyond the third decade of life. For purposes of this exercise, it suffices to use a relatively simple exposure scenario in which an adult with no previous exposure to lead is exposed for several years (20 years was used for definiteness) to a constant ambient level of lead. For the typical adult as defined by the ICRP's biokinetic model for lead, an uptake rate from environment to blood of about 7 μg day^{-1} yields the blood lead concentration observed in normal controls. Although the route of entry of lead into the body is not important here, it is assumed for definiteness that lead is taken into the body only by ingestion, that the ingestion rate is 35 μg day^{-1}, and that 20% of lead entering the small intestine is absorbed to blood. Alternatively, one could assume that the ingestion rate is 70 μg day^{-1} and that 10% is absorbed from the small intestine, for example, although there is a slight difference in model predictions in this case because the gastrointestinal absorption fraction is applied to endogenous secretions into the gastrointestinal tract as well as to ingested lead. The particular gastrointestinal absorption fraction used as a baseline case does not affect the main results of this test, however, provided gastrointestinal absorption remains well below 100%.

With the derived exposure scenario, model parameter values representing various isolated aspects of lead metabolism were then varied, one at a time, to determine the nature and extent of changes in lead metabolism required to explain the mean blood lead concentration observed in hemochromatosis homozygotes (5.6 μg dl^{-1}). It was found that the mean blood lead concentration observed in hemochromatosis homozygotes can be achieved if any one of the following conditions occurs throughout the period of exposure:

(i) The gastrointestinal absorption fraction for lead is about 1.5 times that in normal controls.
(ii) The fractional uptake of lead by red blood cells from plasma is about 1.6 times that in normal controls.
or
The removal half-time from red blood cells to plasma is about 1.6 times that in normal controls.
(iii) Renal clearance of lead is only one-third of that in normal controls.

The mean blood lead concentration observed in hemochromatosis *heterozygotes* can be explained by the qualitative changes indicated in items (i)–(iii) but with smaller quantitative changes from the baseline case. In addition, the blood levels in heterozygotes (but not in homozygotes) can be explained by the assumption that:

(iv) Biliary secretion of Pb is virtually eliminated.

Even radical changes in the level of osteoclastic resorption of bone, exchangeability of bone lead, uptake or loss of lead by soft tissues, or losses of lead via minor excretion pathways (for example, perspiration) did not explain the elevated blood levels in either group. Of course, the elevated blood levels observed in either homozygotes or heterozygotes can be explained by various combinations of the qualitative changes indicated in items (i)–(iv).

The explanation that the gastrointestinal absorption fraction for lead in homozygotes is approximately 1.5 times that in normal control subjects is consistent, at least qualitatively, with the observation that absorption of iron and cobalt may be increased two- to three-fold in homozygotes. Barton and co-workers[1] concluded that none of the alternate explanations (items (ii)–(iv)) are consistent with available data. For example, there is no evidence of altered rates of uptake or loss of lead or related metals by erythrocytes in hemochromatosis, and neither iron deficiency nor iron overload affects iron excretion in rats[12]. Thus, model predictions support the explanation of increased absorption of lead from the gastrointestinal tract in hemochromatosis.

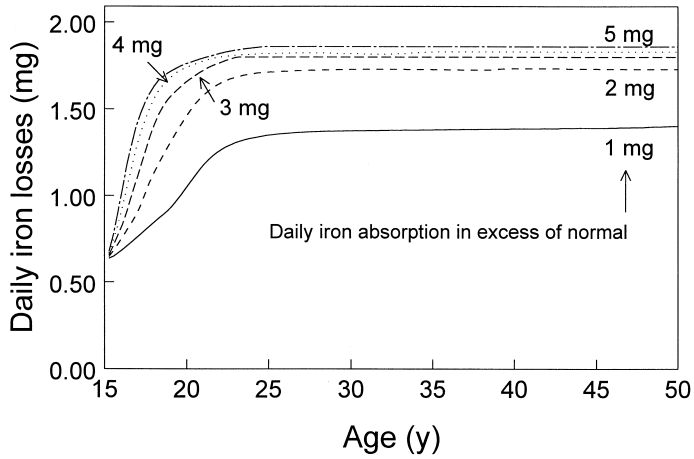

Fig. 16.4. Model predictions of daily losses of iron from the body in hemochromatosis, for various rates of absorption of iron to blood.

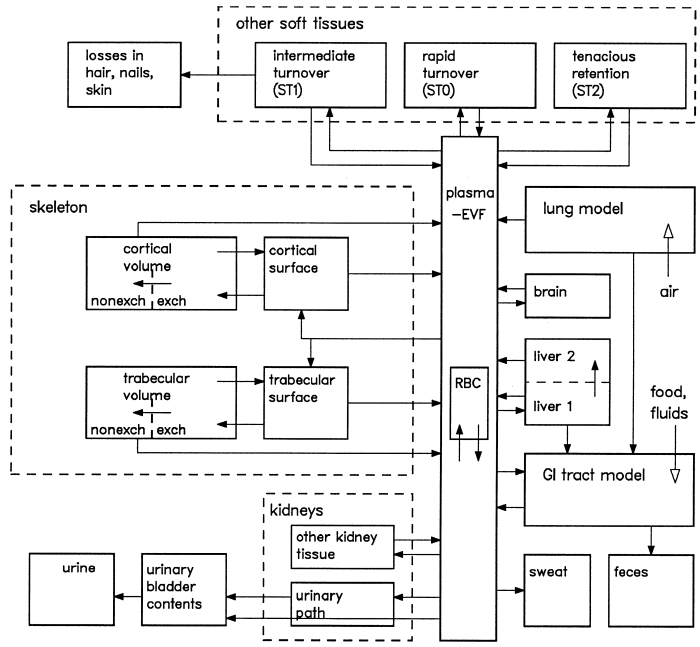

Fig. 16.5. Structure of the biokinetic model for lead[14].

Conclusions

Mathematical models can be used to organize and reduce available data on normal and abnormal kinetics of iron and, depending on the detail and realism of the models, may reveal implications of those data not otherwise apparent. To realize the potential of computer models as clinical tools for diagnosis and treatment of hemochromatosis and other iron-related diseases, however, a dedicated effort must be made to increase the detail and realism in current biokinetic models for iron and physiologically related metals.

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17

Expression of iron overload in hemochromatosis

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Introduction

The amount of body iron stores is a major determinant of clinical outcome in patient with hemochromatosis. Patients with greater iron overload, as assessed by hepatic iron concentration (HIC) and/or the amount of total iron removed by phlebotomy (IR), have an increased prevalence of diabetes, cardiomyopathy and hypogonadism[1–4] and an increased risk of mortality[5]. HIC greater than 400–500 μmol/g is an important risk factor for hepatic fibrosis and cirrhosis[6, 7]. In contrast, arthropathy is unrelated to iron stores[1, 4, 8] and, unlike other clinical manifestations, is not significantly improved by phlebotomy therapy[1]. It is generally accepted that the liver is the first site of iron deposition in persons with hemochromatosis, and that other organs are involved later[3, 9]. Previous studies have suggested that there may be a threshold in the liver for iron accumulation [3, 4, 10]. Mandelli et al.[3] showed that the increases of HIC became progressively smaller until HIC plateaus at a value of ~350 μmol, after which iron accumulation progresses in extrahepatic sites as shown by the increase of both serum ferritin concentration and IR, and by the higher frequency of clinical extrahepatic manifestations excluding arthropathy.

Factors affecting the expression of iron overload

The degree of iron overload and the related clinical complications vary widely in hemochromatosis[1, 2, 8, 9], and both acquired and genetic factors influence phenotypic expression of the disease.

Most clinical complications increase with age[1, 7, 8] and with body iron stores[1, 4, 5, 7]. Previous studies have shown the age-dependent nature of iron accumulation in homozygous hemochromatosis patients[6, 11, 12]. Based on this finding, an age-related index, e.g., the hepatic iron index (HIC/age in years = HII) has been proposed[6], and is reliable in differentiating heterozygotes and patients with alcohol-dependent iron overload from hemochromatosis homozygotes[6, 11, 12]. More recently, the relationship between HIC and age was questioned by Loreal et al.[7] who found the relationship only in younger patients, but not in the whole group of patients studied. Similar negative results were observed in a collaborative French–Canadian study of 410 homozygous hemochromatosis patients of all age ranges[1], and in 152 Italian patients in whom iron stores were assessed by IR (Fig. 17.1). Age dependency of iron overload has been reported by Crawford et al.[13], who showed higher HIC values in older than in younger siblings from Australia, a conclusion not reached in a study of three hemochromatosis families from Canada[14]. Data from Italian hemochromatosis families which demonstrate that older siblings have higher amount of iron overload and clinical complications than younger ones are displayed in Fig. 17.2.

These results suggest that: (a) the iron accumulation rate is not constant throughout the life of hemochromatosis patients. In agreement with this hypothesis is the demonstration that intestinal iron absorption decreases with progressive iron accumulation in hemochromatosis[15]; and (b) the iron accumulation rate is variable among single hemochromatosis patients; it ranged from 110 to 880 mg Fe/year in 47 Italian male patients, as assessed by the ratio of IR to age (IR/age)[4]. This second hypothesis does not exclude the former, and is supported by the presence of interfamilial heterogeneity and intrafamilial homogeneity of phenotype expression[13, 16]. The wide variability of iron accumulation among hemochromatosis patients is also shown by the highly variable rate of increase of serum ferritin concentration after iron depletion[17]. These data suggest that the iron accumulation rate differs markedly in

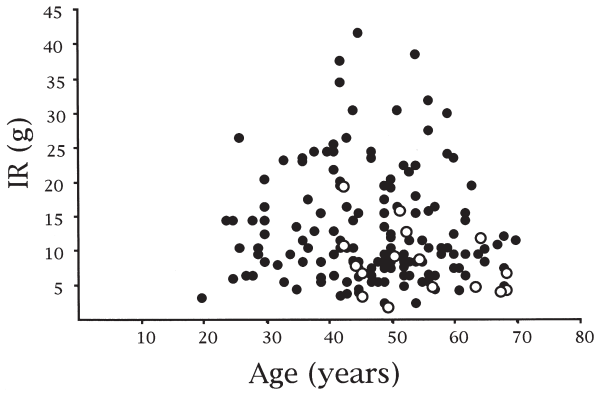

Fig. 17.1. Relationship between total iron removed by phlebotomy (IR) and age in 152 Italian patients with homozygous hemochromatosis (❍: women; ●: men). (Data from Fargion et al.[18]).

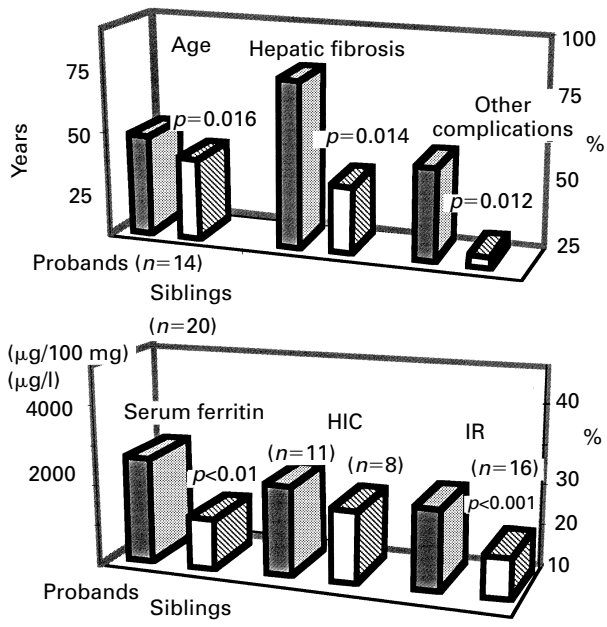

Fig. 17.2. Mean age, frequency of hepatic fibrosis and other clinical complications (diabetes, cardiopathy and hypogonadism), levels of serum ferritin, hepatic iron concentrations (HIC) and total iron removed by phlebotomy (IR) in Italian male hemochromatosis probands and in their younger HLA identical male siblings.

hemochromatosis patients depending on genetic or acquired factors or both.

Environmental factors

Several environmental or acquired factors have been implicated in determining the variable phenotype expression of hemochromatosis, e.g., physiological (in women) and pathological blood losses and blood donations, and dietary iron content.

Sex

Previous studies demonstrated that clinical manifestations of iron overload predominate in male homozygotes[7, 8]. In probands there is a higher proportion of men than women in all populations studied, ranging from 2:1 to 8:1[1, 5, 8, 18], whereas in patients discovered through family study the proportion is similar to the expected 50%[1, 8, 19]. The difference in clinical expression of the disease between males and females has been attributed, in large part, to the lower magnitude of iron stores and levels of iron absorption in female than in male probands[10, 12, 13, 19], the accumulation rate of iron being four-fold greater in men than in women[12]. Among affected siblings discordant for sex, females showed lower liver iron concentration than their male siblings[13]. Similarly, in affected siblings of different sex, we found that total iron removed by phlebotomy was markedly lower in females than in males, unless amenorrhea was present (Fig. 17.3). Menses double or triple the daily iron losses, and the total iron requirement for a normal singleton pregnancy has been estimated at 800 mg (range 500–1400 mg)[20]. Because homozygous subjects accumulate approximately 300–500 mg storage iron per year, homozygous menstruating women may have relatively little iron overload and approximately 6% of them have normal serum ferritin concentrations and transferrin saturation values[10]. On the other hand, the expression of iron overload in women can be very severe. This is the case in the juvenile form of hemochromatosis that similarly affects both sexes and is characterized by the development of heart failure and hypogonadotropic hypogonadism, usually before the age of 30[21].

Dietary iron content

Excess iron cannot be accumulated unless the diet provides more available iron than is required to replace obligatory losses and is required for growth, pregnancy, and lactation. The assumption that the amount of iron in the diet may influence the phenotypic expression of hemochromatosis is based on the evidence that heme iron is readily absorbed and promotes the absorption of non-heme iron, whereas non-heme iron has a low bioavailability and its absorption is influenced by other dietary ingredients[20, 22]. The great per capita consumption of meat, for example, has been proposed as a cause of the high prevalence and relative early age of presentation of fully devel-

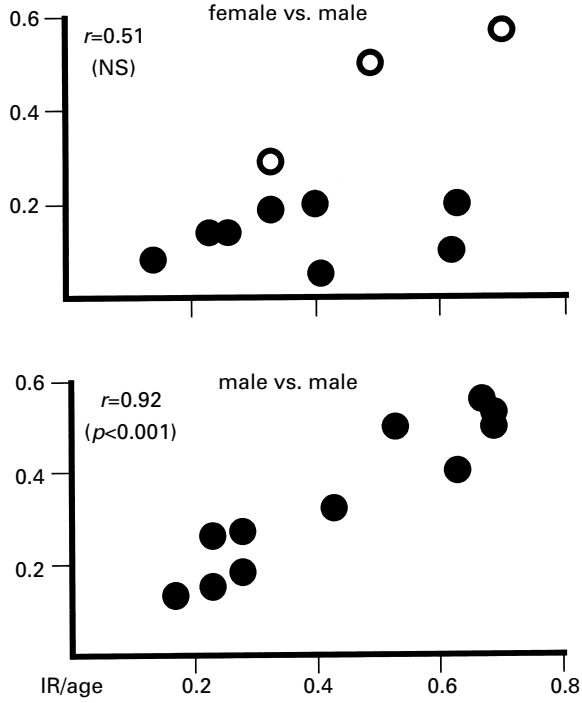

Fig. 17.3. Correlation of the ratio of total iron removed by phlebotomy (IR) and age (IR/age) between Italian hemochromatosis probands and HLA-identical siblings of opposite and same sex (open circles: women who develop amenorrhea before age 40).

oped iron overload disease in Australian patients with hemochromatosis[9]. However, the large variation of iron overload could not be accounted for by meat consumption or alcohol intake in 41 Australian homozygous patients[13]. A study performed in a large, healthy Australian population indicated that diet, in particular meat consumption, was an important factor influencing the concentration of serum ferritin in women but not in men[23]. Other studies designed specifically to evaluate the role of dietary iron in determining the phenotypic variability of hemochromatosis are not available.

Other factors

In the same large population survey[23], blood donation status had the second greatest influence on serum ferritin concentration after sex. In hemochromatosis patients, regular blood donations and pathological blood loss (e.g., from peptic ulcer, hookworm infestation, and chronic inflammatory bowel diseases) delay the expression of the disease in homozygotes[9, 13]. Failure to express iron overload disease, especially in male homozygotes, or failure to reaccumulate iron after a course of venesection therapy, should lead to suspicion of occult blood loss.

Heavy alcohol intake is a common feature in patients with hemochromatosis, although great differences exist in different hemochromatosis populations[7–9]. Whether alcohol ingestion may increase iron absorption is controversial. Even in South African blacks who ingest large amounts of iron from home-made beer brewed in iron pots, the additional presence of an iron-loading gene is needed if iron overload is to develop[24]. There is no evidence that heavy alcohol intake increases the amount of body iron overload in homozygous hemochromatosis[9]. However, chronic high alcohol intake accelerates the development of organ damage[9, 25], influences the distribution of iron by increasing storage in extrahepatic sites[3], and increases the risk of hepatocellular carcinoma in these patients[26]. Similarly, chronic hepatitis B or C infections favor the development of hepatic cirrhosis and hepatocellular carcinoma in hemochromatosis patients, but do not increase total body iron[25, 26]. Other genetic abnormalities that increase iron absorption, e.g., β-thalassemia minor, hereditary spherocytosis, or erythrocyte pyruvate kinase deficiency, may influence the expression of iron overload in persons who also have hemochromatosis[2, 18]. Accordingly, it was recently found that hemochromatosis patients homozygous for the C282Y mutation who also had β-thalassemia trait had very severe iron overload[36]. These aspects are discussed in detail in another section.

Genetic factors

The description of several clearly distinguishable modes of expression in different families first suggested that more than one genetic defect might be responsible for iron overload in hemochromatosis[16]. Analyses of sibling pairs[2, 13] and HLA-A related haplotypes[4, 13] have evaluated the relative impact of genetically determined factors on the phenotypic expression of the disease. The recent isolation of the *HFE* gene[27] has provided an opportunity to analyze the effect of gene mutations on phenotype directly.

Sibling pair studies

The concordance of the severity of iron overload in affected siblings provided strong evidence that the phenotypic expression of this disorder is influenced by genetic factors[2, 13]. Correlation coefficients for HIC, HII and IR/age in siblings of the same and opposite sex are displayed in Table 17.1[2, 13]. The relationship of IR/age in Italian sibpairs is displayed in Fig. 17.3. The correlation coefficient

Table 17.1. Correlation coefficients for indices of iron stores in homozygous hemochromatosis siblings (see also Fig. 17.3)

Siblings	LIC	HII	IR/age
Same-sex pairs	$r=0.81^a$	$r=0.70^a$	$r=0.92^b$
	$p=0.0003$	$p=0.004$	$p<0.001$
Opposite-sex pairs	$r=0.73^a$	$r=0.63^a$	$r=0.51$
	$p=0.06$	$p=0.13$	NS

Note:
[a] Crawford et al.[13]; [b] Camaschella et al.[2].

and the slope of the regression line were greater for same-sex than for opposite-sex sibling pairs, probably due to the lower amount of iron overload in menstruating women[13]. From the results of these studies it was argued that genetic factors are major determinants of phenotype heterogeneity, and that more than one mutation could exist in hemochromatosis.

HLA-A related haplotypes

Several studies demonstrated the presence of a common ancestral haplotype, HLA-A3 linked, in 30 to 50% of the affected chromosomes in all the populations studied[2]. Two independent studies have shown an association between the ancestral haplotype and phenotype expression in hemochromatosis[4, 28]. Australian patients with two copies of the ancestral haplotype have a higher hepatic iron index than those carrying a single copy or none[28]. Italian patients with the ancestral haplotype, even in a single copy, have higher HII, total iron removed (IR), and IR/age, than do patients who lack this haplotype[4]. Recently, Porto et al. found that patients with HLA-A3 had significantly greater iron stores assessed by IR, and a more severe disorder than patients who are negative for HLA-A3[41], confirming the association between ancestral haplotype and phenotype expression in hemochromatosis. These studies were based on the assumptions that patients with the same haplotype have the same mutation, and that the most severe mutation is linked to the ancestral haplotype. This latter assumption was contradicted by the recent demonstration of a single major mutation (C282Y) of the *HFE* gene in homozygous hemochromatosis[27].

The HFE gene

Two mutations have been found in *HFE* gene, as described in details in other sections. The majority of patients are homozygous for the C282Y mutation. The frequency of homozygosity ranged from 64% to 100% in different series[27, 29–33], with a decreasing gradient from northern to southern Europe[30, 31] This suggests that hemochromatosis in southern Europe is more heterogeneous than in northern Europe, as described in other common genetic disorders[31]. The existence of a highly prevalent mutation (C282Y) makes it difficult to explain the phenotypic variability of hemochromatosis, the previous findings on affected sibling pairs, and those on ancestral haplotype-related phenotype expression. The second known mutation of the *HFE* gene, H63D, is present in a minority of hemochromatosis patients, and is associated with a milder form of disease and shows incomplete penetrance[27].

Phenotype expression in C282Y homozygotes

Besides the wide variability found in each genotype, C282Y homozygotes had significantly higher iron indices as compared to compound heterozygotes, and patients heterozygous or homozygous for the H63D mutation[34–36]. However, in a recent screening for hemochromatosis by genetic testing in 7820 blood donors from South Wales, five subjects (three women and two men) homozygous for C282Y mutation were identified who did not have evidence of iron accumulation[37]. Accordingly, a high prevalence of the molecular defect versus the biochemical expression of the disorder have been reported in Australian population[38], suggesting that penetrance of the C282Y mutation is not complete, or that full clinical expression of the disease is modulated either by other genetic or environmental factors in that population. Moreover, discrepant phenotypes have recently been reported in a few families with multiple affected identical siblings, showing individuals homozygous for the C282Y mutation who do not express iron overload[39]. Among patients homozygous for the C282Y mutation, the presence of the ancestral haplotype, even in single copy, was associated with a more severe phenotype[36]. Whether other 6p-linked gene(s) accounts for the association between the ancestral haplotype and more severe expression of the disease remains to be defined[31, 32].

Phenotype expression in C282Y heterozygotes

The frequency of C282Y heterozygotes expressing a homozygous hemochromatosis phenotype ranged between 5 to 10% in the different series[27, 29, 31, 33]. Half of these patients carry the H63D mutation on the other chromosome, suggesting that the two mutations could interact in producing significant iron overload[27, 40].

Phenotype expression in patients without the C282Y mutation

Some authors have suggested that H63D mutation could be only a polymorphic change[32], but some data indicate that its presence is not irrelevant to the development of iron overload. The H63D mutation is in complete linkage disequilibrium with the C282Y mutation, because no chromosome with the C282Y mutation carries the H63D variant[40]. Thus, when data are corrected for the number of chromosomes that do not carry the C282Y mutation, the frequency of H63D is higher in patients than in controls in most of the studies[27, 29, 31, 33]. However, because the H63D mutation, both in the heterozygous and homozygous configurations, is found among normal persons in the population[27, 29, 31, 33], it is likely that this mutation has a variable penetrance[27, 40], or that it has a very mild effect on iron metabolism and is not sufficient per se to give rise to significant iron overload. Patients who are heterozygous or homozygous for H63D and who meet current diagnostic criteria for overt hemochromatosis generally have mild phenotypic expression of disease[34–36]. It is possible that H63D can produce a hemochromatosis-like phenotype only by interacting with other inherited or acquired factors unrelated to hemochromatosis that nonetheless affect iron metabolism.

Patients with a hemochromatosis-like phenotype and the wild-type *HFE* genotype comprised 0–21% of different populations[27, 29–33]. In Italy, the frequency of these patients significantly increased from the north to the south[36], suggesting that the heterogeneity of hemochromatosis in the Mediterranean area could be ascribed in part to the heterogeneous genetic background of Mediterranean populations[31]. Phenotype expression is variable in these patients, but can be very severe[35].

Interaction with additional factors unrelated to hemochromatosis

It has been hypothesized that the interaction of these factors, e.g., β-thalassemia trait, high alcohol intake, and chronic hepatitis, with the *HFE* gene may influence the phenotypic expression of hemochromatosis[2]. Although only β-thalassemia trait seems to increase the severity of iron overload in C282Y homozygotes[36], it is not easy to define the role of these additional factors in patients without two copies of C282Y mutation. Families have been observed with several affected siblings in which the severity of iron overload was not related to the presence of additional factors in at least 50% of the cases[36]. It is possible that, in single cases, the interaction of these additional factors with a single mutation may produce a hemochromatosis-like phenotype (phenocopy).

Among the HLA-A3 positive hemochromatosis patients, Porto et al. found a highly significant positive correlation between iron stores measured as IR and CD4:CD8 ratio[41]. This relationship was not observed in HLA-A3 negative patients. Greater understanding of these results will require additional evaluation of the interrelationships of HLA-A3 positivity, CD4:CD8 ratio, and *HFE* genotype on expression of the clinical phenotype of hemochromatosis.

Among 1058 subjects heterozygous for hemochromatosis, paternal hemochromatosis alleles had a greater effect on the clinical phenotype of female heterozygotes than did maternal alleles, suggesting a parent-of-origin effect of the hemochromatosis gene[42]. This effect has never been described in hemochromatosis, although it is established for several other hereditary disorders[42]. Unlike previous observations in Australian heterozygous females[28], there was no apparent association between phenotype expression and HLA-A3 (the HLA allele associated with the ancestral haplotype) in these heterozygous subjects[42].

Conclusions

Despite major advances in the comprehension of the genetic basis of hemochromatosis and iron overload, several questions remain unresolved. The discovery of a single major mutation (C282Y) in the *HFE* gene does not explain the variability of iron overload in C282Y homozygotes. The concordance of disease expression between siblings, in addition to the association between disease severity and the presence of the ancestral haplotype, suggest that other genetic factors are involved in the regulation of iron metabolism interacting with or modulating *HFE* gene function. The elucidation of the mechanisms of regulation of iron absorption by the *HFE* protein will provide new insights into the comprehension of genotype–phenotype correlations in hemochromatosis.

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Part IV

Diagnostic techniques for iron overload

18

Liver biopsy in hemochromatosis

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Introduction

Biopsy of the liver is considered by many authorities to be essential when the diagnosis of hemochromatosis is suspected due to clinical or biochemical abnormalities[1, 2]. Others have questioned the need for hepatic biopsy in the evaluation of persons with hemochromatosis. The essential feature of patients with hemochromatosis is increased body iron stores with excessive parenchymal deposition of iron, most easily demonstrated in the liver. The degree of iron overload is influenced by age, sex, previous blood donations or blood loss, alcohol use, oral iron intake, and other factors.

Selection of patients for hepatic biopsy

Clinical features

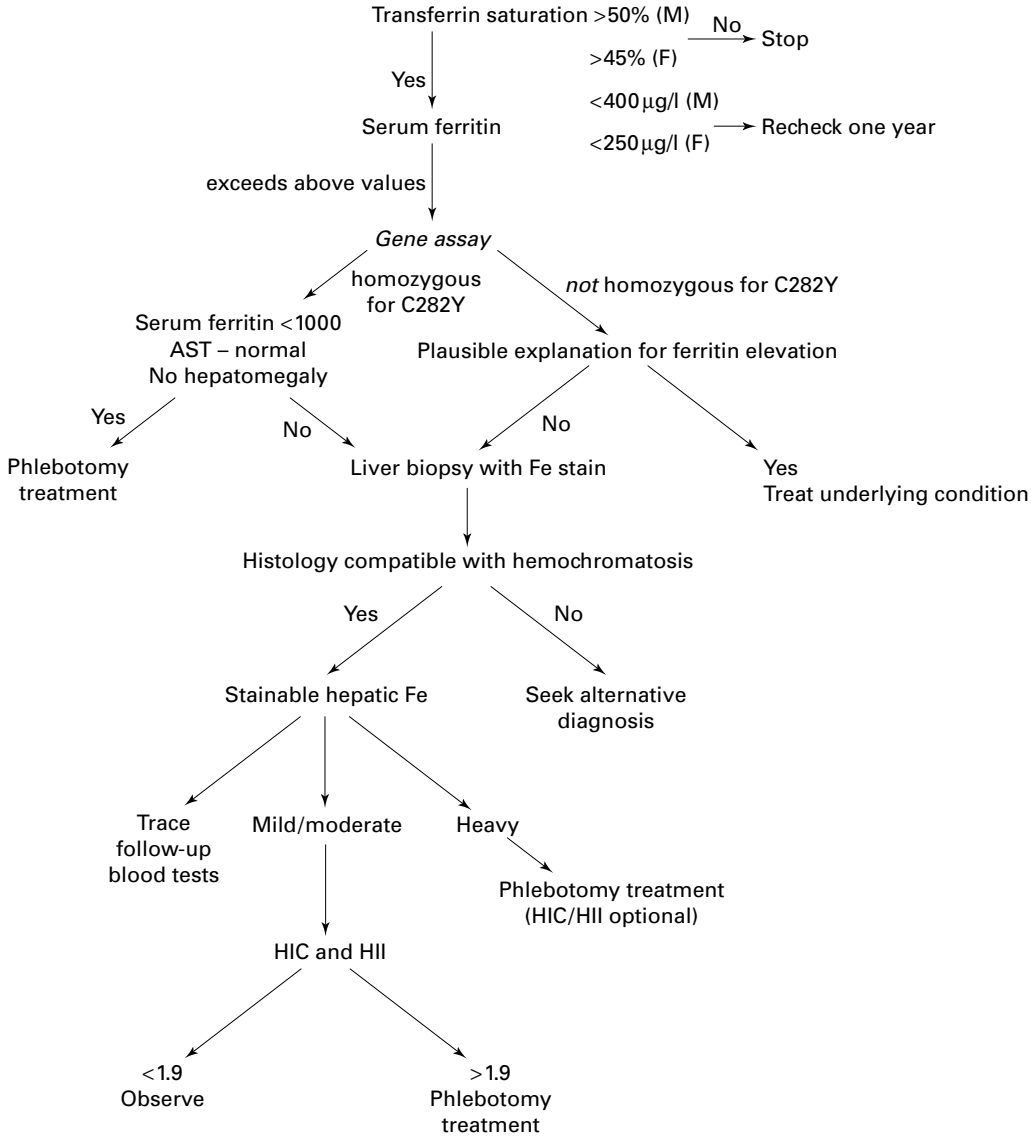
Selecting patients to undergo hepatic biopsy is often the most difficult decision in the management of persons suspected of having hemochromatosis (see algorithm: Table 18.1). This diagnosis should be considered in any patient with unexplained hepatic disease, hepatomegaly, hypogonadism, arthropathy, diabetes mellitus, hyperpigmentation, or cardiomyopathy. Distinguishing hemochromatosis on clinical grounds from hematologic disorders characterized by chronic anemia secondary to ineffective erythropoiesis and iron overload due to increased absorption of iron, and increased iron intake due to medicinal iron or repeated red blood cell transfusions is usually simple.

Laboratory investigation

The serum concentrations of liver-related enzymes (e.g., aminotransferases and alkaline phosphatase) are usually normal or mildly increased in hemochromatosis. Unexplained increases in their serum concentrations should prompt further investigation for iron overload[3, 4]. The transferrin saturation and serum ferritin concentration are useful indicators of hemochromatosis and body iron stores, but the serum iron concentration per se is not. In most patients with hemochromatosis, the transferrin saturation exceeds 55% in men and 50% in women. False-negative values are uncommon. It is unusual to observe a normal transferrin saturation value in a patient with significant iron overload, although this can occur in young persons, patients with a history of blood loss, or in those with acute or chronic inflammatory illnesses. Several factors can 'falsely' elevate the transferrin saturation value, including the use of medicinal iron or estrogen preparations, injury of the liver due to alcohol ingestion, nonalcoholic steatohepatitis, and hepatitis C. Mild or moderate elevations of transferrin saturation occur in approximately 10% of hemochromatosis heterozygotes[5]. Patients with advanced hepatic disease usually have diminished serum transferrin concentrations (and serum iron-binding capacities) due to impaired hepatic synthesis of transferrin.

The serum ferritin concentration is correlated fairly well with body iron stores and therefore is usually markedly elevated in persons with advanced hemochromatosis. It is estimated that 1 μg/l of serum ferritin concentration corresponds to 7–8 mg of stored iron[6]. Elevations of serum ferritin concentration also occur commonly in persons without significant iron overload, making the decision of whether to perform hepatic biopsy difficult. Ferritin is an acute-phase reactant, the blood serum concentrations of which may be elevated in many inflammatory conditions. Hepatic disorders are often accompanied by high serum ferritin concentrations in the absence of iron overload, including forms of hepatic injury characterized by active inflammation and elevation of aminotransferase levels

Table 18.1. Algorithm for evaluation and treatment in suspected hemochromatosis



Notes: AST: aspartate aminotransferase. HIC: hepatic iron concentration. HII: hepatic iron index.

(e.g., alcoholic and non-alcoholic steatohepatitis, viral or drug-induced hepatitis, and ischemic injury). Therefore, alternative explanations for elevation of serum iron parameters (transferrin saturation value and serum ferritin concentration) should always be considered before proceeding with hepatic biopsy for suspected hemochromatosis. The serum ferritin concentration may also be mildly increased in individuals who are heterozygous for hemochromatosis alleles, or in young homozygotes. In the latter, the serum ferritin concentration tends to rise progressively with age, an observation that may be useful in the decision to proceed with biopsy[7].

The hemochromatosis-associated gene *HFE* has already attained an important role in confirming the diagnosis of hemochromatosis in some patients, and analysis of *HFE*-associated mutations may influence the clinician's decision whether to perform hepatic biopsy[8].

It is difficult to set specific biochemical guidelines for performing hepatic biopsy in persons suspected to have hemochromatosis; factors such as age, family history, and

the patient's overall medical condition must also be considered. In general, transferrin saturation values >55% in men and >50% in women and serum ferritin concentration >400 ng/ml in men and >250 ng/ml in women are reasonable indications for performing hepatic biopsy. A morning serum iron concentration and iron-binding capacity should be measured after the patient fasts overnight; abnormal values should be confirmed on at least one additional occasion.

If the transferrin saturation or serum ferritin concentration is elevated to a lesser degree than cited above, the most reasonable approach is to monitor serum iron parameters at yearly intervals and proceed with biopsy only if they rise progressively. This approach should detect significant iron overload before tissue damage occurs. However, some hemochromatosis homozygotes have lesser elevations of serum iron parameters than those cited above[9]. In such cases, excess hepatic iron deposition is usually minimal. From the practical standpoint, hepatic biopsy is not recommended in the absence of evidence or iron overload, as indicated by blood biochemical parameters.

Do all patients with suspected hemochromatosis require hepatic biopsy? Prior to the advent of genotypic testing, most authorities recommended hepatic biopsy in all but young patients with mildly abnormal serum iron parameters, normal serum concentrations of hepatic enzymes, and no history of alcohol abuse or viral hepatitis. The information to be gained from evaluation of liver tissue obtained through biopsy includes assessment of the amount and distribution of hepatic iron and determination of the degree of fibrosis. There is mounting evidence that homozygosity for the C282Y mutation is indicative of hemochromatosis. Thus, assessment of hepatic iron deposition may no longer be essential for the diagnosis of hemochromatosis in all instances. Still, the microscopic evaluation of liver tissue provides the most direct means of determining the degree of hepatic fibrosis and the presence of cirrhosis. Recently reported evidence suggests that severe hepatic fibrosis is unlikely when the serum ferritin is <1000 μg/l, the serum AST concentration is normal and there is no evidence of hepatomegaly[10]. If these observations are confirmed, an appreciable portion of those patients who are homozygous for the C282Y mutation may not require hepatic biopsy. This recommendation does not apply to individuals with non-C282Y-linked iron disorders. In most clinical situations not meeting the above criteria, a single liver biopsy is warranted to confirm the diagnosis of hemochromatosis, to exclude other hepatic conditions and to assess hepatic iron deposition and the degree of tissue damage.

Methods of liver biopsy

Technique and risk of the biopsy procedure

Percutaneous needle biopsy of the liver is performed commonly in most medical centers. Because the procedure entails a slight risk for adverse events, it should be performed only after consideration of the potential information to be gained. Hepatic biopsy is usually performed under local anesthesia via a transthoracic approach; ultrasound guidance reduces the rate of complications and is particularly helpful in obese patients or when the hepatic margins are difficult to delineate by percussion[11]. In hepatic biopsies performed at the Mayo Clinic from 1968 to 1988, McGill and co-workers[12] found the risk of significant hemorrhage in patients with non-malignant disease was 0.16% (i.e., 1–2 per 1000 procedures) and of fatal outcome 0.04% (i.e., 4 per 10000 procedures). In a large multicenter review of hepatic biopsies performed from 1973 to 1983, Piccinino et al.[13] reported a similar frequency of serious complications in persons with non-malignant conditions, but only 0.004% (i.e., 4 per 100000 procedures) had a fatal outcome. Janes and Lindor[14] reported a Mayo Clinic experience with 405 outpatient hepatic biopsies performed from 1989 to 1991. 3.2% of patients were admitted to hospital for post-biopsy observation, primarily due to abdominal pain or orthostatic hypotension. Two required blood transfusions and none required surgical intervention or chest tube placement; there were no fatalities. Because these studies vary in several aspects, it is difficult to compare the results directly. To our knowledge, the risks of hepatic biopsy performed specifically for suspected hemochromatosis have not been assessed directly.

The transjugular route is an alternative approach to hepatic biopsy, especially in persons with decompensated hepatic disease with serious coagulopathy. However, such circumstances are not encountered often in hemochromatosis. Major complications occur in approximately 2% of persons who undergo transjugular hepatic biopsies[15]. The requirement that the procedure be performed under fluoroscopic control in a catheterization laboratory adds considerably to the expense of this biopsy technique.

Triage of hepatic biopsy specimens

Although important information can be obtained from both histological and biochemical examination of liver, it is not advised to measure iron in liver by biochemical methods without performing a histological examination. False-negative biochemical results can occur if unrecognized non-representative tissue, such as skeletal muscle or

hepatic capsule, is submitted. False-positive results can occur if truly increased hepatic iron is present predominantly in macrophages, rather than hepatocytes. Three potential triage options for hepatic tissue exist: (i) sending tissue for biochemical examination only; (ii) obtaining separate specimens for biochemical and histological examinations; and (iii) obtaining a single specimen for histological examination, with or without subsequent biochemical determination using the remaining paraffin-embedded tissue. In our opinion, submitting hepatic biopsy tissue for biochemical iron determination without histological examination is inadvisable due to the potential for false-positive and -negative values. Furthermore, this approach does not provide information regarding the presence of additional types of hepatic injury, iron-related damage of hepatocytes, or of cirrhosis, dysplasia, or iron-free foci. Submitting separate biopsy specimens for biochemical determination and histological examination is acceptable, although false-negative biochemical results due to non-representative tissue can occur. Correlation of the biochemical value with the estimated histochemically demonstrable iron content can serve as a useful control. With this approach, it is preferable that the pathologist request (or not request) the biochemical determination in an algorithmic fashion. In our experience, many unnecessary quantitative iron determinations are performed despite negative or minimally positive histochemical iron stains; this makes the likelihood of finding significant iron overload using a biochemical technique almost nil. It is recommended submitting the entire biopsy specimen in formalin for histological examination[16], assuming that a biopsy core of at least 8–10 mm in length is obtained. Sufficient tissue for quantitative iron determination usually remains after sections are cut for histologic evaluation. With this approach, the pathologist can assess the likelihood of hemochromatosis through histological examination and request biochemical measurement of hepatic iron concentration only when it is likely to provide useful information. If the tissue is non-uniform with respect to iron deposition, the pathologist can ensure that a representative sample is used for biochemical determination by indicating the appropriate areas directly on the tissue block.

Handling of biopsy specimens

Formerly, it was recommended that a portion of the hepatic biopsy specimen be placed in an iron-free container for submission to the laboratory for determination of tissue iron concentration and that another, formalin-fixed portion be sent to the pathologist for histological evaluation. The use of routine formalin fixation and paraffin embedding does not affect the accuracy of the biochemical measurement for tissue iron[17]. However, saline immersion causes leaching of iron from specimens and should be avoided. The pathologist should examine the specimen microscopically prior to submitting it for measurement of hepatic iron concentration, thereby avoiding unnecessary measurement of hepatic iron concentration and assuring that a representative specimen is submitted for biochemical analysis. A tissue specimen of at least 3 mm (i.e., a cylinder 1 mm in diameter and 3 mm in length) is required for biochemical analysis. In all instances, the specimen submitted for histological evaluation should be stained (with appropriate controls) using Perls' Prussian blue, trichrome, and hematoxylin and eosin techniques.

Interpretation of histologic findings and chemical analysis

Histologic findings in hemochromatosis

Histological evaluation of hepatic tissue in hemochromatosis is essential to identify inappropriate sampling, to disclose complicating conditions such as alcoholic hepatic injury, to judge distribution of hepatic iron (hepatocellular vs. macrophage), and to determine the presence or absence of cirrhosis to judge prognosis and potential risk for hepatocellular carcinoma.

A hallmark of hemochromatosis is the deposition of hemosiderin in hepatocytes and biliary epithelium. Hemosiderin is insoluble and particulate, and appears granular with iron stains[18]. With hematoxylin and eosin staining, hemosiderin is tan or brownish granular pigment that tends to appear along bile canaliculi within the liver cell plates (Fig. 18.1). Alternatively, iron may be deposited in the form of soluble ferritin that imparts diffuse, non-granular light blue staining of hepatocyte or macrophage cytoplasm (Fig. 18.2). Iron deposited as ferritin is non-specific and is not characteristic of hemochromatosis. Early in the course of hemochromatosis, hemosiderin is deposited in periportal (zone 1) hepatocytes (Fig. 18.3). With progressive iron accumulation, midzonal (zone 2) and centrilobular (zone 3) hepatocytes and biliary epithelium accumulate iron progressively. Inflammation or fatty degeneration are not features of uncomplicated hemochromatosis; their presence suggests the occurrence of alcoholic hepatic injury or chronic hepatitis C. Individual hepatocytes eventually accumulate lethal amounts of iron and undergo 'sideronecrosis.' The released iron locally is then taken up into macrophages; however, hepatocellular

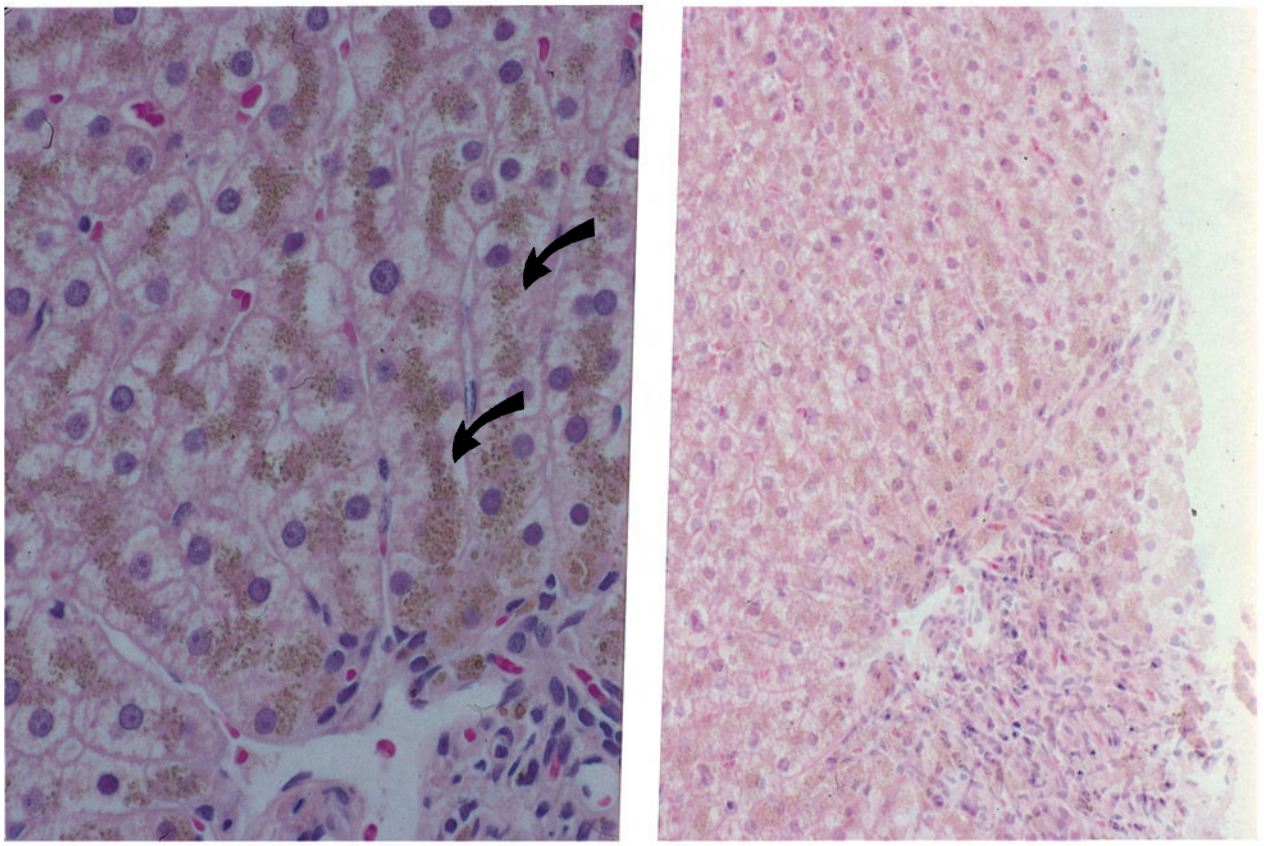


Fig. 18.1. Hemosiderin in precirrhotic homozygous hemochromatosis (hematoxylin and eosin staining). The iron-positive particles appear along the bile canaliculi within the liver cell plates (arrows) and, in hemochromatosis, always involve periportal (zone 1) hepatocytes (bottom, left frame) with variable involvement of the remaining hepatocytes (see text). Iron stains are recommended, because lesser degrees of iron deposition may be overlooked with hematoxylin and eosin staining alone (×225; ×45).

iron continues to be most evident (Fig. 18.4). By contrast, iron deposition in hematologic disorders occurs primarily in cells of the reticuloendothelial system (Kupffer cells) and, when present in large quantities, 'spills over' into the hepatocytes to a lesser degree. Thus, the finding of iron deposited primarily in Kupffer cells does not suggest the diagnosis of hemochromatosis. However, quantification of hepatic iron may be useful in assessing the need for treatment of patients who have non-hemochromatosis iron overload. With continuing iron accumulation and sideronecrosis, progressive fibrosis and cirrhosis may occur. Initial evidence of iron-related fibrosis is often associated with 'threshold' hepatic iron concentrations that are 7–8 times normal, although this is highly variable. Pre-cirrhotic portal and periportal fibrosis has a 'holly leaf' configuration. Hepatic cirrhosis in hemochromatosis appears bland; fine fibrous tissue septae surround regenerative nodules. Another histological feature described in livers of persons with hemochromatosis is the iron-free focus. This is characterized by a localized, usually round, collection of dysplastic-appearing hepatocytes that have less stainable iron than surrounding hepatocytes[19]. The iron-free focus is purported to represent an early step in the development of hepatocellular carcinoma. The clinician and the pathologist should be aware that approximately one-third of patients with hemochromatosis-associated cirrhosis ultimately develop hepatocellular carcinoma[19].

Hemosiderosis in conditions other than hemochromatosis

Hepatic hemosiderin deposition can occur in a wide variety of disease states other than hemochromatosis. In general, thorough clinical evaluation and careful histologic examination can distinguish these conditions from hemochromatosis, although biochemical analysis of hepatic

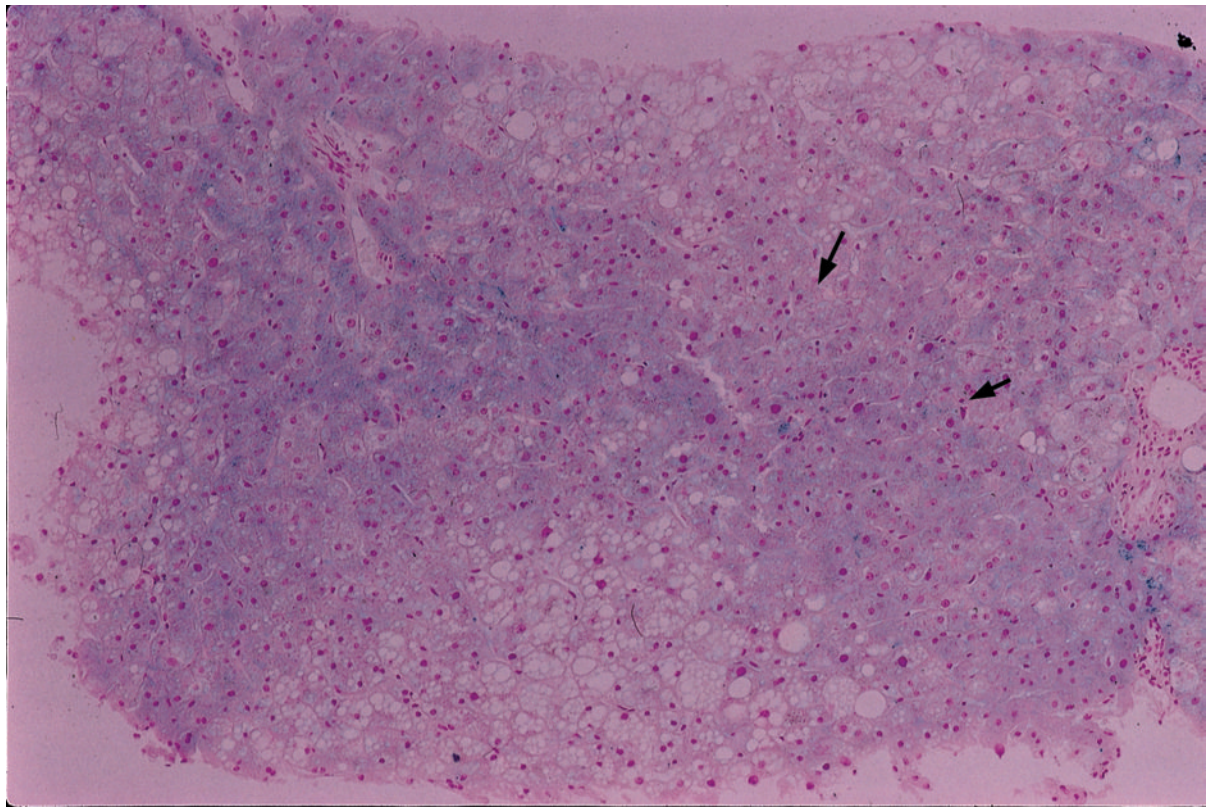


Fig. 18.2. Diffuse ferritin-like iron (Perls' Prussian blue staining). Areas of hepatic parenchyma (arrows) show a subtle, diffuse-type iron. This form of iron is non-specific, not characteristic of hemochromatosis, and should not prompt quantitative iron determination (×133).

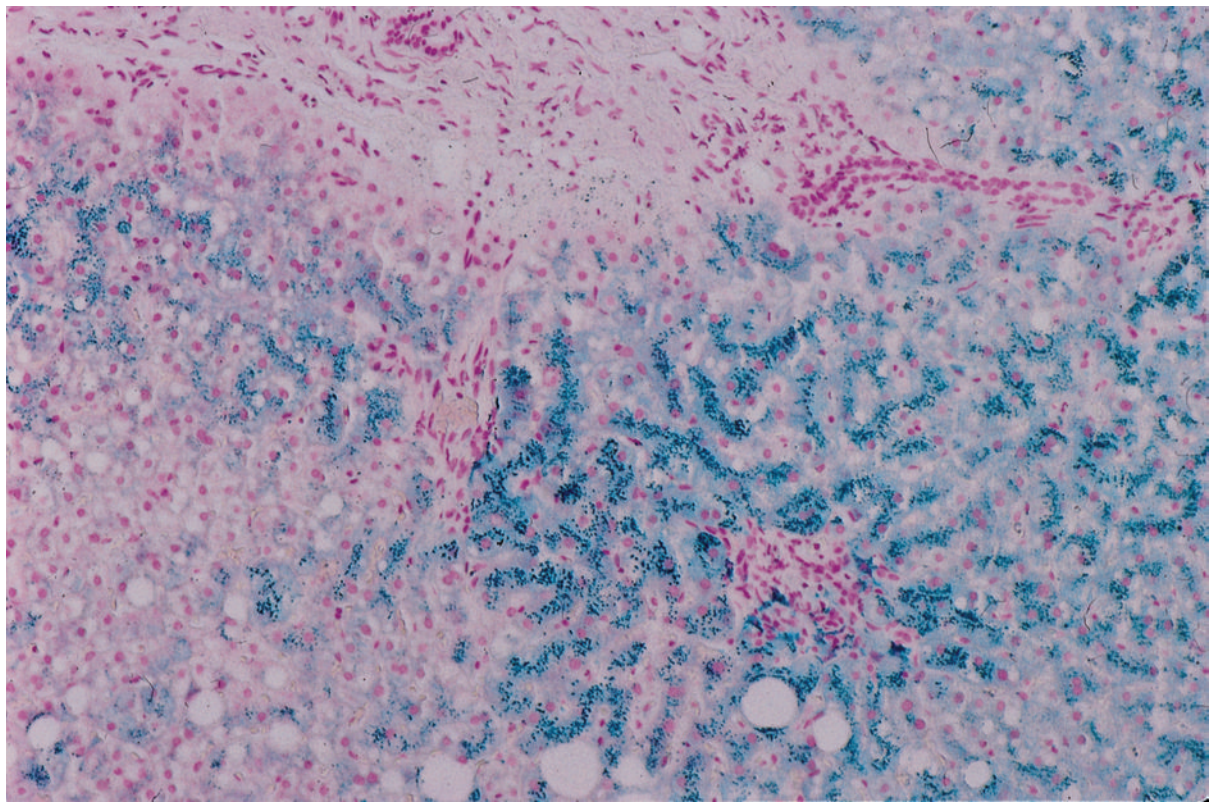


Fig. 18.3. Moderate hemosiderin deposition in precirrhotic homozygous hemochromatosis. Zone 1 hepatocytes are predominantly involved, biliary hemosiderin is not evident, and fibrosis has not yet occurred, all indicating relatively early, precirrhotic disease (10367 μg Fe/g dry weight; iron index 3.2) (Perls' Prussian blue staining; ×133).

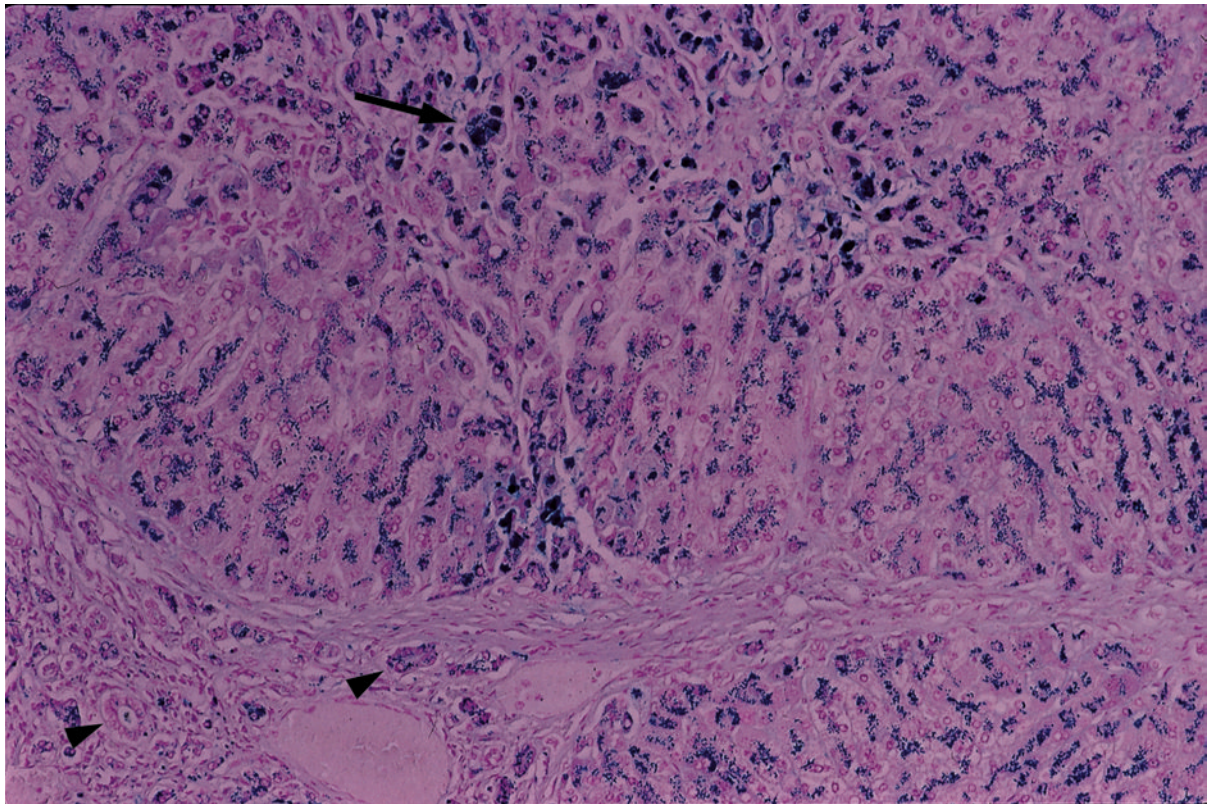


Fig. 18.4. Marked hemosiderosis and cirrhosis in homozygous hemochromatosis. Although most is in hepatocytes, some Kupffer cell (arrow) and biliary iron (arrowheads) is also present (Perls' Prussian blue staining; ×133).

tissue iron (see below) or genotyping may be necessary in ambiguous cases. A differential diagnosis of iron pigment is available elsewhere[21].

One of the most common non-hemochromatosis causes of hemosiderosis is alcoholic steatohepatitis. Histological features include fatty infiltration and varied degrees of inflammatory reaction. Generally, stainable hepatic iron is only mildly increased in such cases, but at times it is sufficient to cause confusion with hemochromatosis. Measurement of hepatic iron concentration usually allows one to distinguish these conditions. The mechanism whereby mild hemosiderosis occurs in conditions other than hemochromatosis is unknown[22]. Increased iron absorption may occur in persons who develop alcoholic disease of the liver[23], but this has not been generally supported by measurements of iron absorption after ingestion of alcohol[24]. However, increased iron absorption has been demonstrated in a small number of chronic alcoholic men[25]. Some persons with mild hepatic iron deposition are probably hemochromatosis heterozygotes, because approximately 10% of Caucasians of European descent are so affected; mild iron overload is manifested in about one-tenth of these individuals. Patients with non-alcoholic steatohepatitis seem to be less prone to develop concurrent hemosiderosis[26].

Transfusions and chronic hemolytic disorders commonly lead to hepatic hemosiderin deposition. Because excess iron in these disorders tends to accumulate in Kupffer cells, it is easily distinguished from the iron deposition in hepatocytes characteristic of hemochromatosis (Fig. 18.5). After hemolysis, iron tends to be deposited in both hepatocytes and Kupffer cells, and thus demonstration of hemolysis by laboratory means is helpful in distinguishing hemolysis-related hemosiderosis from hemochromatosis. Chronic viral hepatitis is sometimes accompanied by hepatic hemosiderin deposition[27] consisting of diffuse, ferritin-like iron that appears largely in Kupffer cells, both of which findings are dissimilar to those of hemochromatosis (Fig. 18.6). Accumulation of hemosiderin is fairly common in cirrhosis of all etiologies. Ludwig et al. noted stainable hemosiderin in 32.4% of 447 cirrhotic livers and increased chemically determined iron in 20.3%, including 8.5% in which the level was in the hemochromatosis range (hepatic iron index >1.9)[28]. It was

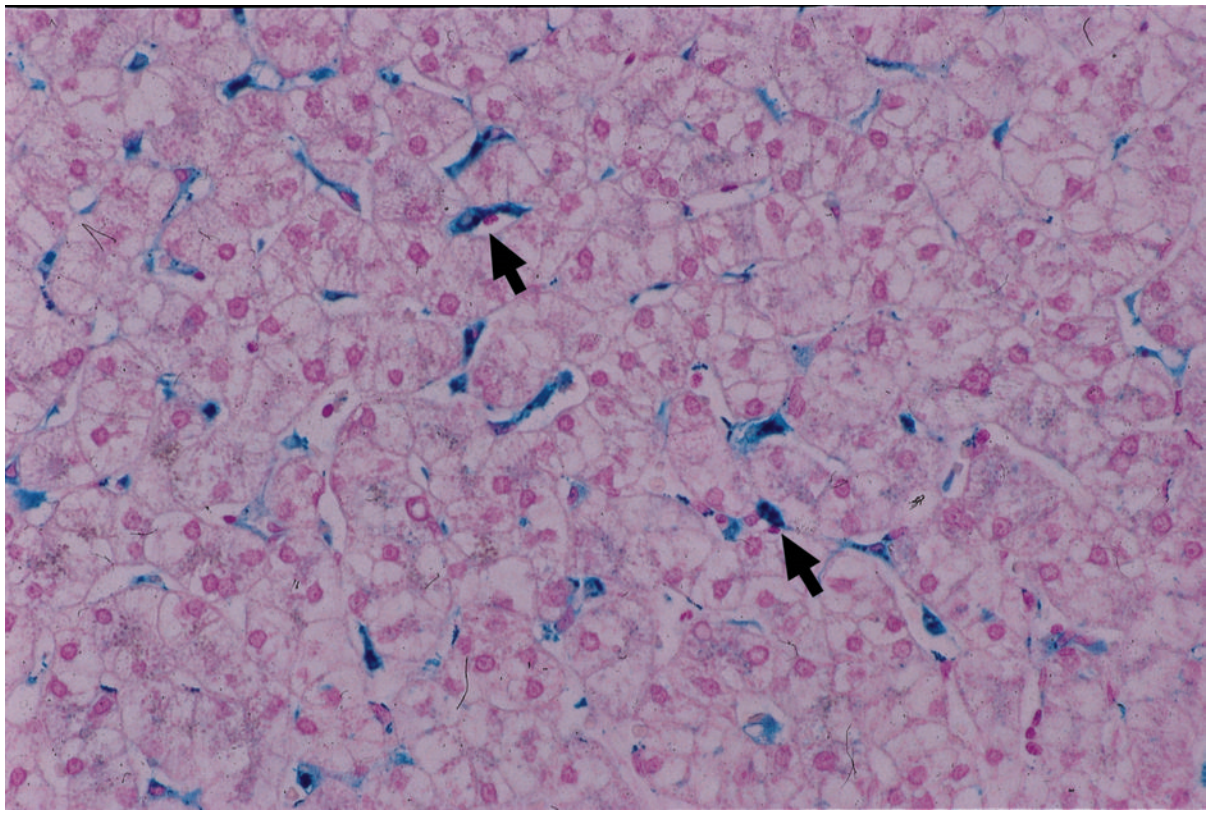


Fig. 18.5. Kupffer cell hemosiderosis. The presence of hemosiderin in Kupffer cells alone (arrows) is typical of mild transfusion hemosiderosis, is non-specific, and should not prompt further consideration of hemochromatosis (Perls' Prussian blue staining; ×240).

thought that homozygous hemochromatosis was the cause of cirrhosis in only five cases. Although some of the other cases could have been attributed to incidental hemochromatosis heterozygosity, it seems clear that iron deposition can occur in hepatic cirrhosis as a secondary phenomenon, although its etiology is unclear. Patients with biliary cirrhosis seem to be less prone to accumulate iron than do patients with non-biliary cirrhosis. In cases of cirrhosis with iron deposition, one is usually able to determine whether homozygous hemochromatosis is present using traditional means of assessment; genotyping may be helpful in equivocal cases.

Chemical analysis of hepatic iron

Methodology

The measurement of iron concentration in hepatic biopsy specimens has been a valuable diagnostic tool that has also broadened the concept of hemochromatosis. Tissue iron analysis must be performed in a qualified laboratory where rigorous control of processing and analytical procedures is followed to assure accurate results. The analysis is performed by inductively coupled plasma/mass spectroscopy. The results are reported in μmol/g dry weight or in μg/g dry weight. Normal values in the Mayo Metals Laboratory are 7–43 μmol/g dry weight (400–2400 μg/g dry weight) in men and 2–29 μmol/g dry weight (100–1600 μg/g dry weight) in women. The variation of repeated determinations on the same test sample is approximately 5%.

Interpretation of hepatic iron concentration

Despite the somewhat subjective nature of histochemical assessment of hepatic iron deposition, the results of this semiquantitative technique correlate reasonably well with the hepatic iron concentration measured using chemical methods[29]. This is especially true at higher hepatic iron concentrations, which are almost always associated with grade 3 or 4 stainable iron, although occasional instances of disparity are encountered. The significance of moderate degrees of hepatic iron overload (grade 2) may be difficult to interpret, particularly in persons 30–60 years of age. In such cases, measurement of hepatic iron concentration is of particular value (see algorithm). Although the hepatic

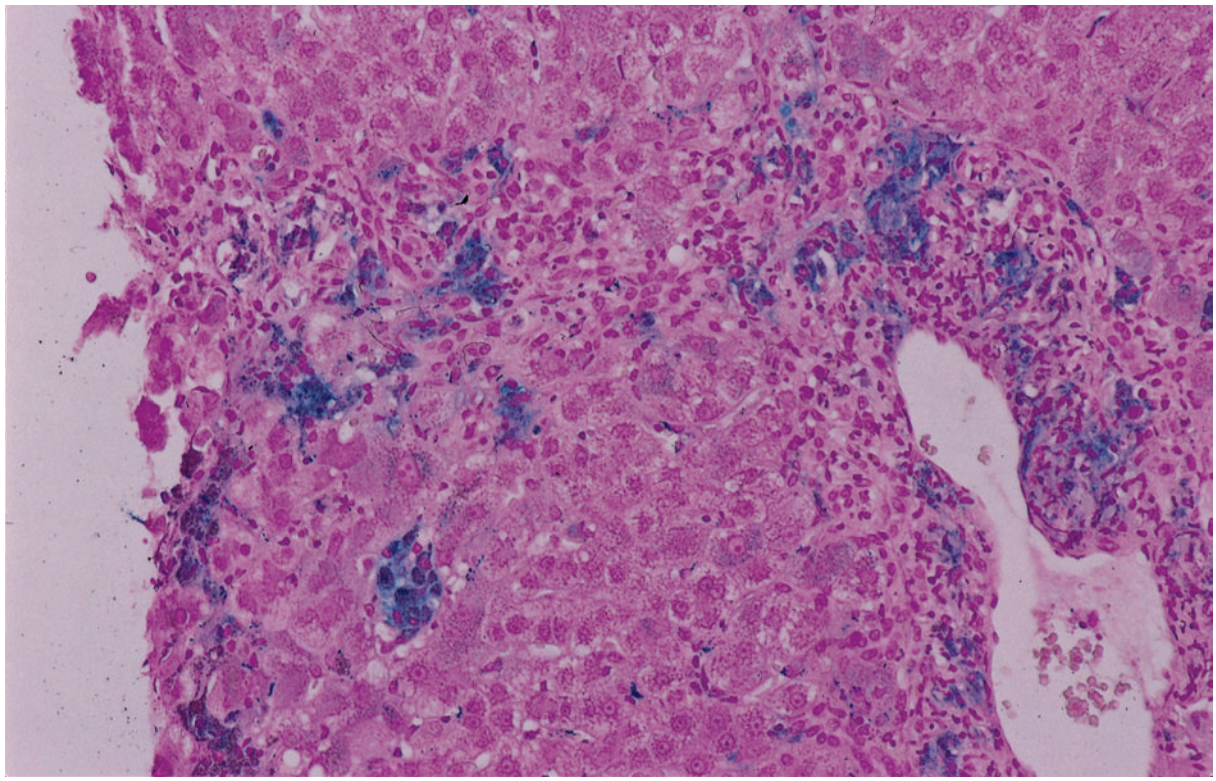


Fig. 18.6. Kupffer cell hemosiderosis in chronic hepatitis C. Compare with iron staining in Figs. 18.3–18.5 (Perls' Prussian blue staining; ×150).

iron concentration provides useful diagnostic information, it is not necessary to perform this measurement on every biopsy specimen obtained for suspected hemochromatosis in routine clinical practice. For instance, if the pathologist observes little stainable iron, measurement of hepatic iron concentration is unnecessary. Conversely, if there is histologic evidence of heavy iron deposition, measuring the hepatic iron concentration may be superfluous.

Heavy iron overload, defined arbitrarily as hepatic iron concentration $>10\,000\ \mu g/g$ dry weight or iron stores >10 g estimated by quantitative phlebotomy, is indicative of hemochromatosis; iron accumulation of this degree rarely occurs in other conditions. With the advent of HLA typing, however, it has become apparent that hemochromatosis homozygotes, especially young individuals and premenopausal women, may have iron overload of minimal degree. Furthermore, only a portion of genotypically defined homozygotes manifest disease-related morbidity with advancing age[9].

The concept of the hepatic iron index, introduced by Bassett et al. in 1986[30], is useful, especially in the interpretation of mild or moderate degrees of hepatic iron overload. The index is calculated by dividing the hepatic iron concentration in μmol/g dry weight by the patient's age in years. Because the hepatic iron concentration is often reported in μg/g dry weight, such values must be divided by 55.8 to convert them to μmoles/g dry weight. The utility of the hepatic iron index is based on the observation that hepatic iron concentration rises progressively with age only in hemochromatosis homozygotes. A hepatic iron index of ≥ 1.9 is consistent with homozygous hemochromatosis, whereas values <1.9 may be associated with heterozygous hemochromatosis, alcoholic disease of the liver, or other conditions not accompanied by significant iron overload. Borderline values may be difficult to interpret and a number of caveats exist regarding the use of the hepatic iron index (see below). In ambiguous cases, genotyping may be helpful.

Although the validity of the hepatic iron index has been confirmed in several studies, there are occasional instances where available evidence and clinical judgment support another interpretation. First, determination of hepatic iron concentration and hepatic iron index for diagnostic purposes should be restricted to cases in which the histologic distribution suggests hemochromatosis, i.e., hepatocellular

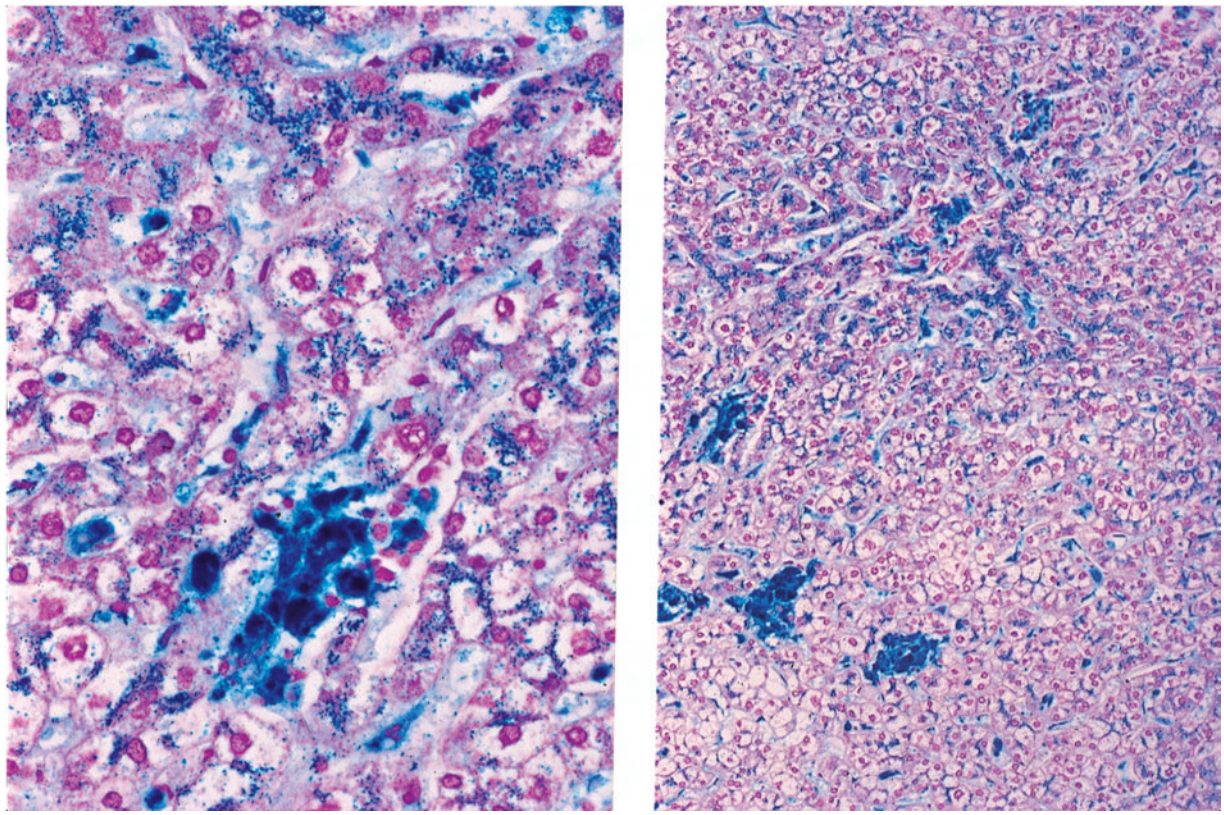


Fig. 18.7. Marked transfusion hemosiderosis in an 18-year-old patient with acute non-lymphocytic leukemia. The marked iron deposition mimics that of hemochromatosis; however, the degree of Kupffer cell iron is unusual for hemochromatosis with a similar degree of iron overload (see Fig. 18.3). In this case, iron quantitation studies (8815 μg Fe/g dry weight; hepatic iron index 8.8) could have mistakenly led to a diagnosis of hemochromatosis had the history and iron distribution not been considered.

iron predominates over Kupffer cell iron, and a reasonable clinical explanation for iron overload does not exist. Transfusion hemosiderosis, particularly in younger individuals, can be associated with hepatic iron indices greater than 1.9 (Fig. 18.7). Second, the applicability of the hepatic iron index to the study of children has not been demonstrated and thus the index should be used in this group of patients with utmost caution, if at all. Third, the index was originally applied to cases of early hemochromatosis and must be interpreted cautiously in patients with advanced chronic hepatic disease wherein exceptions to validity of the index occur frequently (Fig. 18.8)[28]. Last, sampling variations sometimes occur (see below).

Because measurement of hepatic iron concentration is not always available, Deugnier et al.[31] investigated the utility of a histologic hepatic iron index based on detailed and systematic grading of iron deposits in various locations of the hepatic lobule. In their hands, this index differentiated heterozygous and homozygous patients, but patients with conditions other than hemochromatosis were not evaluated. This method, or the computerized assessment of hepatic iron described by Olynyk et al.[32], can be performed on a standard histology section of liver stained with Perls' Prussian blue technique. The findings from both methods correlate reasonably well with the chemical measurement of hepatic iron and support the opinion that determination of hepatic iron concentration may not be essential in every case. A pathologist, experienced in assessing hepatic iron deposition, should assist the clinician in making this decision.

Potential difficulties in interpretation of hepatic biopsy and hepatic iron concentration

There are potential problems with measurement of hepatic iron concentration that can give rise to spurious results. The distribution of iron, although generally uniform in non-cirrhotic livers, can be inhomogeneous in cirrhotic livers. Irregular distribution is unlikely to affect the final interpretation if the hepatic iron concentration is very high, but it could cause misinterpretation when

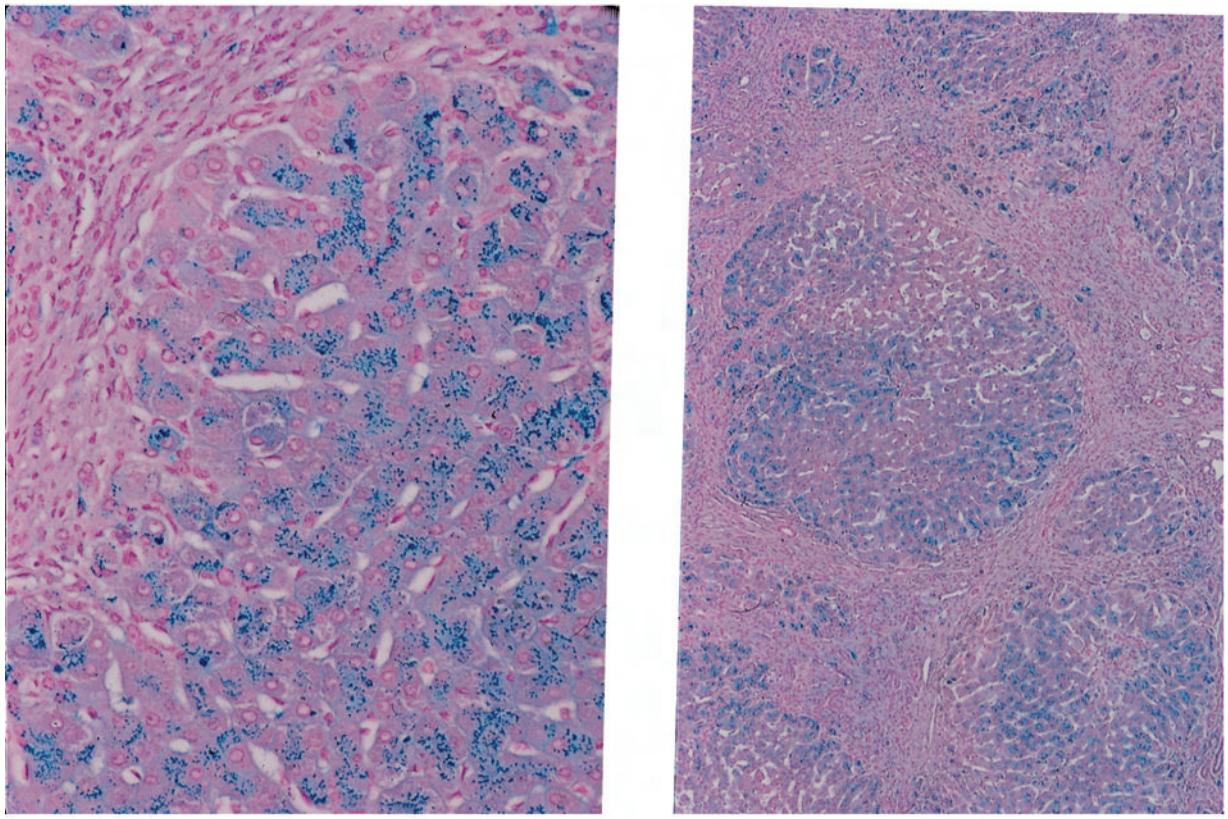


Fig. 18.8. Hemosiderosis in alcoholic cirrhosis (hepatic iron concentration 3645 μg/g dry weight of liver; hepatic iron index 1.7). The amount of iron is much less than required to cause cirrhosis. The non-specific hemosiderin deposition is secondary to alcoholic cirrhosis or incidental heterozygosity for a hemochromatosis allele, but hemochromatosis homozygosity is unlikely.

hepatic iron overload is moderate. A biopsy specimen that consists largely of scar tissue could lead to underestimation of the degree of iron deposition.

The finding of hepatic siderosis of mild or moderate degree in a patient with histological evidence of cirrhosis should be interpreted with caution, because mild iron overload is unlikely to be the cause of the cirrhosis. A threshold level of hepatic iron concentration of approximately 12 times the upper limit of normal is required for the production of significant fibrosis[29]. This was observed when hepatic iron concentration was measured in livers explanted in the course of orthotopic liver transplantation[28]. In several livers, the hepatic iron index was mildly elevated (≥ 1.9), but the amount of hepatic iron appeared to be insufficient to have caused cirrhosis, and the explanation for the siderosis was uncertain. However, earlier hepatic biopsy specimens available in some cases showed that cirrhosis was present before excess iron deposition occurred, thus providing evidence against the diagnosis of hemochromatosis.

The occurrence of siderosis in alcoholic disease of the liver is a common and often vexing problem. In such patients, serum iron parameters are often elevated and stainable hepatic iron may appear greater than the hepatic iron concentration would suggest. The hepatic iron concentration, however, rarely exceeds two or three times the upper limit of normal[22]. However, alcoholic patients with heavy iron overload (i.e., hepatic iron concentration greater than five times normal) are usually hemochromatosis homozygotes[33]. Alcohol probably works in synergy with iron to aggravate tissue injury and may cause cirrhosis in the hemochromatotic liver earlier than would occur with iron overload alone[30, 34, 35]. The hepatic iron index is useful in categorizing patients with features of alcoholic disease of the liver and hepatic siderosis. Hepatic siderosis in hematologic disorders and viral hepatitis is common, but is usually easily distinguished from that of hemochromatosis on clinical and routine histologic grounds.

Alternatives to hepatic biopsy

Occasionally, performing hepatic biopsy may not be advisable in patients with suspected hemochromatosis due to patient reluctance, advanced age, or associated medical conditions. In such cases, it is reasonable to proceed with phlebotomy treatment to achieve iron depletion. DNA-based analysis of *HFE* gene mutations is particularly useful in evaluating such patients, but it is not thought it should supplant hepatic biopsy. It is important to quantify the amount of iron removed (~200 mg of iron per 500 ml of blood) for retrospective estimation of total body iron stores. Demonstrating total iron stores of more than 5 g generally supports a diagnosis of homozygous hemochromatosis; lesser amounts are difficult to interpret. Occasionally, neither biopsy nor quantitative phlebotomy is feasible in patients with advanced, decompensated hepatic disease. Computed tomography or magnetic resonance imaging can be of diagnostic help if the procedure provides evidence for or against severe hepatic iron deposition[36, 37]. Due to their expense, these imaging modalities are not recommended for routine diagnostic evaluation. Analyses of *HFE* mutations will aid in clarifying the diagnosis in many of these uncertain situations.

Acknowledgment

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19

Histochemistry of iron and iron-associated proteins in hemochromatosis

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Introduction

Of the transition and heavy metals important for normal metabolism, iron is the most prevalent. In living organisms, iron is almost always present as the ferrous (Fe^{2+}) or ferric (Fe^{3+}) form. Because each of these ionic species is highly reactive, most iron in biological systems is bound to transport, regulatory, or storage proteins, or exists as a component of iron–porphyrin complexes or metalloproteins. Histochemical analysis of iron and iron-associated proteins in normal human and animal tissues permits an understanding of normal iron absorption and metabolism, and the distribution of iron and its chemical forms among cell types and subcellular organelles.

In hemochromatosis, the absorption of iron is inappropriately great for body iron content. Because mechanisms to excrete iron are limited, most persons with hemochromatosis eventually develop iron overload. Histochemical techniques can be used to study abnormal iron absorption and metabolism, to localize pathologic iron deposits (including those not otherwise detectable using other routine methods of analysis), to assess the severity of iron overload in tissues, and to assist in the histologic differentiation of hemochromatosis from other iron overload disorders. This chapter reviews the chemical and physical basis of the histochemistry of iron and iron-associated proteins, and discusses the utility and significance of these findings in hemochromatosis. The broader use and findings of similar histochemical techniques applied to studies of iron overload disorders in animals and iron metabolism in cultured cells are described in Chapters 13, 14, 47, 48 and 49.

Histochemistry of iron and iron-associated proteins

Iron

At the light microscopic level, the basis of iron histochemistry is the ability of iron to form highly colored, insoluble reaction products with many reagents. Although numerous techniques can be used to demonstrate iron for general histological purposes[1], the acid ferrocyanide reaction is the most useful for research and diagnostic purposes[2]. In routine clinical pathology practice, Perls' acid ferrocyanide technique is preferred for evaluation of the liver and other tissues because it is more sensitive, specific, and easily applied than other methods to visualize iron[3]. The reaction of acid ferrocyanide and non-heme ferric iron produces ferric ferrocyanide, an insoluble, intensely blue, and relatively permanent reaction product (Prussian blue). Because most non-porphyrin-bound iron in mammalian tissues exists in the ferric form, the acid ferrocyanide reaction selectively identifies most iron other than that in hemoglobin, myoglobin, and related substances. Although little non-porphyrin-bound ferrous iron exists in mammalian tissues, it can be visualized by microscopy using acid ferricyanide (Turnbull's blue) reagent[4]. At the ultrastructural level, iron is detected readily by its electron density, particularly when studied in specimens that have not undergone treatment with lead citrate, uranyl acetate, or other electron-dense contrast reagents.

Intensification techniques can enhance the staining of unbound iron and iron ligands of proteins, thus increasing the sensitivity of histochemical analyses at the light and electron microscopic levels. Perls' Prussian blue and Turnbull's blue reagents accomplish this because additional iron is added to native cellular iron by the

precipitation of ferro- and ferricyanide moieties, respectively. The stain precipitate produced by both techniques is presumed to be a form of ferriferrocyanide that by electron diffraction has a single crystal structure with a high degree of symmetry[5]. Silver proteinate can intensify the staining of ferriferrocyanide deposits in hematopoietic and intestinal cells at the ultrastructural level[6]. In light microscopic studies, the use of diaminobenzidine to enhance ferric ferrocyanide stain deposits (diaminobenzidine black technique) could permit greater understanding of small, but increased, quantities of iron in the brain and certain other tissues that occur in persons with hemochromatosis[7, 8]. Sequential or 'double' staining techniques involving iron histochemistry are also helpful in studying iron deposition in cell subpopulations. For example, combined immunocytochemical and Prussian blue staining reveals the preferential deposition of iron in specific subpopulations of endocrine epithelial cells in hemochromatosis[9, 10]. Other sequential staining techniques that identify iron or iron-binding substances and lysosome components in myeloid cells may provide new insights into abnormal iron metabolism in hemochromatosis[11, 12]. To semi-quantify stainable iron deposits, several histochemical grading systems have been used to analyze the liver, pancreas, heart, and neutrophils in hemochromatosis[13–20].

Iron-associated proteins

Non-immunologic methods

Because most native iron in tissues is protein-bound, staining iron using routine histochemical methods permits localization of the functional, corresponding protein. Further, the topography, size, and intensity of stain deposits suggest the identity of the iron-associated protein. For example, iron normally bound to transferrin or lactoferrin can be localized by light and electron microscopy using acid ferrocyanide staining, and this can be readily distinguished from ferritin iron[21]. Functional, non-ferritin iron-binding substances that are unsaturated with native iron can be treated with iron nitrilotriacetate in tissue specimens to saturate their iron-binding sites. The iron can then be visualized using acid ferrocyanide to display iron-binding reactivity, a technique employed to study transferrin, lactoferrin, and other iron-binding substances in hematopoietic cells and enterocytes[22, 23]. The specificity of the reaction sequence for lactoferrin is significantly enhanced when specimens are pre-treated in acidified media to remove iron bound to transferrin before addition of the acid ferrocyanide reagent[11, 12, 20]. The cytoplasmic iron-binding reactivity in mature neutrophils thus demonstrated corresponds largely (if not entirely) to lactoferrin, and can be semi-quantified using a cytochemical scoring technique[20]. The presence of transferrin and lactoferrin receptors on cells can be inferred by detecting the corresponding proteins or their respective iron ligands using immunohistochemical methods or acid ferrocyanide technique, respectively. In some circumstances, the binding of transferrin or lactoferrin to cell surfaces may depend on the protein's degree of saturation with iron[24–26].

The iron storage protein ferritin and aggregates of partially degraded, iron-rich ferritin (hemosiderin) are readily identified by their iron content using acid ferrocyanide in light microscopic and ultrastructural specimens. At the ultrastructural level, ferritin particles are observed in four forms: (i) as free molecules in the cytosol, sometimes in a crystalline pattern; (ii) in multilamellar arrays; (iii) in single membrane-bound organelles (siderosomes); and (iv) in aggregates without a limiting membrane (clusters)[27]. Other technical aspects of iron identification and localization in biological materials by transmission electron microscopy have been reviewed[28]. Individual molecules are easily identified by their polyhedral configuration, size, and electron-dense iron cores, even without the application of iron staining techniques. However, the ferritin iron cores differ in normal subjects and in hemochromatosis patients when analyzed by transmission electron microscopy. Those isolated from patients with secondary iron overload have α-FeOOH crystal structures, whereas in hemochromatosis, hemosiderin iron cores consist of amorphous ferric oxide[29, 30]. Ferritin receptors occur on the surfaces of hepatocytes, and are presumed to occur on the surfaces of monocytes and macrophages[31]. It is probable that iron bound to other proteins is also detected by acid ferrocyanide and FeNTA techniques, but a positive identification of the proteins may require parallel analyses using other histochemical and biochemical techniques. Hemoglobin, myoglobin, and other iron–porphyrin compounds can be visualized in histological specimens using a variety of non-immunologic techniques that do not depend directly on iron histochemistry[32, 33].

Immunologic methods

The availability of polyspecific and monoclonal antibodies to iron transport, regulatory, and storage proteins provides the basis for their localization and identification. Many of these antibodies are available commercially, including a monoclonal anti-transferrin receptor antibody used for diagnostic purposes (anti-OKT9®; Ortho Diagnostics; Becton-Dickinson Corp.). Particular methods of fixation are often needed, depending on the protein to be detected, the specimen under study, and the staining reagents used. A variety of direct and indirect immunohistochemical

methods can be combined with the corresponding antibody reagents to produce reaction products that are detectable using immunoperoxidase-diaminobenzidine[22, 34–37], immunofluorescence[38], immunogold[39], or immunoferritin[21, 22] sequences. Routine light, fluorescence, or electron microscopy are used to detect reaction products in tissue preparations. When single-cell suspensions are studied, flow cytometry can be used to analyze subpopulations of cells for their expression of surface transferrin receptors, lactoferrin, surface lactoferrin receptors, and cytoplasmic lactoferrin. The relative intensity of fluorescence can also be measured using microdensitometry techniques[40]. Proteins that have been visualized directly or indirectly by immunohistochemistry include transferrin, transferrin receptor, lactoferrin, lactoferrin receptor, ferritin (heavy and light chains), ferritin receptor, and the wild-type product of the major histocompatibility (MHC) class I hemochromatosis-associated *HFE* gene. Using similar methods, other proteins implicated in iron metabolism can also be analyzed, and it should be possible to distinguish between normal and mutant proteins. In general, immunohistochemical reactions are highly specific when antibody reagents are carefully prepared and used. However, their reaction products are more difficult to localize at the subcellular level than those corresponding to iron alone.

Radionuclide techniques

The most common of these methods is based on the administration of exogenous radioiron (^{55}Fe or ^{59}Fe) to an experimental subject, and its subsequent radiodecay in tissue specimens. Detection of the radioiron is accomplished by high-resolution radioautography[41–43]. Because ^{55}Fe undergoes beta-decay, it is the preferred isotope for radioautography. A film emulsion with which tissue sections are coated is developed after an appropriate period of incubation; silver grains indicate the presence of radioiron atoms. These methods are very sensitive and specific, but localize iron within cells less well than histochemical methods, and visualize only those tracer atoms that have decayed during the incubation period. Because these techniques involve exposure of the investigator and study subject to radioactivity, and require that tissue samples be removed for study thereafter, the use of radionuclide techniques in human studies is limited. Although histologic, radioautographic studies of iron absorption and metabolism in hemochromatosis are not reported, techniques using radioiron have contributed significantly to our understanding of intestinal absorption of iron and its utilization by bone marrow cells.

The stable isotopes of iron are ^{54}Fe, ^{56}Fe, ^{57}Fe, and ^{58}Fe. For absorption measurements, current technology permits use of ^{54}Fe, ^{57}Fe, and ^{58}Fe. The relative expense of stable isotope studies is great, and the stable isotopes are unsuitable for iron absorption measurements in situations in which the incorporation of iron into erythrocytes is abnormal. Further, stable isotope analysis does not appear to have been used for histochemical studies, and it is unlikely that stable isotope use will supplant the use of ^{55}Fe and ^{59}Fe, except in pregnant women and children[44]. Electron probe X-ray microanalysis permits the positive identification of iron and measurement of its concentration in subcellular organelles, regardless of its isotopic composition[27, 45].

Nucleic acid methods

Two histochemical methods based on nucleic acid chemistry have potential application in the study of hemochromatosis. In situ hybridization detects specific RNA present in histological samples. Typically, ^{35}S- or ^{32}P-labeled riboprobes generated by in vitro transcription of a cDNA using viral RNA polymerase are hybridized to tissue fixed on a microscope slide. Thereafter, radioautography is performed, and silver grains overlying cells that contain specific RNA are detected. Fluorescence in situ hybridization (FISH) is a general method to assign chromosomal location, gene copy number (both increased and decreased), or chromosomal rearrangements. Biotin-, digoxigenin-, or fluorescent-labeled nucleotides are incorporated into specific cDNA probes by nick-translation, and hybridized with solubilized, fixed metaphase cells. The copy number of specific chromosomes or genes is determined by counter-staining with fluorescein isothiocyanate-labeled avidin or other detector reagents. The number and location of detected fluorescent spots correlates with gene copy number and chromosomal location. The method also allows chromosomal analysis in interphase cells, allowing extension to conditions of low cell proliferation. With the discovery of hemochromatosis-associated mutations on 6p[46] and other mutants responsible for abnormal iron metabolism, it is likely that use of these methods will reveal new insights in the pathogenesis of hemochromatosis and other hereditary iron overload disorders.

Histochemical observations in hemochromatosis

Iron absorption

No results of histochemical studies of iron absorption in hemochromatosis appear to have been reported, but it is

likely that the sequence of inorganic iron absorption visualized with histochemistry will resemble that of normal persons and animals. Cytochemical and high-resolution radioautographic methods have been used to study the absorption of inorganic iron in the small intestine of normal humans[47] and rodents[41–43]. Normally, inorganic iron is absorbed almost entirely by the small intestine, with a gradient of duodenum > jejunum > ileum, and villus tip > lateral villus > crypt. In the early phase of iron absorption, iron is found almost exclusively in the absorptive cells and lamina propria. Goblet cells, Paneth cells, argentaffine cells, and undifferentiated cells do not take up significant amounts of iron[42]. However, the staining of goblet cells for transferrin, lactoferrin, and ferritin may increase after iron administration[47]. The uptake of radioiron by the brush border and terminal web of duodenal enterocytes is rapid, and ferrous iron appears to be converted to a ferric form[43]. Thereafter, iron is detected throughout the absorptive cells, especially rough endoplasmic reticulum and adjacent cytoplasm, and over areas rich in free ribosomes. Within ten minutes, iron appears in the basal and lateral membrane, lateral intercellular spaces, basal membrane, lamina propria, vascular endothelium, and macrophages in the lamina propria[41–43]. Little iron appears in association with the mitochondria, Golgi apparatus, or nucleus[43].

The absorption of heme iron, like that of inorganic iron, is maximal in the duodenum and is enhanced by iron deficiency[48–50]. Heme iron absorption is increased in hemochromatosis also, but to a lesser extent than is that of inorganic iron[50, 51]. The pathway of heme iron absorption has been studied in normal humans[50, 51], and has been visualized in dogs[35]. Intact hemoglobin-iron enters absorptive enterocytes by endocytosis[35]. In membrane-bound organelles, hemoglobin is degraded and iron is released by the action of heme oxygenase[52]. Converted to an inorganic form, the iron is subsequently transported to the lateral intercellular space where the absorptive process appears to be completed in a manner similar to that of absorbed inorganic iron[35]. However, it is possible that other mechanisms also exist for absorption of hemoglobin iron.

There is a paucity or absence of ferritin bodies within enterocytes and macrophages in the apical villous stroma of the small intestines of persons with hemochromatosis[53–55]. Often likened to the histochemical picture seen in the small intestine of iron-deficient subjects, this constellation of abnormalities in hemochromatosis is possibly due to the decreased tendency to store iron in monocytes and macrophages (see below)[56]. Paradoxically, submucosal plasma cells in the duodenum in hemochromatosis contain iron within lysosomes and free cytoplasmic ferritin[57]. In persons with hemochromatosis, histochemically demonstrable ferritin in the intestine and intestinal mucosal H- and L-ferritin fail to rise in parallel with serum ferritin concentrations[56, 58], and mRNA for both H- and L-ferritin components is inappropriately low[59]. To date, however, studies of ferritin genes have failed to demonstrate mutations that could account for hemochromatosis[60], and thus explain the abnormalities of ferritin observed in enterocytes, monocytes, and macrophages.

Although transferrin occurs in the bile[61, 62], transferrin mRNA has not been identified in rat or human gastrointestinal mucosal cells[59, 61, 63]. Surface transferrin receptors in enterocytes have been localized by immunocytochemistry to the basolateral but not microvillous cell membranes[64–66]. In preterm rats, there is a high level of duodenal transferrin receptor along the full length of the crypt-villous axis, but this is reduced toward the villous tip soon after birth[67]. The density of transferrin receptors remains high in the small intestinal crypts in animals of all ages[67]. Because there is no correlation between iron absorption and transferrin receptor expression in either neonates or adult animals, it is presumed that the receptors are involved in the transport of iron away from the intestine[68]. For these and many other reasons, it is improbable that transferrin participates in the transport of iron from the intestinal lumen or through the absorptive cell in an abluminal direction in normal persons (or in those with hemochromatosis)[69]. Further, duodenal transferrin and transferrin receptor staining using immunofluorescence in persons with hemochromatosis and in normal control subjects does not differ significantly[66]. Nonetheless, the receptors could represent a means by which enterocytes procure iron for their own metabolism[67, 70] or excrete excess iron. Detection of transferrin or its excess native iron burden in hemochromatosis by immunologic and acid ferrocyanide techniques, respectively, should permit analyses of its interactions with specific enterocyte basolateral membrane receptors.

Lactoferrin coats the luminal surface of the small intestine and binds to specific lactoferrin receptors located on the apical cytoplasm. Thus, lactoferrin may participate in the mucosal uptake and absorption of iron (and manganese) in normal infants and adults[71–73]. The process in adults probably does not occur by pinocytosis[72]. In adults with hemochromatosis, there are lower concentrations of lactoferrin in duodenal fluid than in normal subjects, and exogenous human apolactoferrin inhibits radioiron absorption in normal subjects, but not in those with hemochromatosis[74]. However, the pertinence of these observations to iron absorption and development of iron overload in hemochromatosis is unclear.

On the distal aspect of 6p, there is a major histocompatibility class I-like gene designated as *HFE*, two mutations of which are associated with hemochromatosis[46]. It has been proposed that wild-type *HFE* protein is expressed on the luminal aspect of enterocytes where it binds β_2-microglobulin and an iron chelator[46, 75]. The distribution of the wildtype *HFE* gene product determined by immunohistochemical methods in normal control subjects has been described[76]. In the gastrointestinal tract, normal immunoreactive *HFE* protein is expressed in most epithelial cells. In non-polarized epithelial cells and leukocytes, there is staining of either the entire plasma membrane or absence of staining. In most polarized epithelial cells, the immunoreactivity appears to be restricted to the basolateral membranes, e.g., gall bladder and bile ductular epithelium. In the duodenum, jejunum, and ileum, there is a unique pattern of intracellular, perinuclear staining of epithelial cells, particularly those in the crypts[76]. This histochemical observation in the small intestine seems to contradict some biochemical data about the *HFE* protein. Wild-type *HFE* protein binds β_2-microglobulin, whereas the C282Y mutant protein, but not the H63D mutant protein, abrogates this interaction[77]. Although the wild-type and H63D *HFE* proteins are said to be expressed on the cell surface, the C282Y mutant *HFE* protein is localized exclusively intracellularly. This is the first functional consequence of the C282Y mutation to be recognized, but the manner in which this abnormality of *HFE* cell surface expression leads to increased iron absorption is unknown. Mice with a genetic knockouts for the β_2-microglobulin or hemochromatosis gene also develop iron loading[75, 78, 79]. There are no reports that confirm the perinuclear localization of *HFE* in enterocytes[76]; the assignment of a perinuclear location to other iron-associated proteins[80] has subsequently been shown to be due to fixation artefact. The iron chelator thought to bind to the *HFE* gene product and β_2-microglobulin in human or mouse enterocytes[75] has not been identified. Further, recent data indicate that the *Nramp2* gene product (not the *HFE* gene product) may be responsible for the transport of iron across the microvillous membrane[81].

Iron-associated proteins and intermediate iron metabolism

In hemochromatosis, transferrin in plasma and serum is nearly always saturated to an abnormally great degree with iron[82], but is electrophoretically normal and has normal affinity for iron[83–85]. Studies of perfused rat livers indicate that the uptake of transferrin-bound iron is less than 2%, but that up to 75% of non-transferrin-bound iron (NTBI) is removed[86–88]. Detection of transferrin and its excess native iron burden by immunologic and acid ferrocyanide techniques should permit further analysis of its interactions with specific cell surface receptors. Although the transferrin gene has been cloned[89], there are no reports to date of possible mutations in the gene or its regulatory elements that could account for unusual cases of hemochromatosis. Taken together, these data suggest that there is not a common, primary abnormality of transferrin in hemochromatosis.

In untreated persons with hemochromatosis, the transferrin receptor is usually not detected on the surfaces of hepatocytes by histochemical or biochemical methods, in contrast with that of normal persons[90–92]. After iron depletion using therapeutic phlebotomy, expression of surface transferrin receptor increases[92]. The serum concentration of transferrin receptors in hemochromatosis is also decreased when iron overload is present, and increases after therapeutic phlebotomy[93, 94]. A corresponding phenomenon occurs in duodenal mucosal cells[58]. Taken together, these observations suggest that alterations of transferrin receptor expression are due to iron overload, that fundamental transferrin receptor function is normal[95, 96], and that abnormal transferrin receptor presentation on cell surfaces and in the plasma is an indirect effect of hemochromatosis-associated mutations.

In normal persons, monocytes have transferrin receptors demonstrable by immunohistochemical and other methods[25, 26], but lack receptors for apotransferrin[24]. In some patients with hemochromatosis, the percentages of peripheral blood monocytes that express transferrin receptors is high[95]. Using histochemical methods, the increased iron saturation of serum transferrin in hemochromatosis should permit visualization of transferrin on cell surfaces that express specific surface transferrin receptors, particularly monocytes, macrophages, and erythroid cells[97]. The gene for transferrin receptor has also been cloned[98], and mRNA for transferrin receptor is inappropriately increased in hemochromatosis[59]. Evaluation of the gene and its iron-responsive elements could demonstrate abnormalities to account for presently unexplained cases of hemochromatosis.

Non-transferrin-bound iron (NTBI) refers to low-molecular weight iron complexes, primarily iron citrate, that form as a consequence of saturation of the binding capacity of transferrin[99]. Of the total serum iron in persons with hemochromatosis, the NTBI component is markedly increased in comparison with that in normal persons[100, 101]. In the rat, NTBI is rapidly removed as it passes through hepatic sinusoids. Autoradiographic studies using ^{55}Fe reveal that NTBI perfused via the portal vein is taken up

Table 19.1. Peripheral blood neutrophil lactoferrin in hemochromatosis

Method of lactoferrin measurement	Hemochromatosis subjects	Normal control subjects
Radial immunodiffusion	6.8 ± 1.4 (S.D.) μg/10^6 cells (n = 4)[104]	7.7 ± 1.9 μg (S.D.) μg/10^6 cells (n = 36)[104]
Radioimmunoassay	2.43 ± 1.05 (S.D.) μg/10^6 cells (n = 17)[105]	3.45 μg/10^6 cells[108]
Cytochemical scoring	382 ± 5 (S.E.M.) (n = 15)[106 b]	410 ± 2 (S.E.M.) (n = 22)[20 b]
Indirect immunofluorescence + flow cytometry[a]	77.7 ± 12.2 (S.E.M) % positive (n = 4)[107 b]	97.1 ± 0.3 (S.E.M) % positive (n = 41)[109]

Notes:

[a] These data are expressed as the mean ± 1 S.E.M. of determinations made in subjects with untreated hemochromatosis. PHBR is a measure of the homogeneity of fluorescence intensity of total cell (surface + cytoplasm) immunostaining for lactoferrin. The PHBR of 4.8 ± 1.9 (S.E.M.) for neutrophils isolated from men with untreated hemochromatosis was similar to that of normal control subjects (4.4 ± 0.4).

[b] These data correspond to men with untreated hemochromatosis.

primarily by hepatocytes[86, 87]. It is probable that a gradient is produced, such that central areas of hepatic acini are exposed to lower concentrations of iron than periportal areas. These features are consistent with the anatomical distribution of hepatic iron seen in hemochromatosis, i.e., a predominantly hepatocellular iron loading with a portal to central gradient. Because this iron is cleared rapidly and efficiently by the liver, and its uptake is not diminished by iron loading[87], the prompt removal of NBTI iron from the hepatic circulation in rats could account for the fact that hepatic iron loading precedes iron accumulation in other organs in hemochromatosis[102]. In rats, the hepatic uptake of NTBI is mediated by a membrane carrier and occurs by an electrogenic mechanism[88]. With increased hepatic iron storage in hemochromatosis, the number of siderosomes in hepatocytes increases at the apparent expense of lysosomes. In severe iron overload, most of the iron is stored in residual bodies[27]. This sequence is reversed by therapeutic phlebotomy[27].

Lactoferrin, synthesized by neutrophils, is localized in their cytoplasmic granules by a variety of histochemical and immunocytochemical techniques[22, 39]. After its release from neutrophils, lactoferrin can transport iron to monocytes and macrophages that express surface lactoferrin receptors[26, 103]. In hemochromatosis, the lactoferrin content of peripheral blood neutrophils (regardless of the presence or absence of hepatic cirrhosis) is slightly decreased (Table 19.1). Histochemical and flow cytometric analysis of neutrophils in persons with hemochromatosis suggests that the decreased lactoferrin content of these cells could be explained by a lactoferrin-deficient subpopulation of neutrophils. Whether this is due to decreased lactoferrin synthesis, to neutrophil degranulation, or to other causes is unknown. However, this implies that the decreased iron deposited in monocytes and macrophages in hemochromatosis could be related to diminished iron transport to these storage cells by lactoferrin released into the plasma by neutrophils. In iron deficiency and after phlebotomy, neutrophil lactoferrin concentrations are reduced in persons with hemochromatosis[104]. This could be explained by structural, biosynthetic, structural, and functional changes in neutrophils that occur in iron deficiency[36, 110, 111]. The possible significance of this in persons with hemochromatosis undergoing iron depletion therapy is unknown.

Peripheral blood monocytes from normal persons display weak to moderate lactoferrin immunoreactivity that appears as diffuse cytoplasmic staining or as crescentic or beaded surface positivity. Normal peripheral blood lymphocytes, particularly B-lymphocytes, have weaker immunofluorescence indicative of lactoferrin, usually displayed as faint surface fluorescence beading[25]. In hemochromatosis, surface and cytoplasmic lactoferrin immunoreactivity in both of these cell types is less than that observed in normal control subjects (Table 19.2). This could be an indirect consequence of the decreased quantities of neutrophil lactoferrin in hemochromatosis, or to other abnormalities in lactoferrin metabolism. The uptake and processing of lactoferrin-bound iron into macrophages isolated from hemochromatosis patients is similar to that from normal subjects[105]. Defective interaction of lactoferrin with cells of the reticuloendothelial system could, therefore, explain the increased iron absorption characteristic of hemochromatosis. There are no reports of lactoferrin concentrations in the external secretions of persons with hemochromatosis. Whether lactoferrin, with or without bound iron, alters resistance to certain bacterial infections (Bullen, Chapter 36), or participates in joint injury[112, 113] in hemochromatosis is also unknown. Although the human lactoferrin gene has been cloned[114],

Table 19.2. Peripheral blood mononuclear cell lactoferrin in hemochromatosis[a]

	Cell surface lactoferrin, % positive cells	Total cell lactoferrin, % positive cells (PHBR)
Hemochromatosis monocytes ($n=4$)	6.0 ± 6.0	6.8 ± 2.3 (2.6)
Normal control monocytes ($n=22$)	n.d.	28.7 ± 3.6 (1.0 ± 0.1)
Hemochromatosis lymphocytes ($n=4$)	3.4 ± 3.4	9.0 ± 8.3 (3.1)
Normal control lymphocytes ($n=19$)	n.d.	13.1 ± 1.3

Note:

[a] These data are expressed as the mean $\pm$ 1 S.E.M. of determinations made in four male subjects with untreated hemochromatosis[107]. PHBR is a measure of the homogeneity of fluorescence intensity of total cell (surface + cytoplasm) immunostaining for lactoferrin. Monocyte-rich cell isolates were prepared using adherence techniques or elutriation; lymphocyte-rich isolates consisted of non-adherent mononuclear cell isolates. Peripheral blood lymphocytes consist of approximately 80% T-cells and 20% B-cells[109]. n.d., not done.

there are no mutations in the gene or its regulatory elements in persons with hemochromatosis reported.

Ferritin synthesis within monocytes and macrophages occurs after their uptake of iron transported by transferrin or lactoferrin. The stainable iron in monocytes from patients with hemochromatosis exists as a few, fine granules suggestive of iron-laden ferritin in the cytosol[115]. In contrast, large, confluent hemosiderin granules occur in monocytes in transfusion hemosiderosis. In both disorders, however, the ferritin content of monocytes is increased[116–118]. The rate of ferritin synthesis in monocytes isolated from hemochromatosis patients is normal or slightly decreased[117, 119, 120]. In hemochromatosis patients with normal or elevated serum ferritin concentrations, there is early release of iron from reticuloendothelial iron stores[121]. These results could be explained by a failure of the conversion of intracellular soluble ferritin to insoluble hemosiderin in monocytes and macrophages, and could account for the hyperferremia and decreased macrophage storage iron observed in hemochromatosis[121, 122]. Accelerated ferritin release by mononuclear cells also has implications for the altered control of intestinal iron absorption in hemochromatosis.

Hepatocytes express surface ferritin receptors[123, 124], and take up ferritin released from Kupffer cells rapidly in vitro[31]. The ability of monocytes and macrophages to internalize ferritin imply that these cells also have surface ferritin receptors[31]. The applicability of these observations to abnormal iron metabolism in hemochromatosis remains unexplored.

HFE immunoreactivity has not been detected in hepatocytes, endocrine or exocrine pancreatic cells, gastric mucosal cells, or the cells of Brunner's glands in the duodenum, although relatively large amounts of iron are often found in these locations in untreated patients with hemochromatosis[76]. Outside of the gastrointestinal tract, little or no staining for *HFE* immunoreactivity was visualized in the brain, neutrophils, macrophages, and lymphocytes[76]. However, the function(s) of normal *HFE* protein at any of these sites, and the immunocytochemical distribution of the C282Y variant of *HFE* protein believed to be characteristic of many patients with hemochromatosis, has yet to be elucidated. A 54–kDa protein isolated from human intestinal microvillus membranes and important for iron uptake by microvillus membrane vesicles in vitro[125] is highly expressed in the duodenum and liver of patients with hemochromatosis[126]. The subcellular localization of this protein indicates that it is also a candidate for participation in iron transport in normal subjects and in persons with hemochromatosis.

The distribution of iron-associated surface receptors or cytoplasmic iron-binding substances is not uniform among cells in the same organ. Approximately one-half of human and rat enterocytes contain natively unsaturated iron-binding substance(s) in a similar intracellular distribution[23]. These siderophilic enterocytes and the relative intensities of their cytochemical stain deposits are distributed in gradients directly correlated with known iron absorption capacities (duodenum > jejunum > ileum, and villus tip > lateral villus > crypt)[23]. The siderophilia could be due to transferrin[23], but there has been no high-resolution ultrastructural analysis of the distribution of cytoplasmic immunoreactive transferrin within the enterocyte cytoplasm to confirm or deny this. Similarly, this iron-binding reactivity could be due to non-transferrin, non-lactoferrin, non-ferritin, iron transport substances[23]. In the anterior pituitary and pancreatic islets in hemochromatosis, preferential uptake of iron occurs in gonadotroph cells and B cells, respectively[9, 10]. In human parenchymal and non-parenchymal liver cells, there is heterogeneous distri-

bution of transferrin receptors[40]. At least in the case of hepatocytes, this histologic pattern of iron distribution may be due to gradients of non-transferrin-bound plasma iron created in the liver[86–88]. Subpopulations of blood monocytes express natively bound lactoferrin and lactoferrin receptors[25, 26]. Taken together, these findings suggest that binding or uptake of iron, other metals, and metal-associated proteins may occur only in subpopulations of many cell types. However, the biochemical and physiologic explanations for these observations remain to be elucidated.

Iron deposition in tissues

In most histological studies of iron deposition in hemochromatosis, the study subjects were presumed or proven to be homozygous for hemochromatosis gene(s). Most surveys have been performed using acid ferrocyanide staining technique, wherein the terms iron and hemosiderin become synonymous. Early investigators primarily studied specimens obtained at autopsy from persons (mostly males) who were not treated with therapeutic phlebotomy. This work forms the basis of much of the histologic study of iron deposition performed in persons with hemochromatosis, and has been summarized in great detail[127]. More recent studies are based largely on specimens removed for diagnostic purposes, often from persons with less severe iron overload than those reported in previous decades.

The iron deposits in target organs in hemochromatosis are identifiable at the light and ultrastructural levels, usually in ferritin and hemosiderin. In untreated homozygotes, iron-laden ferritin is readily visible as brownish pigment in many tissues on gross inspection or in hematoxylin and eosin-prepared sections, especially in the liver. In many organs, extracellular iron deposits are commonly observed in persons with advanced iron overload. However, non-transferrin-bound iron is present in the plasma of untreated patients, and could enter cells by pathways demonstrated in vitro or in vitro[86, 87, 128]. This implies that acid ferrocyanide staining in hemochromatosis tissues also visualizes iron unassociated with ferritin or other well-described proteins. With the exception of small intestinal enterocytes, excess iron is deposited first and predominantly in epithelial cells; among these, the greatest quantities are usually observed in hepatocytes. The epithelia of the pancreas and other endocrine organs, cardiac myocytes, and synovial lining cells also accumulate relatively large quantities of iron. However, histochemical techniques of sufficient sensitivity reveal that virtually all cells (except monocytes, macrophages, and small intestinal enterocytes) in untreated adults with hemochromatosis appear to contain excess iron that they attempt to store in ferritin. The severity of iron overload in persons with hemochromatosis varies widely according to age, sex, dietary and hormonal factors, the occurrence of homozygosity or heterozygosity for hemochromatosis-associated genes, the specific mutation present, and the degree of therapy to achieve iron depletion[129, 130]. With few exceptions[131], the tissues of persons who have completed iron depletion therapy are expected to reveal little or no excess stainable iron. Accordingly, the staining intensity of pathologic iron deposits also varies widely among affected persons, and no amount of stored iron, great or small, can permit ascertainment or exclusion of the diagnosis of hemochromatosis.

Gastrointestinal tract

In persons with hemochromatosis, hemosiderin sometimes occurs in the submucosal connective tissue of the gingivae, both as extracellular deposits and in lymphatics. There appear to be no descriptions of iron histochemistry in the esophagus. In the stomach, deposits of ferric iron occur frequently in the glandular epithelium, particularly in the crypts, where iron is often present in the apical portion of cells. Hemosiderin is sometimes present in the subjacent lamina propria. In the duodenum, the cells of Brunner's glands often contain relatively large deposits of ferric iron. Lesser quantities of ferric iron are sometimes visualized in glandular cells of the jejunum, particularly those in crypts[127]. In most of the small intestine, however, there is a paucity or absence of stainable iron within enterocytes and in macrophages of the apical villous stroma and lamina propria[53–55]. Ferric iron is rarely present in the muscularis of the small intestine, although lymphocytes in submucosal lymphoid follicles and Peyer's patches sometimes contain hemosiderin. In the colon, iron detectable by light microscopic technique appears as scanty deposits in submucosal connective tissue cells or submucosal lymphoid nodules. Throughout the abdomen, the visceral and parietal peritoneum lack stainable iron, although hemosiderin is often visible in subperitoneal tissues[127].

Liver

Perls' Prussian blue stain is the most sensitive and specific stain technique for the study of hepatic iron deposition in hemochromatosis[3]. Increased stainable hepatocyte iron in a periportal distribution, at least in the early stages, characterizes hemochromatosis[3,131]. Iron deposition first occurs in siderosomes either around the nucleus or aligned along bile canaliculi, away from capillaries[3]. Cytoplasmic ferritin molecules in hepatocytes appear as

Table 19.3. Histologic grading of hepatic iron in hemochromatosis[a]

Scheuer grade	Gross appearance of slide[b]	Microscopic appearance
0	pink to red	no blue granules (at 100× or 45O× magnification)
1	pink to red	blue granules in <5% of hepatocytes or diffuse faint blue (at high magnification)
2	faint purple	blue granules present in 5–10% of hepatocytes (periportal)
3	purple	abundant blue granules present in ≤40% of hepatocytes (periportal accentuation with central sparing)
4	deep blue	abundant blue granules in >40% hepatocytes (decreased load centrilobularly)

Notes:

[a] This table is adapted from ref. [134].

[b] The actual grading is based on the microscopic appearance alone.

diffuse, faint blue staining. Larger amounts of stored iron appear as coarsely granular, blue-black deposits of hemosiderin. As the severity of hepatic iron overload progresses, otherwise normal small bile duct epithelial cells frequently accumulate iron. In heavily iron-loaded patients, the Kupffer cells sometimes contain large numbers of coarse hemosiderin granules, although the predominant cell type containing excess iron remains the hepatocyte. Intravascular lymphocytes sometimes reveal diffuse, bluish staining[132]. Arterial, venous, and lymphatic endothelia and walls sometimes contain iron deposits. Unlike the splenic capsule, that of the liver typically contains little or no visible iron[127].

Hepatic fibrosis appears to be related to iron deposition within portal structures, including bile ducts, and correlates well with the presence of necrotic hemosiderin-laden hepatocytes (sideronecrosis)[16]. In cirrhotic livers, fibroblasts usually contain large amounts of iron also, but much of the iron in connective tissue is extracellular. Similarly, numerous pseudo-bile canaliculi are nearly always heavily pigmented with hemosiderin. In a minority of patients with hemochromatosis, usually those with hepatic cirrhosis, the amount of stainable iron visualized in small foci of apparently regenerating hepatocytes is usually less than in older hepatocytes (iron-free foci)[3]. The deposition of hemosiderin in hepatocytes has therefore been interpreted to be a function of age of hepatocytes[133]. However, cells in these areas may show features suggestive of dysplasia[14].

Histologic grading of hepatic iron deposits

The semi-quantitative estimate of iron deposition in hepatocytes using acid ferrocyanide-stained sections is an important diagnostic parameter. In early hepatic iron overload, molecules of ferritin free in hepatocyte cytosol impart a diffuse blue tint; in more advanced iron overload, hemosiderin is visualized as dense, blue–black granules. The histochemical grading system of Scheuer[13], or some adaptation thereof, is the most widely used[13, 135]. This method is simple and deals only with hepatocyte iron; adaptations made to the original scoring criteria permit greater objectivity[134] (Table 19.3; Chapter 18). Many hemochromatosis homozygotes, particularly middle-aged or older males, have grade 3 or 4 hepatocyte iron. The hepatocyte iron grade in persons heterozygous for a hemochromatosis gene is usually 0–2[17, 134], although 1–3% of these persons eventually develop systemic iron overload[135]. Although other histologic grading systems are perhaps more accurate and include assessments of non-hepatocyte iron, they are more difficult to apply[14–17]. Each system yields histochemical grades of hepatic iron deposits that correlate reasonably well with quantities of iron measured using biochemical analysis or atomic absorption spectrometry. The Brissot system[14] correlates better with chemical analysis than the Scheuer system[13], probably because the former includes non-hepatocyte iron. Histologic grading and chemical analysis of hepatic iron are complementary, and maximum information is available when results of each are correlated[134]. In particular, these parameters are closely associated with the presence or absence of cirrhosis, and the relative degree of iron overload that must be alleviated by therapeutic phlebotomy[134]. Image analysis facilitates grading the hemosiderin deposits[136], but the methodology is impractical for routine diagnostic use.

Pancreas, salivary glands, and lacrimal glands

In the pancreas of untreated patients, hemosiderin is distributed uniformly throughout acinar and ductular epithelium, usually in relatively large quantities[18, 127]. The islets of Langerhans often contain stainable iron, and combined Prussian blue and anti-insulin-peroxidase–anti-

peroxidase staining techniques reveal that iron is deposited selectively in pancreatic B cells[10]. A similar phenomenon occurs in transfusion iron overload[137], suggesting that pancreatic B cells possess an unusual iron transport mechanism, or contain cellular components with high avidity for iron. In cases in which pancreatic fibrosis has occurred, siderosis of the fibrous tissue, sometimes severe, may be present; hemosiderin is uncommonly discernible in blood vessels[18, 127]. A histologic grading method for evaluating pancreatic hemosiderin deposition has been proposed[18]. In salivary glands, glandular tissue usually contains hemosiderin deposits. Typically, these are greater in serous than in mucous glands, and are greater in acinar than in ductal epithelium[127]. Smaller quantities of stainable iron are occasionally observed in connective tissue and vasculature. In lacrimal glands, hemosiderin deposits have been present in the few cases reported[127].

Endocrine and reproductive organs

In many untreated patients, the pituitary contains hemosiderin deposits present chiefly in the anterior lobe[138]. Combined immunocytochemical and Prussian blue staining of the anterior pituitary reveals coarse, dark, iron-positive granules in many gonadotroph epithelial cells that are immunoreactive for luteinizing hormone; a minority of thyrotroph, corticotroph, or somatotroph cells contain iron[9]. The pars intermedia usually contains hemosiderin, both intra- and extracellular, as does vascular endothelium. The pars nervosa contains stainable iron less frequently and in smaller quantities than the anterior pituitary[127, 138].

In the adrenal glands, the zona glomerulosa of the cortex is the initial and main site of hemosiderin deposition. In the zona fasciculata, the amount of stainable iron is usually slight, and even less is found in the zona reticularis. The medulla often contains iron in small amounts in medullary cells, connective tissue cells, eosinophil cells, and capillary endothelium. Hemosiderin may also occur in connective tissue cells of the capsule and in subcapsular lymphatic vessels, or as extracellular iron in the same microanatomical areas. Connective tissue and vasculature within the glands sometimes contain stainable iron, usually in small amounts[127].

Hemosiderin occurs in the thyroid gland in most cases, sometimes in relatively great amounts, and usually in glandular epithelium and parafollicular cells. In some cases, the colloid stains diffusely blue and is sometimes punctuated with particulate iron; no staining is observed in other cases. In fibrous tissue, iron deposits occur in small amounts, or are not present. Vasculature within the gland may display ferric iron staining positivity in vessel walls or endothelium. The parathyroid glands are often deeply pigmented by gross inspection, and most parathyroid epithelial cells contain abundant hemosiderin[127].

Deposits of ferric iron are present in the testes in most untreated men with iron overload. Much of this iron is observed in endothelium and vascular musculature, often as coarse granules. Hemosiderin also occurs in the capsule, seminiferous tubules, interstitial cells of Leydig, and connective tissue, and as extracellular deposits in connective tissue. However, iron is usually not visualized in germinal epithelium. In the epididymis, hemosiderin deposits may be found in vascular endothelium, efferent ducts, and connective tissue, although iron deposition in the seminal vesicles appears to be uncommon[127]. In the prostate, stainable iron is present in the glandular epithelium, connective tissue, and vascular endothelium in approximately one-half of cases[127]. Little data exist on iron deposition in the ovary, uterus, and oviducts; much of this lack of information appears to be related to the paucity of female subjects in early case series.

Heart

Hemosiderin is found consistently in the cytoplasm of cardiac myocytes in untreated persons with severe iron overload[127, 129, 130]. In some cases, all myocardial fibers are filled with hemosiderin. The topographic distribution of iron deposits is variable, although they appear most consistently in subepicardial myocytes[127, 139, 140] and least frequently in the sinus node[141]. When a histologic grading method was used to semi-quantify iron deposits, the ventricles and interventricular septa contained the greatest amounts, the atrioventricular node, bundle branches, and atria had intermediate quantities, and relative sparing of the sinus node was confirmed[19, 140, 142]. Within myocytes, iron is deposited as fine grains at both poles of the nucleus that taper in a spindle-shaped formation along the longitudinal axis of the cell[127]. In extreme cases, the myocyte may be replaced by a mass of fine pigment granules contained within the sarcolemma, although little morphologic evidence of damage to myocytes at the light microscopic level is found. Small quantities of coarse hemosiderin granules are sometimes present in connective tissue within lymphatic vessels, fibroblasts, and mononuclear cells, and extracellularly. The coronary arteries are rarely affected, although the adventitia, endothelium, perithelium, and histiocytes of the heart, the aorta, and other great vessels sometimes contain small quantities of stainable iron[127].

Skin and breast

Skin biopsies taken from hyperpigmented areas may show several abnormalities (Chevrant-Breton, Chapter 27). The

major factor contributing to abnormal pigmentation is increased melanin in the basal layer of the epidermis, although hemosiderin deposits occur in the epidermis in approximately one-third of cases. Hemosiderin may be present both extracellularly and within macrophages, particularly around blood vessels and in the basement membrane of the sweat glands. Iron-positive granules may also be present in epithelium of the sweat glands and, occasionally, of the basal layer of the epidermis. In contrast to the sweat glands, sebaceous glands contain stainable iron in fewer cases, and the intensity of staining is less. Hemosiderin deposits have been described in the epithelium of the male breast, but no extensive description of iron deposition in the female breast has been published[127].

Bones, joints, skeletal muscle, tendons, and ligaments

There are no substantial studies of iron deposition in bones in hemochromatosis. In most untreated persons with hemochromatosis, iron in hemosiderin is preferentially deposited in synovial lining cells, but also occurs in synovial connective tissue and cartilage ([127]; Schumacher, Chapter 24). In contrast, hemosiderin deposits due to hemarthroses of various etiologies tend to occur in macrophages and connective tissue[45]. Among hemochromatosis patients requiring hip or knee replacement surgery, stainable iron is nearly always intracellular, and is not related spatially to crystal deposits[45]. Although occasional ferritin-like particles have been visualized adjacent to apatite crystals in affected joints, iron has not been demonstrable by energy dispersive elemental analysis[45, 143, 144]. In a minority of cases, hemosiderin deposits occur in striated myocytes taken from a variety of sites; there appear to be no reports of iron deposition in tendons and ligaments[127].

Hematopoietic tissues

In the bone marrow, deposits of hemosiderin visualized with acid ferrocyanide staining in uncomplicated cases of hemochromatosis are not increased. However, closely packed, uniformly sized granules of hemosiderin in smear or squash preparations have been described frequently[145]. Ultrastructural analysis reveals that these granules consist of membrane-bound ferritin and hemosiderin located primarily in sinus-lining endothelial cells, and their heterotopic iron storage appears to increase with increasing severity of iron overload[144]. In blood monocytes and marrow macrophages, iron deposits exist as free cytoplasmic ferritin and as ferritin and hemosiderin within lysosomes (siderosomes), but do not increase with progressively severe iron loading[111, 145]. Plasma cells in the marrow (and in other locations) also contain heterotopic iron stores in some cases[57, 145]. In patients in whom increased iron deposition in macrophages or erythroblasts is observed, iron overload disorders other than hemochromatosis or the coincidental occurrence of hereditary and acquired forms of anemia characterized by ineffective erythropoiesis should be suspected.

In the spleen, stainable iron is usually present, but less so than in the liver and pancreas. In the pulp, all cell types may contain iron. Macrophages usually contain the greatest quantities; lymphocytes sometimes have diffuse, faint blue cytoplasmic staining. The splenic capsule, in contrast to that of the liver, frequently contains iron deposits in the connective tissue cells and smooth muscle cells. The trabeculae usually contain iron deposits that are sometimes calcified. The vessels, particularly larger ones, are more frequently affected in the spleen than in glandular organs. Iron deposits are observed in endothelium, elastic layers, and perivascular tissue, but the splenic sinuses and Malpighian bodies are relatively free of hemosiderin[127].

The lymph nodes usually contain hemosiderin, sometimes in great quantities, especially in nodes contiguous to parenchymal organs that also contain much stored iron, e.g., liver and pancreas. The iron, largely extracellular, appears in subcapsular sinus and lymphatic spaces. Nodal microvasculature may appear to be composed of iron. Although the macrophages and multinucleate giant cells of lymph nodes often contain iron, the lymphocytes within follicles are often spared the deposition of iron[127].

In the thymus, numerous stromal cells may contain hemosiderin, especially epithelial cells of the central parts of thymic rests that later form Hassal's corpuscles. However, the corpuscles and round cell layers usually lack stainable iron. Monocytes, macrophages, lymphocytes, and plasma cells in the liver, heart, submucosal connective tissue of the small intestine (including submucosal lymphoid follicles and Peyer's patches), and other organs sometimes contain faint diffuse cytoplasmic iron positivity[127].

Urinary tract

The kidneys contain little or no stainable iron in most cases. When present, hemosiderin deposits usually occur with greatest frequency and intensity in the tubules, particularly the convoluted tubules. Hemosiderin, occurring as fine or coarse granules, is also deposited in glomerular loops and capillary endothelium; Bowman's capsule is affected less frequently than the remainder of the glomerulus. Curiously, the minimal iron deposition in the kidney in hemochromatosis contrasts sharply with the substantial deposits of iron observed in kidneys of β_2-microglobulin-deficient mice[75]. Little stainable iron is usually visualized

in renal connective tissue and vasculature. There appear to be no reported observations on iron deposition in the ureter in hemochromatosis. In the urinary bladder, hemosiderin occurs in the 'gland' cells and in endothelium of occasional small arteries. In the urethra, stainable iron has been described in the gland cells and in the lining membrane[127].

Respiratory tract

In the tonsils, stainable iron is often present in mucous epithelium, lymphocytes, and connective tissue. In the pharynx, epiglottis, and trachea, the mucous glands may be markedly siderotic. Epiglottic chondrocytes and the mucous membrane may also contain small quantities of finely granular hemosiderin. In the trachea and bronchi, stainable iron is sometimes visualized in the mucous membrane, in mucous glands, and in chondrocytes. In the lungs, relatively small quantities of ferric iron are visualized in alveolar walls or within alveolar spaces (sometimes in alveolar macrophages) in a majority of cases. The pulmonary arterioles and capillaries occasionally reveal iron deposits, but visceral pleura and intrapulmonary connective tissue usually lack stainable iron[127].

Central nervous system and eye

In the brain, epithelial cells of the choroid plexus almost invariably contain striking hemosiderin deposits[127, 146–148]. Cells of area postrema of the medulla oblongata and of the olfactory bulbs usually contain stainable iron also[146–148]. Extracellular iron deposits are sometimes found scattered throughout the leptomeninges and extracellularly within the pia-arachnoid, but ependymal cells lining the ventricular system, neurons, and neuroglial cells do not appear to contain hemosiderin[148]. Stain intensification methods such as diaminobenzidine black are useful to study the small amounts of normal iron in the brains of animals[7, 8], but there appear to be no published studies of the application of such techniques to the analysis of iron deposition in the brain in hemochromatosis. This leaves open the possibility that iron deposition in brain cells is greater or more widespread in hemochromatosis than is presently appreciated. Reports of iron deposition in the spinal cord, peripheral nerves, ganglia, and other peripheral nervous system structures appear to be lacking.

In the eye, traces of ferric iron can be visualized in the corneal epithelium, sclera, nonpigmented ciliary epithelium, iris pigment epithelium, peripapillary pigment epithelium, and fibroblasts; some iron is extracellular. These correspond to the rust-colored pigmentation of the bulbar conjunctivae (most marked at the inferior limbus and extending onto the cornea), eyelid borders, insertions of the recti muscles, sclerae, and peripapillary regions observed in a minority of patients[149, 150].

Neoplasms in persons with hemochromatosis

Primary hepatic cancers occur in 20–35% of persons with hemochromatosis who have hepatic cirrhosis[134]. Regardless of their histologic pattern, the parenchymal cells of most of these cancers lack stainable iron at the light microscopic level[3, 127, 151], and this appears to be unrelated to iron depletion therapy. The tumor stroma, however, is occasionally siderotic[151]. In some patients without primary hepatic cancer (with and without hepatic cirrhosis), small foci of apparently regenerating hepatocytes contain less stainable iron than do surrounding hepatocytes (iron-free foci)[3]. Although this has been interpreted as a function of hepatocyte age[133], the parenchymal cells of iron-free foci may be dysplastic[14]. Adenomata of the thyroid[152, 153], adenocarcinomas of the lung, breast, stomach, colon, and pancreas, and blasts of acute non-lymphoblastic leukemia in untreated persons with hemochromatosis also appear to lack iron staining at the light microscopic level[154, 155]. However, the broad significance of reduced or absent stainable iron in neoplastic cells in persons with hemochromatosis is not known.

Differential diagnosis of hemochromatosis

The histologic changes associated with hemochromatosis are almost, if not entirely, attributable to iron overload. However, iron overload in hemochromatosis is not invariably associated with structural or functional abnormalities in target organs, particularly in young persons. Because these persons are likely to have preventable or reversible abnormalities if diagnosed and treated appropriately, the pathologist must recognize that no amount of stainable iron, great or small, can reliably include or exclude the diagnosis of hemochromatosis. In hemochromatosis and other disorders, brown pigment in tissues may represent hemosiderin, hemofuscin, melanin, or anthracosis. Perls' Prussian blue staining is necessary to verify the presence of iron. When excess stainable iron is identified in specimens obtained by biopsy, surgery, or post-mortem examination, it is important to review the causes of systemic and organ-specific iron overload.

Several hereditary iron overload disorders other than hemochromatosis have now been characterized in humans with respect to tissue and cell iron deposition, and each must be considered in the differential diagnosis (Table 19.4). The systemic iron overload due to dyserythropoietic anemias, erythrocyte transfusion, and increased

Table 19.4. Human hereditary disorders that cause iron overload[a]

	Gene affected; Chromosomal location	Sites of increased iron deposition
Hemochromatosis[b]	*HFE*; 6p	Parenchymal cells of liver, pancreas, other endocrine organs, heart, joints, many other sites
African iron overload	?	Macrophages; parenchymal cells of liver, pancreas, heart, other sites
Atransferrinemia	transferrin; 3q	Parenchymal cells of liver, pituitary, heart, thyroid
Hereditary aceruloplasminemia	ceruloplasmin; 3q	Basal ganglia; parenchymal cells of liver and pancreas
Dyserythropoietic anemias[c]	various	Parenchymal cells of liver, heart, pancreas, endocrine organs; macrophages
Friedreich's ataxia	frataxin; 9q	Cardiac myocytes; other sites not well studied
Melanesian iron overload	?	Parenchymal cells of liver; other sites not well studied

Notes:

[a] This table is adapted from Carmel et al.[155]. Hallervorden-Spatz disease (20p13) and Zellweger syndrome are autosomal recessive disorders associated with excess iron deposition. In the former, this occurs in basal ganglia; in the latter, excess iron is found in the liver (mainly Kupffer cells), spleen, kidneys, and lungs. Whether these disorders represent primary abnormalities of iron metabolism is uncertain.

[b] This applies to Caucasians with 'classical' HLA-linked hemochromatosis. Other Caucasians appear to have a clinically indistinguishable form of hemochromatosis due to other mutation(s) of uncertain chromosomal location(s). Many persons with porphyria cutanea tarda develop mild or moderate hepatic iron overload in association with inheritance of a C282Y mutant *HFE* allele. Juvenile hemochromatosis, a rare disorder, may also be hereditary, but appears to be unrelated to *HFE* mutations. The cause and heritability of neonatal hemochromatosis have not been clearly established, but the disorder seems unrelated to *HFE* mutations.

[c] These disorders include severe beta-thalassemia, hereditary forms of sideroblastic anemia, pyruvate kinase deficiency, some variants of hereditary spherocytosis and other inherited erythrocyte membrane protein defects, and congenital dyserythropoietic anemia, types I and II.

dietary iron intake can mimic hemochromatosis. A recently described non-HLA-linked syndrome of normal serum transferrin saturation, excess hepatocyte iron deposition, and diabetes mellitus may be relatively common, although its etiology remains obscure[156]. Alcoholism and hepatitis C often result in increased stainable iron in the liver[134]. Increased iron deposition in organs other than the liver can be readily distinguished histologically and clinically from hemochromatosis. Hemosiderosis of the lung can occur due to several primary and secondary pulmonary causes, e.g., Goodpasture's syndrome and mitral stenosis, respectively. Renal iron deposition, unusual in hemochromatosis, is usually due to chronic intravascular hemolysis, e.g., sickle cell trait and paroxysmal nocturnal hemoglobinuria. Hemorrhage due to many causes can result in local iron deposition, especially in macrophages, in most tissues. Altogether, iron overload due to hemochromatosis and non-hemochromatosis causes is not uncommon. Each disorder that causes increased tissue iron deposition must be diagnosed correctly to permit appropriate treatment and genetic counseling[130].

In routine surgical and anatomical pathology practice, sections stained with hematoxylin and eosin, other routine techniques (especially Mallory's trichrome), and Perls' Prussian blue stains should be prepared and reviewed when the diagnosis of hemochromatosis or iron overload is considered. The tissue most often examined is a specimen of liver obtained by biopsy[134]. Among American Caucasians undergoing hepatic biopsy, 8.5% had increased stainable iron, and 3.4–4.6% percent of hemochromatosis homozygotes[154, 157]. Thus, demonstrating any increased acid ferrocyanide-reactive iron localized primarily in hepatocytes, often in a periportal distribution, strongly suggests the diagnosis of hemochromatosis in Caucasian patients. The occurrence of sideronecrosis and iron in bile ductular cells also suggests the diagnosis of hemochromatosis, although these findings typically occur in patients with advanced iron overload. Coincidental liver disease occurs in approximately 15% of hemochromatosis homozygotes diagnosed during routine medical care delivery[154]. Micronodular cirrhosis with increased stainable hepatocyte iron without findings typical of Laennec's cirrhosis, chronic viral hepatitis, autoimmune hepatitis, or other primary liver disease is often due to hemochromatosis.

The deposition of excess iron and the histologic changes

typical of iron overload occur in target organs other than the liver in hemochromatosis. The presence of hemosiderin in gastric, duodenal, or jejunal glandular epithelial cells observed in biopsy or surgical specimens should prompt further consideration of the diagnosis of hemochromatosis. In contrast, hemosiderin deposition in macrophages of the lamina propria usually results from the absorption of heme iron from gastrointestinal hemorrhage[54]. In addition to the liver, the pancreas, anterior pituitary, heart, thyroid, and lymph nodes may become fibrotic due to severe iron overload[127]. Endomyocardial specimens obtained by biopsy may have stainable ferric iron, and a Perls' Prussian blue stain should be performed on specimens from all patients who undergo endomyocardial biopsy to evaluate cardiomyopathy[140]. The arthropathy of hemochromatosis can antedate hepatic disease due to iron overload by many years, and joint tissues may be obtained by biopsy or at joint replacement surgery. Calcium pyrophosphate deposition (pseudogout) and splitting at the interface between articular cartilage and subchondral bone, in addition to the occurrence of stainable ferric iron in synovial lining cells, are characteristic histologic changes that provide clues to the diagnosis of hemochromatosis[45]. Testicular specimens obtained by biopsy may reveal atrophy of the seminiferous tubules and scanty mitoses, absent spermatozoa and spermatids, thickening of the tubular walls, and Leydig cells reduced in numbers or absent[158]. Examination of blood and marrow sometimes provides hints to the diagnosis of hemochromatosis. Many hemochromatosis homozygotes have mild macrocytosis of uncertain etiology, even in the absence of hepatic dysfunction or other common causes of enlarged erythrocytes[159]. Prussian blue staining of peripheral blood or buffy coat smears may reveal bluish cytoplasmic staining of monocytes. Although increased iron deposition in bone marrow is not typical of uncomplicated hemochromatosis, the presence of increased macrophage or erythroblast iron or other cytologic changes in marrow cells may indicate the coincidental presence of hereditary or acquired anemia characterized by ineffective erythropoiesis[134].

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Computed tomography and magnetic resonance imaging in the diagnosis of hemochromatosis

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Introduction

The diagnosis of hemochromatosis can be made in different circumstances: (i) in symptomatic individuals who present with clinical features suggestive of the disorder; (ii) in persons who are discovered to have abnormal serum iron tests; or (iii) in persons who are evaluated in the context of a family study. The insufficient sensitivity and specificity of serum iron tests (transferrin saturation and serum ferritin concentration) sometimes do not permit a definite diagnosis to be made until a hepatic biopsy has been performed. Histologic examination of the liver is an important element in the diagnosis of hemochromatosis[1], because: (i) it reveals hepatic iron overload and permits an assessment to be made of the distribution of iron in the liver (typically, iron deposition occurs in hepatocytes with periportal and perilobular distribution)[2]; (ii) it permits biochemical measurement of hepatic iron concentration to be performed. A hepatic iron index (hepatic iron concentration/age ratio) >1.9 is helpful in establishing a diagnosis of hemochromatosis homozygosity[3]; and (iii) it provides assessment of the degree of fibrosis which is of major prognostic significance.

Due to the physical properties of iron, iron overload is responsible for modifications in computed tomography attenuation and magnetic resonance signal intensity of the liver. This has promoted interest in developing these principles as a quantitative method to assess hepatic iron concentration. In this chapter, the focus is on the radiologic, non-invasive diagnosis of iron overload and on providing additional information about radiologic features of hepatocellular carcinoma arising in patients with hemochromatosis. Non-specific signs of hepatic disease (e.g., radiological signs of hepatic cirrhosis or portal hypertension) are not addressed.

Detection and quantification of liver iron overload

There is an imperfect correlation of serum iron tests (serum iron concentration, transferrin saturation, and serum ferritin concentration) with the degree of excess body iron. Biochemical determination of the hepatic iron concentration is the method of reference for quantification of iron overload[4]. However, measuring the hepatic iron concentration requires that a hepatic biopsy be performed. Although relatively safe, this is an invasive procedure associated with significant morbidity and an estimated mortality rate of 0.015% to 0.030%. Therefore, there is a need for accurate, non-invasive methods to detect and quantify hepatic iron content.

Computed tomography (CT) scanning

Due to its density, iron increases X-ray attenuation in the liver and other organs affected by iron overload. This phenomenon was identified during initial clinical experiences with CT scanning. Iron is responsible for a spontaneous hyperdense liver image that produces an abnormal visualization of the portal and hepatic vascular structures (Fig. 20.1(*a*); 20.1(*b*)). This property has been studied in the setting of non-invasive iron quantification in liver. Both single- and dual-energy techniques have been employed.

Single-energy CT scanning

The basic data of CT scans are represented by a CT number expressed in Hounsfield units (HU) that represents the degree of X-ray attenuation in a given volume. The attenuation coefficient depends on the density of the material studied, and on variables such as the energy of incident X-ray beam[5]. The measurements must be obtained in several operator-defined regions of interest drawn in the liver, with

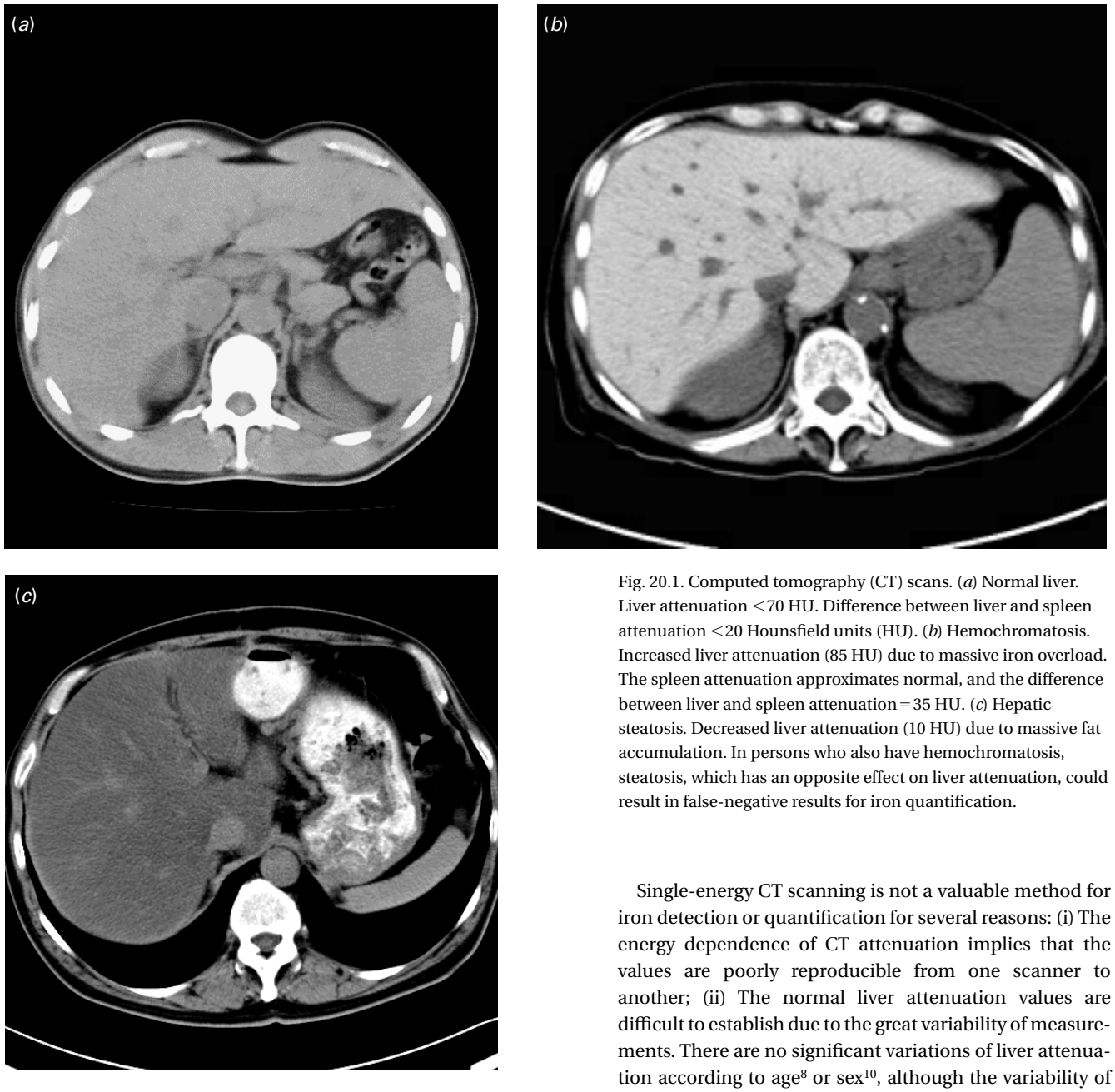

Fig. 20.1. Computed tomography (CT) scans. (*a*) Normal liver. Liver attenuation <70 HU. Difference between liver and spleen attenuation <20 Hounsfield units (HU). (*b*) Hemochromatosis. Increased liver attenuation (85 HU) due to massive iron overload. The spleen attenuation approximates normal, and the difference between liver and spleen attenuation = 35 HU. (*c*) Hepatic steatosis. Decreased liver attenuation (10 HU) due to massive fat accumulation. In persons who also have hemochromatosis, steatosis, which has an opposite effect on liver attenuation, could result in false-negative results for iron quantification.

avoidance of hilar vascular structures and artifacts. All studies report an increase in the liver attenuation coefficient in both primary[6–9] and secondary iron overload. This increase is correlated with hepatic iron content, and the correlation is more perfect than the correlation observed between serum ferritin concentration and hepatic iron concentration[6, 7, 9]. The attenuation values decrease during therapeutic phlebotomy and return to normal[9].

Single-energy CT scanning is not a valuable method for iron detection or quantification for several reasons: (i) The energy dependence of CT attenuation implies that the values are poorly reproducible from one scanner to another; (ii) The normal liver attenuation values are difficult to establish due to the great variability of measurements. There are no significant variations of liver attenuation according to age[8] or sex[10], although the variability of measurements are greater in older patients. The metabolic state of the liver and the degree of hydration also influence the measurements. The upper limit of normal value has been estimated at 70 HU in most studies[7–12]. It is also possible to take into account the difference between liver and spleen attenuation, which in normal conditions and subjects is <20 HU[12], but this does not improve results[7]. (iii) The specificity of a liver attenuation coefficient increase for the diagnosis of iron overload is good[7, 9], although some instances of false-positive results have been reported[6, 13].

An increase in liver attenuation coefficient, due to iodine storage, has been reported in prolonged treatment with high doses of amiodarone[14] and in few cases of hepatic glycogenosis, due to glycogen accumulation in hepatocytes. (iv) The most important problem has been that the sensitivity of the technique remained insufficient, and precluded its routine use as a non-invasive means for iron quantification. When patients with moderate iron overload were studied, CT scanning was unable to detect hepatic iron overload less than five times the upper normal value of hepatic iron concentration[6, 7]. (v) Moreover, steatosis, a common finding (associated with alcoholism, diabetes mellitus, and hyperlipidemia), decreases liver attenuation (Fig. 20.1(*c*)), and can result in false-negative results when measurements of iron overload are sought. For these reasons, CT scanning technique is no longer used in iron quantification. However, the fortuitous discovery of a high liver attenuation in a CT scan performed for reasons other than to investigate iron overload is an important sign that suggests the diagnosis of hemochromatosis.

Dual-energy CT scanning

The limits of single-energy CT scanning led to the development of technical procedures that would increase the sensitivity for and minimize the influence of fat in estimating hepatic iron content. The rationale of dual-energy CT scanning is to isolate the components of high atomic number in a material by subtracting the attenuation of an image obtained at low kilovoltage from the attenuation of an image obtained at high kilovoltage. The curves expressing the relationship of attenuation as functions of kilovoltage are very close to one another for atoms of low atomic number, but the curves for atoms of high atomic number are widely separated at low kilovoltage. The subtraction of two images performed at two different kilovoltages (e.g., 90 kV and 130 kV) therefore minimizes contrast due to low atomic number atoms (e.g., carbon, hydrogen, and oxygen) and isolates the contrast due to high atomic number atoms (e.g., calcium and iron). This method has been used particularly for quantification of the calcium content of osteoporotic bones. The theoretical advantage of this technique for iron quantification is that it eliminates the error due to the presence of fat.

There are few in vivo studies of the non-invasive quantification of hepatic iron. In 1980, Chapman et al. used this method with energies of 110 kV and 140 kV to study eight patients with hemochromatosis, and found a better correlation between the hepatic iron content determined by dual-energy CT scanning and the biochemical hepatic iron concentration than the correlation obtained with single-energy CT scanning[15]. Goldberg et al. used energies of 80 kV and 120 kV to study the livers of iron-overloaded dogs, and obtained an excellent correlation with hepatic iron concentration[16]. Duvauferrier et al. studied 18 patients and found that dual-energy CT scanning was a better predictor of mild iron overload, especially in patients who also had hepatic steatosis[17]. Further development of this technique was hampered by the increasing availability of magnetic resonance imaging that appeared to be more promising.

Magnetic resonance imaging (MRI)

Iron is a paramagnetic substance that becomes strongly magnetized when placed in a magnetic field. This is responsible for a shortening of relaxation times that is more pronounced on T2 (transverse) than on T1 (longitudinal) relaxation time, leading to a decrease in signal intensity of the liver (and other organs with iron deposits) on T2-weighted images[18, 19]. The T2 relaxivity of ferritin, which is due to the iron core rather than to the apoferritin protein shell, does not appear to be sufficient to account for the extreme decrease in T2 times observed in hepatic iron overload. In vitro spectroscopy experiments using solutions of $FeCl_3$ suggested that low-molecular-weight cytosolic iron has much greater relaxivity[19, 20] and plays a major role. The most likely explanation is that inorganic iron is in close contact with the water proton. Because the relaxation enhancement effect decreases as a function of the sixth power of distance, the effect of iron-laden ferritin is considerably diminished. The sequences used to detect iron overload are not significantly influenced by the triglyceride content of the liver[21]. Therefore, in contrast with single-energy CT scanning, hepatic steatosis does not decrease the sensitivity of the technique.

In vivo MRI studies showed inaccuracy of the T2 calculation due to the extreme decrease of signal intensity related to heavy iron overload, leading to low signal-noise ratio. Therefore, signal intensity measurements were preferred[13, 19, 22, 23]. Preliminary studies used spin echo images[6, 13, 22], but the sensitivity of these sequences was insufficient to detect $<$3.5–5 times the upper normal hepatic iron concentration value (36 μmol/g dry weight of liver). The use of high magnetic field strength[23, 24] with a magnet operating at 1.5 tesla or the use of gradient-echo T2*-weighted sequences have greater sensitivity and detect iron overload corresponding to 80–300 μmol/g dry weight of liver[25, 26]. Gradient-echo sequences are available on every MRI unit. With a TR of 120 ms and a 128 $\times$ 256 matrix, the acquisition time is approximately 15 seconds, allowing breathhold acquisitions. The flip angle must be reduced (approximately 20°) to decrease the T1-weighting of these sequences. The TE selected for gradient-echo

sequences must be 'in phase' (i.e., approximately a multiple of 7 divided by the field strength, expressed in tesla), because when using 'out of phase' TE, chemical shift artifacts can reduce liver signal intensity if hepatic steatosis is present. According to the degree of iron overload, different sequences must be used[26]. Highly T2*-weighted GRE sequences (obtained with a TE of 14 ms at 1.5 tesla, 21 ms at 1.0 tesla, or 30 ms at 0.5 tesla) give the best results for detection or quantification of mild overload (<100 μmol/g dry weight of liver). Proton-density gradient-echo sequence (TE = 4 ms at 1.5 tesla, 7 ms at 1.0 tesla, or 15 ms at 0.5 tesla) give the best results in cases of moderate iron overload (hepatic iron concentration 100–250 μmol/g dry weight of liver). Less sensitive sequences, such as short TE spin-echo sequences, are accurate for massive iron overload with hepatic iron concentrations >250 μmol/g dry weight of liver.

The signal intensity of the liver must be compared with a reference value. The use of reference phantoms is difficult and not compatible with routine use[19, 26]. Therefore, liver signal intensity must be compared with the signal intensity of other organs on the same image not affected by iron overload and that will be used as a reference. The spleen is iron-loaded in some instances. Measurement of fat signal intensity is sometimes difficult in thin patients[26], and there is a greater variability in measurements[27]. Therefore, liver/muscle ratios are used by most authors. The liver/muscle ratio decreases as hepatic iron overload increases. No accepted reference range exists for values of SI (L/M) and threshold values differed among studies from 0.5[6] to 0.8[25] to 1.0[26]. Schematically, a liver signal intensity less than that of muscle indicates the presence of hepatic iron overload (Figure 20.2(*a*); 20.2(*b*)). For precise quantification, liver and muscle signal intensities must be measured using operator-defined regions of interest. Liver signal intensity is averaged from several regions of interest in the right lobe of the liver. Muscle signal intensity is measured on paraspinal muscle.

Decreased liver signal intensity due to iron overload is not specific for hemochromatosis. Transfusion iron overload, a relatively common cause of iron overload, is easily recognized by clinical history. Although hemochromatosis is responsible for parenchymal iron overload involving the liver, pancreas, heart, and endocrine glands, chronic erythrocyte transfusions can result in predominantly reticuloendothelial iron overload involving mainly the spleen, bone marrow, and sinusoidal (Kupffer) cells of the liver[28]. Therefore, transfusion iron overload decreases the signal in spleen and the liver; in hemochromatosis, the spleen signal is usually normal. However, in some cases of hemochromatosis, usually persons with massive iron overload, there is a diffuse decrease in the signal of the spleen. A low pancreatic signal in a patient with hemochromatosis could indicate hepatic cirrhosis[29], but this finding needs to be confirmed in larger studies. Many pathological conditions other than hemochromatosis and transfusion iron overload can induce hepatic iron overload, including porphyria cutanea tarda, inefficient erythropoiesis[30], end-stage cirrhosis[31, 32], iron overload with polymetabolic disorders[33], and chronic viral hepatitis[34]. Few studies have addressed the question of whether iron distribution that could be measured using magnetic resonance imaging will permit a distinction of hemochromatosis to be made from these other conditions (grouped under the generic term hemosiderosis). The only study that included such patients could not compare their features with those of persons with hemochromatosis, because all the hemochromatosis patients studied had massive iron overload that precluded further conclusions[35]. Finally, some cases of focal iron deposits had been reported. First, segmental wedge-shaped volumes of decreased liver signal can be related to disturbances of portal vein circulation (thrombosis or arteriovenous shunts) caused by hemodynamic factors or mild iron deposition in hepatocytes. Second, large regenerative nodules in otherwise iron-free cirrhotic livers may accumulate iron, and some of them can develop internal iron-poor foci of hyperplasia or malignancy[36]. These two conditions are very different from the diffuse hepatic iron overload encountered in hemochromatosis.

Diagnosis of hepatocellular carcinoma

Hepatocellular carcinoma is a frequent complication of hemochromatosis, and accounts for 30–45% of deaths of hemochromatosis patients in most recent studies. Major risk factors include age >45 years, male sex, underlying cirrhosis (although some cases have been reported in non-cirrhotic livers), and association with other carcinogenic factors such as alcoholism and hepatitis virus infection[37]. Hepatocellular carcinoma is present at the time of diagnosis in approximately 5% of persons with hemochromatosis (personal observation). MRI can detect small, focal cancerous lesions in cases of massive hepatic iron overload, because the decrease in liver magnetic resonance signal intensity leads to a high contrast between the tumor (devoid of iron) and the non-tumorous adjacent liver (Figure 20.3)[38]. The high frequency of malignant transformation of iron-free foci justifies the regular screening of male hemochromatosis patients >45 years of age who have hepatic cirrhosis. This screening relies on regular

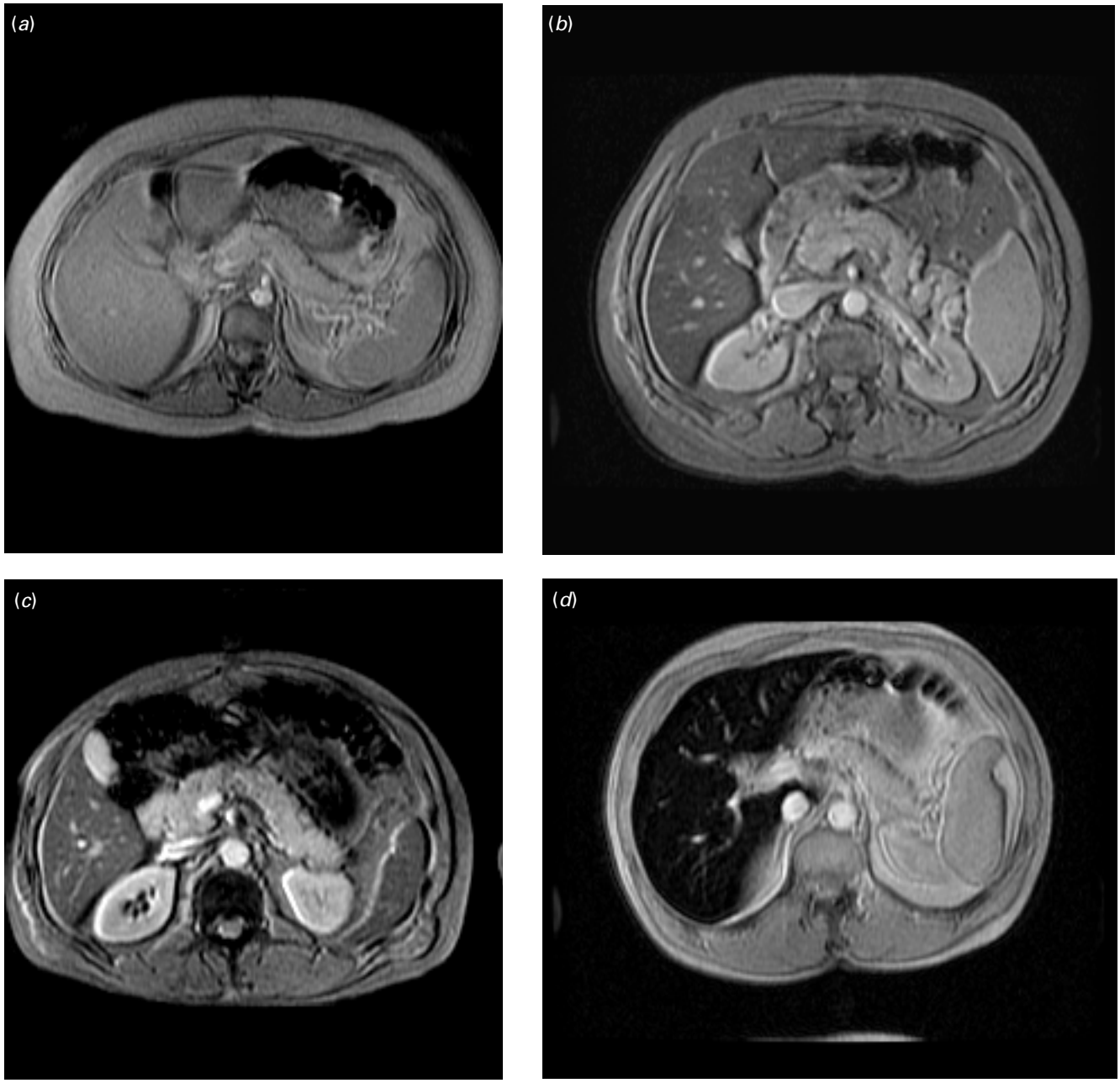

Fig. 20.2. Magnetic resonance imaging (MRI). (*a*) Normal liver. Signa 1.5 tesla (GEMS, Milwaukee). T2* weighted-gradient-echo sequence (TR = 120 ms; TE = 14 ms; flip angle = 20°). The liver signal intensity is greater than the paraspinal muscle signal intensity. (*b*) Hemochromatosis. Mild iron overload (hepatic iron concentration = 80 μmol/g dry weight of liver; upper limit of normal = 36 μmol/g dry weight of liver). Same parameters as described for Fig 20.1(*a*). The liver signal intensity is less than the paraspinal muscle signal intensity. The signals of the pancreas and spleen are normal. (*c*) Hemochromatosis. Moderate iron overload (hepatic iron concentration = 146 μmol/g dry weight of liver). (*d*) Hemochromatosis. Severe iron overload (hepatic iron concentration = 352 μmol/g dry weight of liver). The great decrease of the hepatic signal is responsible for the appearance of a 'black' liver.

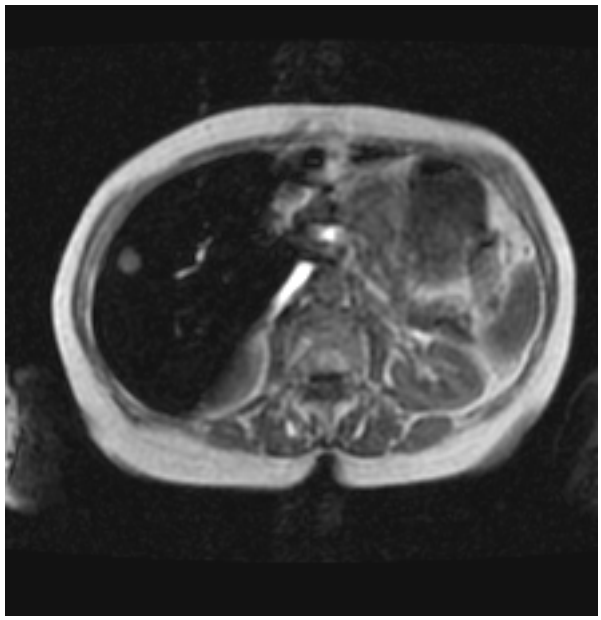

Fig. 20.3. Magnetic resonance image (MRI) of a small hepatocellular carcinoma complicating hemochromatosis. There is high contrast between the tumor (devoid of iron) and the adjacent liver (massively iron overloaded).

ultrasound examinations of the liver and measurements of the serum concentration of alpha-fetoprotein.

Conclusions

MRI is a promising tool for the detection and quantification of hepatic iron overload when liver/muscle signal intensity ratio and gradient echo sequences are used on high-field magnets. The correlation of magnetic resonance signal and hepatic iron concentration is much better than with the serum ferritin concentration. The wide utilization of the method is limited by the difficulty in reproducing results from one magnet to another. Some large multicenter studies are necessary to establish a standardized method that could promote MRI scanning technique as a replacement for performance of hepatic biopsy. However, even with the most sensitive sequences, the threshold of iron detection is ~80 μmol/g dry weight of liver (about twice the normal value), and a normal signal therefore does not permit exclusion of the diagnosis of hemochromatosis in young individuals who have little or no excess hepatic iron. In addition, the future of imaging modalities will probably be modified by the recent discovery of the *HFE* gene[39], mutations of which are encountered in 60–96% of patients believed otherwise to be hemochromatosis homozygotes. Thus, DNA-based genetic testing can establish the diagnosis of homozygosity for the hemochromatosis-associated C282Y mutation of the *HFE* gene. However, genetic testing cannot determine the degree of expression of the clinical phenotype of hemochromatosis (iron overload). Therefore, further study of diagnostic strategies is needed to define the role of MRI scanning in the diagnosis and management of hemochromatosis.

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Part V

Complications of iron overload

21

Mechanisms of iron toxicity

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Introduction

Approximately 1 in 250 individuals of northern European descent have hemochromatosis, making it one of the most common inherited disorders[1–3]. In hemochromatosis, there is a pathologic expansion of body iron stores due to an increase in the absorption of dietary iron. Cellular uptake of circulating excess iron results in increased formation of ferritin and hemosiderin, especially in the parenchymal cells of several organs (e.g., liver, heart, pancreas). At high tissue iron levels, these organs show evidence of cellular damage accompanied by functional insufficiency[1–3]. Although best studied in the liver, pathological studies also support the association of iron overload and tissue injury in other organs. The liver is a major site of excess iron accumulation, and massive deposition of iron within hepatocytes can lead to the development of fibrosis and cirrhosis[1–4]. Excess iron can be stored as ferritin in both the cytoplasm and lysosomes, or as hemosiderin in lysosomes. The hepatic concentration of iron is an important factor in determining hepatotoxicity, because removal of excess iron by phlebotomy or chelation results in clinical improvement[1–3].

This chapter will describe the mechanisms of toxicity induced by iron overload, focusing on two major hypotheses: the oxidative injury hypothesis and the lysosomal injury hypothesis. These hypotheses concerning iron-induced damage are not mutually exclusive, because there is evidence of oxidative damage to lysosomal lipids that may contribute to lysosomal injury in iron overload. Evidence of damage to cellular organelles and to DNA after iron overload will also be summarized. There are several recent reviews of various aspects of iron-induced toxicity[5–13].

Oxidative injury hypothesis

The oxidative injury hypothesis postulates that iron overload in vivo can result in the formation of oxyradicals, with resultant damage to cellular constituents and impairment in cellular function. This section summarizes how iron catalyzes the formation of oxyradicals, the potential cellular targets of these radicals, and the evidence that iron can cause free radical production in vivo.

Iron catalyzes the formation of oxyradicals

Abundant evidence now exists from in vitro experiments that iron can catalyze the production of oxyradicals, when iron is available in a redox-active form (for review see ref.[14]). Two important radicals that can result from these reactions are lipid radicals and hydroxyl radicals. One type of reaction promoted by iron that leads to the formation of lipid radicals is the decomposition of preformed lipid hydroperoxides[14]. Ferrous (Fe^{2+}) iron chelates can react with lipid hydroperoxides (LOOH) to form alkoxyl radicals ($LO^{\bullet}$):

$$LOOH + Fe^{2+} \rightarrow Fe^{3+} + OH^{-} + LO^{\bullet}$$

Similarly, ferric (Fe^{3+}) iron chelates can react with lipid hydroperoxides to form peroxyl radicals ($LOO^{\bullet}$):

$$LOOH + Fe^{3+} \rightarrow Fe^{2+} + H^{+} + LOO^{\bullet}$$

Both alkoxyl and peroxyl radicals can propagate the chain reaction of lipid peroxidation by extracting hydrogen atoms from nearby lipids (Fig. 21.1), or can react with other cell constituents.

Iron can also catalyze the production of hydroxyl radicals through Fenton or Haber–Weiss chemistry[14]. In the Fenton reaction, the hydroxyl radical ($OH^{\bullet}$) is produced from hydrogen peroxide (H_2O_2):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

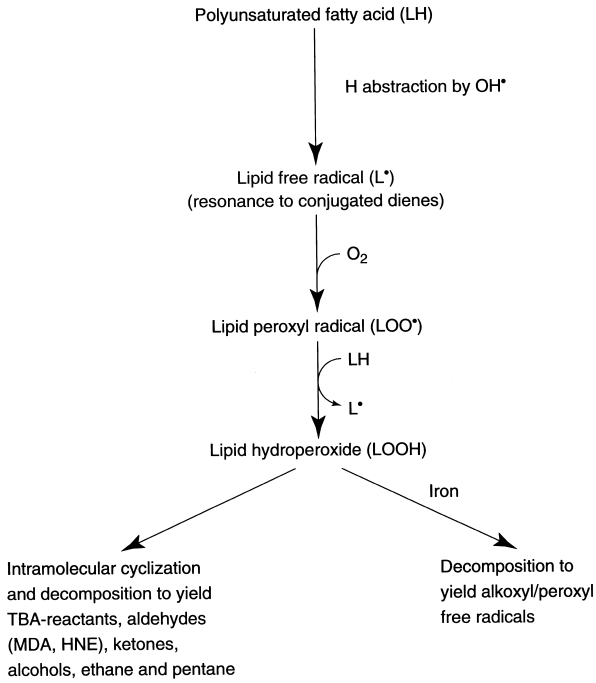

Fig. 21.1. Lipid peroxidation of a polyunsaturated fatty acid in a membrane lipid. The hydroxyl radical ($OH^{\bullet}$) formed by iron-catalyzed reactions may attack a polyunsaturated fatty acid of a membrane lipid (LH) to yield a carbon-centered lipid radical ($L^{\bullet}$). Intramolecular rearrangement of the double bonds of this radical results in conjugated dienes. $L^{\bullet}$ adds oxygen to yield a lipid peroxyl radical ($LOO^{\bullet}$), and $LOO^{\bullet}$ can attack another LH, generating a new $L^{\bullet}$ and a lipid hydroperoxide (LOOH). LOOH is susceptible to cleavage by iron chelates, generating alkoxyl and peroxyl radicals. Many breakdown products result from lipid peroxidation, including the reactive aldehydes, MDA and HNE[14, 19].

In the iron-catalyzed Haber–Weiss reaction, the superoxide radical ($O_2^{\bullet -}$) reduces ferric iron that reacts with hydrogen peroxide by Fenton chemistry to yield the hydroxyl radical:

$$Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$
$$\text{Net reaction: } O_2^{\bullet -} + H_2O_2 \xrightarrow[\text{Fe catalyst}]{} O_2 + OH^{\bullet} + OH^{-}$$

Spin trapping experiments have confirmed that hydroxyl radicals are produced in the livers of experimental animals after administration of iron[15, 16].

The hydroxyl radical is extremely reactive and can attack many cell constituents including lipids, proteins and nucleic acids[14]. Because the polyunsaturated fatty acids of membrane phospholipids are particularly susceptible to oxidative attack, the process of lipid peroxidation has been widely studied after iron overload.

Iron causes lipid peroxidation and oxidant stress in vivo

The process of lipid peroxidation is shown in schematic form in Fig. 21.1. After initiation of lipid peroxidation by hydroxyl radicals or another radical species, a number of products are produced, including conjugated dienes, lipid hydroperoxides and a large number of lipid breakdown products[14]. The latter include the reactive aldehydes malondialdehyde (MDA) and 4-hydroxynonenal (HNE). MDA and HNE can react with proteins and the resulting adducts can serve as markers of lipid peroxidation.

Substantial evidence has now been accumulated that demonstrates that iron overload in experimental animals can result in hepatic lipid peroxidation in vivo (Table 21.1). A number of lipid peroxidation products have been detected in the liver including conjugated dienes, thiobarbituric acid-reactants (TBA-reactants), HNE, fluorescent products, and protein adducts of MDA. High concentrations of iron in the liver seem to be required to produce lipid peroxidation[17–19], suggesting that the normal storage pathways must be overwhelmed before iron is available to catalyze oxyradical production.

Although oxyradicals have the potential to cause damage to lipids, proteins, carbohydrates and DNA, cells contain cytoprotective mechanisms (antioxidants, scavenging enzymes, repair processes) whose action counteracts the effects of oxyradical production[14]. Thus, the net effect of oxyradicals on cellular function depends on the balance between radical production and these cytoprotective systems. Vitamin E (α-tocopherol) is an important lipid-soluble antioxidant, that acts to quench lipid peroxidation; in doing so, vitamin E is oxidized[20]. Although water-soluble, ascorbate also plays an important role in the defense against lipid peroxidation through the regeneration of vitamin E from oxidized vitamin E[20]. Levels of both of these critical antioxidants can be decreased under prooxidant conditions. In experimental iron overload, hepatic and plasma vitamin E concentrations are low[21, 22], and plasma ascorbate concentrations are also decreased[21].

Although there is substantial evidence in experimental animals that iron overload can induce oxidative stress and depletion of antioxidants, there are few data that address this issue in patients with hemochromatosis. Young et al.[23] reported that the mean plasma level of TBA-reactants was increased in a group of 15 patients with hemochromatosis, compared to the mean level in 15 matched healthy controls. Concurrent measurement of the plasma concentrations of vitamin E and ascorbate revealed that these were decreased in the patients with hemochromatosis. However, the iron studies on these patients indicate that,

Table 21.1. Hepatic lipid peroxidation in experimental iron overload

Observations	References
Conjugated dienes	17, 18, 40, 42, 48, 50, 80
TBA-reactants	39, 46, 81–86
HNE	87
MDA-protein adducts	46, 85, 88
Fluorescent products	85, 88, 89

on the average, they were not iron depleted at the time of antioxidant determination. This point is emphasized by the finding of von Herbay et al.[24] that plasma vitamin E concentrations were decreased in a subgroup of patients with hemochromatosis designated as having 'high hepatic iron overload' on the basis of serum iron concentrations (> 180 μg/dl), transferrin saturation values (>50%) and plasma ferritin concentrations (>450 μg/l), but not in those with less severe iron loading. These results suggest that lowering the levels of iron is, in itself, associated with beneficial effects on the antioxidant profile, lending further support to the hypothesis that oxidant stress is involved in the pathophysiology of iron overload. In neither study were analyses of hepatic tissue performed. Therefore, it cannot be ascertained if changes in hepatic lipid peroxidation and vitamin E levels mimic those measured in plasma, but this seems likely based on the findings in experimental animals. Niemela et al.[25] and Houglum et al.[26] have provided direct evidence of hepatic lipid peroxidation in patients with hemochromatosis by detecting increased amounts of MDA-protein adducts and HNE-protein adducts in liver biopsies. After phlebotomy therapy, the levels of these adducts in the liver were significantly reduced[26], indicating that their presence was dependent on elevated hepatic iron levels.

Taken together with the results in experimental animals, these studies in patients with hemochromatosis support the concept that iron overload can induce oxidant stress when the body iron stores are substantially elevated. A question for further study is whether administration of supplemental vitamin E will confer added benefit to conventional phlebotomy therapy for hemochromatosis.

Lysosomal injury hypothesis

Accumulation of intracellular iron in lysosomes is commonly seen in patients with iron overload[27]. It is generally considered that sequestration of iron within lysosomes serves a protective role by removing this redox-active metal from other cytoplasmic components and by providing a route for iron removal from the liver through lysosome-mediated biliary excretion[28]. The lysosomal injury hypothesis proposes that excessive accumulation of iron within lysosomes can lead to lysosomal fragility, impaired lysosomal function, and eventual cellular injury through the release of hydrolytic enzymes and stored iron into the cytoplasm.

When examined at an ultrastructural level, iron-loaded cells have abundant and enlarged lysosomes which contain iron stored in the form of ferritin and hemosiderin[27]. Peters and coworkers[29–32] have proposed that iron overload results in an acquired lysosomal storage disease, wherein iron accumulation in lysosomes increases their fragility, with resultant cell damage. The activities of several lysosomal enzymes, including *N*-acetyl-β-glucosaminidase (NAGA) and acid phosphatase, are increased in hepatic biopsies of patients with iron overload[29, 30]. These increases may result from lysosomal proliferation and distension[32, 33]. When lysosomal fragility was determined in homogenates of these hepatic biopsy specimens, there was a reduction in latent (membrane-enclosed) NAGA activity, without a change in latent acid phosphatase activity[29, 30]. This suggests that there may be two populations of lysosomes in iron-overloaded livers: one population, rich in NAGA, that becomes more fragile, and the other, rich in acid phosphatase, that is relatively unaffected[30]. In patients with hemochromatosis who had their excess iron stores depleted by phlebotomy, the NAGA latency returned to normal, indicating that the increase in lysosomal fragility was iron-dependent[30]. Although it is not known if the lysosomal fragility observed in vitro reflects the situation in vivo, evidence to support this possibility comes from a study in thalassemic patients in which elevated serum NAGA concentrations were positively correlated with the degree of iron overload (as assessed by serum ferritin concentrations)[34]. Stål et al.[33] have performed ultrastructural studies on hepatic biopsy specimens from patients with hemochromatosis, and have observed that the lysosomal volume density in hepatocytes was increased and was correlated with the degree of iron overload. In this study, lysosomal volume density became normal after iron removal by phlebotomy.

One mechanism that may contribute to the increased lysosomal fragility found in iron overload is lipid peroxidation[32, 35]. O'Connell et al.[36] have shown that ferritin iron, and especially hemosiderin iron, are increased in hepatic biopsy specimens from patients with iron overload (approximately 10- and 100-fold, respectively). However, both ferritin and hemosiderin can stimulate lipid

peroxidation in vitro at acidic pH without a reductant[35]. Because lysosomes are an acidic organelle compartment, it is possible that intralysosomal lipid peroxidation initiated by ferritin or hemosiderin occurs in iron overload.

Hemosiderin is approximately 20% as potent as ferritin in stimulating lipid peroxidation in vitro, expressed per unit iron content. On this basis, it has been suggested that conversion of ferritin to hemosiderin may have a relative cytoprotective effect by reducing the ability of iron to stimulate peroxidation[36]. However, because the total amount of hemosiderin increases disproportionately in iron overload, the potential role in toxicity cannot be excluded[32].

Studies in animals with experimental iron overload have also demonstrated an increase in hepatic lysosomal fragility[32, 37–39]. In rats fed a diet containing carbonyl iron, a decrease in latent NAGA activity was seen in homogenates of liver when the hepatic iron concentration was greater than approximately 8000 μg Fe/g dry weight, and the degree of lysosomal fragility correlated with the hepatic iron concentration[38]. Iron overload in this model also increased the activity of the lysosomal enzymes acid phosphatase[40] and NAGA[38, 40] in the liver, similar to the changes seen in hepatic biopsy specimens from patients with iron overload[30]. Biliary iron excretion is increased in iron-loaded rats[38, 41], and it has been proposed that lysosomes serve as key intermediaries in this excretory pathway[28, 38].

Myers et al.[39] have performed a detailed study examining the structure, physicochemical properties, and pH of hepatic lysosomes in rats with dietary iron overload. Lysosomal iron content was increased dramatically, and the lysosomes were enlarged, misshapen, and more fragile. Lysosomal membranes from the iron-loaded group had evidence of increased lipid peroxidation (TBA-reactants), and their fluidity was decreased. The pH of lysosomes within hepatocytes isolated from the livers of iron-loaded rats was increased. The authors concluded that iron-catalyzed peroxidation of lysosomal membrane lipids is likely the mechanism of these structural, physicochemical and functional disturbances.

Although lysosomal changes have been observed in patients with hemochromatosis and in animals with iron overload, it remains to be determined if fragile lysosomes release their contents into the cytoplasm in vivo with resultant cellular injury.

Cellular organelle dysfunction in iron overload

In addition to lysosomes, iron-induced oxyradicals may damage hepatic mitochondria, the endoplasmic reticulum, and the plasma membrane.

Table 21.2. Effect of iron overload in vivo on hepatic mitochondria

Observations	References
Lipid peroxidation	17, 18, 40, 42, 48
Impaired oxidative metabolism	40, 42, 43, 48
Decreased cytochrome C oxidase	43
Decreased Ca^{2+} sequestration	47
Increased Ca^{2+} release	48

Mitochondria

The effects of iron overload on hepatic mitochondria in experimental animals is summarized in Table 21.2. Mitochondrial oxidative metabolism is susceptible to iron-induced impairment. In normal mitochondria, electron transport is tightly coupled to the phosphorylation of ADP; thus, in the absence of ADP (state 4), the rate of oxygen consumption is low, while with ADP present (state 3), the rate of oxygen consumption is increased. Normal mitochondria, therefore, have a high respiratory control ratio (calculated by dividing the state 3 respiratory rate by the state 4 respiratory rate). Hepatic mitochondria from iron-loaded rats show significant decreases in the state 3 respiratory rate and in the respiratory control ratio at moderate degrees of hepatic iron overload[40, 42, 43]. At hepatic iron concentrations at which there were decreases in oxidative metabolism, there was also evidence of conjugated diene formation indicative of mitochondrial lipid peroxidation. These studies suggest that chronic hepatic iron overload in vivo results in an inhibitory defect in the mitochondrial electron transport chain. Similar defects in oxidation are produced in vitro by iron-induced peroxidation of normal mitochondria[44].

The effects of iron overload on mitochondrial respiration are caused primarily by a decrease in cytochrome C oxidase activity[43]. Cytochrome C oxidase is functionally dependent on intact cardiolipin, a mitochondrial phospholipid which contains a high percentage of polyunsaturated fatty acids. Thus, it is likely that lipid peroxidation damages cardiolipin that may impair cytochrome C oxidase activity. Alternatively, direct peroxidative injury to the protein subunits of cytochrome C oxidase might occur concomitantly with mitochondrial lipid peroxidation in iron overload. The impairment in mitochondrial oxidative capacity caused by iron overload could have a deleterious effect on the energy state of the liver. Consistent with this possibility, chronic dietary iron overload in rats results in a substantial decrease in hepatic ATP concentrations[43, 45, 46].

This decrease in hepatic ATP levels may disturb hepatocellular functions and compromise cellular integrity.

Chronic iron overload in rats also results in an impairment in calcium sequestration by hepatic mitochondria[47], that occurs in parallel with a decrease in substrate oxidation. Because mitochondrial calcium sequestration depends on the maintenance of the mitochondrial transmembrane potential, and because a reduction in substrate oxidation will decrease the transmembrane potential, it is likely that the defect in substrate oxidation is at least partly responsible for the altered mitochondrial calcium sequestration. Masini et al.[48] have reported an increase in calcium release from hepatic mitochondria isolated from rats with chronic iron overload. This suggests that an increase in mitochondrial permeability may also play a role in decreasing calcium sequestration. Impairment of mitochondrial calcium sequestration alone may have little effect on the cytosolic free calcium concentration, because mitochondrial calcium uptake is low at normal cytosolic calcium concentrations[49]. However, because mitochondria can accumulate large amounts of calcium when cytosolic calcium concentrations are elevated, a defect in sequestration might potentiate any increase in the cytosolic free calcium concentration resulting from other perturbations in calcium homeostasis. Additional studies to investigate hepatic mitochondrial function in patients with hemochromatosis are needed.

Endoplasmic reticulum

Hepatic microsomal lipid peroxidation in vivo was found in rats with chronic dietary iron overload, when the hepatic iron content exceeded a threshold level[50]. In addition, decreased activities of microsomal cytochrome P-450, aminopyrine demethylase activity, and cytochrome b_5 were associated with microsomal lipid peroxidation in this model[50]. Although this does not establish causality, it seems likely that the loss of these cytochromes was the result of peroxidative damage. Iron-induced peroxidation in hepatic microsomes in vitro causes a reduction in cytochrome P-450 levels, probably due to the destruction of heme in cytochrome P-450[51, 52]. Another microsomal function sensitive to the damaging effects of peroxidation is calcium sequestration[53]. Chronic dietary iron overload in rats resulted in impairment in hepatic microsomal calcium sequestration[47], and this may alter hepatocellular calcium homeostasis and contribute to cell injury.

Bonkovsky et al.[54] have measured antipyrine clearance and hepatic cytochrome P-450 concentrations in patients with various types of iron overload. Although the number of patients studied was small and some variation existed between the groups, no differences were found between iron-loaded patients and controls.

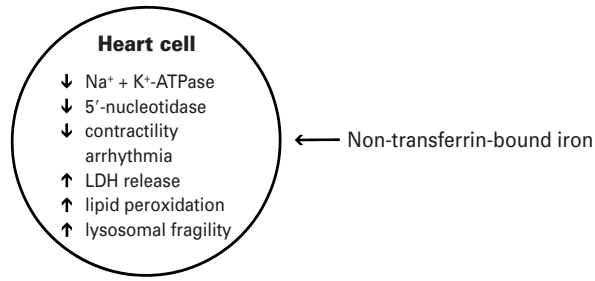

Fig. 21.2. Effects of non-transferrin-bound iron on cultured cardiac myocytes[55–57].

Plasma membrane

Hershko and co-workers have studied the toxicity of non-transferrin-bound iron in rat myocardial cells in culture[55–57]. Exposure of cardiac myocytes to ferric ammonium citrate resulted in lipid peroxidation, decreased contractility, and arrhythmias (Fig. 21.2). These investigators have identified thiol-rich enzymes (such as $Na^+ + K^+$-ATPase and 5′-nucleotidase) in the plasma membrane as possible primary targets of iron toxicity, and suggested that oxidative modification of these enzymes may impair their activity[56, 57]. Because $Na^+ + K^+$-ATPase plays a key role in maintaining the cell membrane potential, a decrease in its activity may contribute to the functional deficiencies observed[56, 57]. Iron exposure also increased lysosomal fragility in these cardiac myocytes, indicating that lysosomal damage may also play a role in myocardial injury caused by iron overload[55].

There are few data concerning the effects of iron overload on liver plasma membranes. Pietrangelo et al.[58] demonstrated a decrease in the content of unsaturated fatty acids in the phospholipids of the plasma membrane fraction in rats with mild iron overload. A similar change was also seen in hepatic mitochondrial membranes, and these changes in membrane composition are consistent with the occurrence of lipid peroxidation[58].

Iron can cause DNA damage in vivo

Patients with long-standing hemochromatosis with hepatic cirrhosis have an increased risk for developing hepatocellular carcinoma[59, 60]. Accordingly, it has been proposed that hepatocellular carcinoma in these patients may be a consequence of iron-induced oxidative damage

to hepatic DNA, combined with the replicative stimulus provided by cirrhotic nodular regeneration. Iron salts produce DNA strand breaks when incubated in vitro with either purified DNA[61, 62] or with isolated rat liver mitochondria[63] or nuclei[64]. In addition, endogenous iron plays a key role in the DNA damage produced by hydrogen peroxide in mammalian cells, because pre-treatment with iron-chelating agents diminishes this damage[65–67]. It is thought that some iron is bound to DNA in vivo and that this iron, in the presence of superoxide and hydrogen peroxide, can catalyze the formation of 'site-directed' hydroxyl radicals that cause DNA damage[67]. Lipid peroxidation products in the presence of iron can also produce DNA damage in vitro through a mechanism that also may involve the hydroxyl radical[68].

The first in vivo evidence that chronic iron overload results in damage to hepatic DNA was provided by a study that showed an increased number of strand breaks in hepatic DNA from iron-loaded rats[69]. The induction of strand breakage in DNA has been associated with initiation and promotion events in chemically-induced carcinogenesis[70, 71]. Increased oxidative damage to hepatic DNA occurs in *Ah*-responsive mice after a single subcutaneous injection of iron-dextran[72]. Oxidative DNA damage was evaluated using 8-hydroxydeoxyguanosine as a marker. DNA templates containing 8-hydroxydeoxyguanosine are misread at the modified base and at adjacent residues[73], and this could result in oxidative mutagenesis[74].

Iron can also cause the transformation of cells. Repeated intraperitoneal injections of rats with the iron chelate ferric nitrilotriacetate (FeNTA) resulted in renal adenocarcinoma with metastases to liver, lung, and peritoneum[9, 10]. Administration of FeNTA increased the amount of 8-hydroxydeoxyguanosine in renal DNA, suggesting that oxidative damage to DNA may play a role in FeNTA-induced renal carcinogenesis[9, 10, 75]. Vitamin E supplementation inhibited FeNTA-induced renal lipid peroxidation, apoptosis, 8-hydroxydeoxyguanosine formation, and the development of cancer[76]. Exposure of cultured rat liver epithelial cells to a high concentration of FeNTA resulted in the appearance of some cells that show morphological transformation, grow in soft agar, and induce metastatic carcinomas when injected into newborn rats[77]. The authors suggested that oxidative damage to DNA catalyzed by FeNTA may play a key role in the rapid neoplastic transformation of these cells. In addition, iron can act as a promoter of already initiated hepatocytes in the development of hepatic cancer[78].

Little information is available concerning DNA damage in patients with iron overload. Carmichael et al.[79] examined hepatic DNA samples from six patients with hemochromatosis, and 'bulky' DNA lesions were detected that contained from 2 to 50 base modifications per 100 million nucleotides. Unexpectedly, the levels of 8-hydroxydeoxyguanosine were not elevated in these samples, suggesting that these 'bulky' DNA lesions may be a more sensitive indicator of iron-induced DNA damage.

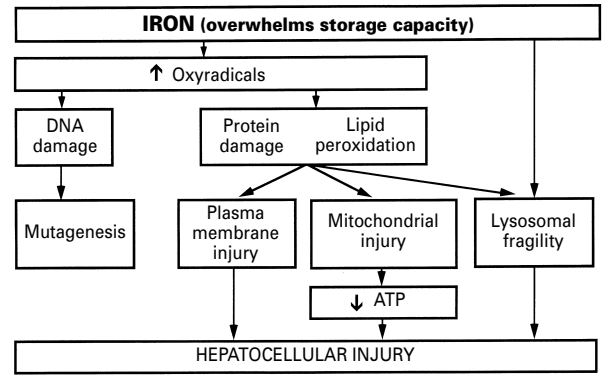

Fig. 21.3. Mechanisms of toxicity induced by iron overload.

Therefore, there is evidence that iron can produce oxidative injury to DNA in experimental animals. This damage might be an important step leading to carcinogenesis, especially when coupled with a proliferative stimulus. Although damage has been detected in hepatic DNA from patients with hemochromatosis, it is not known whether this is the result of an oxidative process.

Conclusions

Several proposed mechanisms of iron-induced toxicity are summarized in Fig. 21.3. It is suspected that free radicals play a role in iron-induced cell toxicity because iron salts have a powerful pro-oxidant action in vitro. In the presence of available cellular reductants, iron in low-molecular-weight forms may play a catalytic role in the initiation of free radical reactions. The resulting oxyradicals have the potential to damage cellular lipids, nucleic acids, proteins and carbohydrates, resulting in wide-ranging impairment in cellular function and integrity. However, cells are endowed with cytoprotective mechanisms (e.g., antioxidants, scavenging enzymes, repair processes) that counteract the effects of free radical production. Thus, the net effect of iron-induced free radicals on cellular function depends on the balance between radical production and cytoprotective systems. As a result, the rate of free radical production must overwhelm the cytoprotective defenses before cellular injury occurs.

Evidence has now accumulated that iron overload in

experimental animals can result in oxidative damage to lipids in vivo, once the concentration of iron exceeds a threshold level. In the liver, lipid peroxidation is associated with impairment of membrane-dependent functions of mitochondria (oxidative metabolism) and lysosomes (membrane integrity, fluidity, pH). Although these findings do not prove causality, it seems likely that lipid peroxidation is involved, because similar functional defects are produced by iron-induced lipid peroxidation in these organelles in vitro. Iron overload impairs hepatic mitochondrial respiration primarily through a decrease in cytochrome C oxidase activity. In iron overload, hepatocellular calcium homeostasis may be impaired through damage to mitochondrial and microsomal calcium sequestration. DNA has also been reported to be a target of iron-induced damage, and this may be related to malignant transformation. Enzymes in the plasma membrane such as $Na^+ + K^+$-ATPase may be key targets of damage by non-transferrin-bound iron in cardiac myocytes. Levels of some antioxidants in the liver are decreased in rats with iron overload suggesting on-going oxidative stress. Reduced cellular ATP levels, lysosomal fragility, impaired cellular calcium homeostasis, and damage to DNA may contribute to hepatocellular injury in iron overload.

There are few data regarding free radical production in patients with iron overload. Patients with hemochromatosis have elevated plasma levels of TBA-reactants and increased hepatic levels of MDA-protein and HNE-protein adducts, indicating the occurrence of lipid peroxidation. Additional investigation will be required to assess oxidant stress and its potential pathophysiologic role in patients with hemochromatosis.

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22

Iron as a carcinogen

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Introduction

Data from human and in vivo and in vitro experimental studies strongly support a role of iron in initiation and promotion of carcinogenesis. This chapter reviews these data and discusses the putative mechanisms whereby iron may act as a carcinogen.

Evidence that iron is involved in carcinogenesis

Human data

Studies performed in the general population and in selected non-hemochromatosis groups suggest that elevation of body iron stores is associated with a moderately increased risk of cancer. Otherwise, a high risk of primary hepatic cancer, especially hepatocellular carcinoma, is well-documented in hemochromatosis patients. Large cohort studies[1–6] have been performed to test for an association between abnormalities of serum iron tests and risks of cancer occurrence and cancer-related mortality (Table 22.1). All but one[5] have concluded that there is a slight to moderate increase in these risks in persons with high serum transferrin saturation or total serum iron-binding capacity. Thus, elevation of body iron stores is associated with increased risks of cancer occurrence and death.

However, these results and conclusions must be interpreted with caution due to the unreliability of serum iron tests in reflecting body iron stores accurately[7]. Day-to-day variations of serum iron concentration, total iron-binding capacity and transferrin saturation were not taken into account; all studies relied on a single determination of serum iron test(s). In addition, confounding factors leading to a decrease or an increase in the values of serum iron tests were not always sought. Important causes of disturbance of serum iron test values without modification in total body iron stores, such as inflammation, bleeding, alcoholism, and hepatic disease were not considered systematically when results were interpreted, introducing further uncertainty about the conclusions of the studies.

Case-control studies[8–13] reveal an association between high serum ferritin concentrations and cancer death or development of cancer, especially primary hepatic carcinoma[8, 9, 12] and colonic adenoma[10, 11, 13]. However, even the highest increases of relative risks were slight. In addition, the same concerns regarding validity of the conclusions are present as those in large cohort studies.

Determining whether blood donors are at reduced risk of cancer could help to answer the question of an association between body iron stores and cancer risk. The risk of developing cancer (all sites combined) was significantly lower in blood donors than in non-donor control subjects[14]. These findings might be consistent with a protective effect of blood donation that lowers body iron stores. Such an effect might have been particularly apparent in Sweden, the general population of which consumes iron-fortified foods. However, donor selection and a lifestyle that may be more healthy in donors could also explain the lower incidence of cancer in Swedish blood donors. It has been suggested that blood transfusion of patients with cancer undergoing surgery may increase the risk of cancer recurrence due to additional iron loading[15]. In addition, several case-control studies indicate that people exposed to iron dust are at increased risk to develop cancers. This was demonstrated for lung carcinoma in iron ore miners[16] and for renal cell carcinoma in workers in iron and steel metallurgy and in persons exposed to asbestos[17].

A relationship between iron and hepatocellular carcinoma is suggested by the more frequent demonstration of hyperplastic hepatocellular foci that are more likely to be pre-malignant lesions in iron-positive (73%) than in

Table 22.1. Serum iron tests[a] and cancer risk in general population and in patients with chronic hepatic disease

References	Population studied	Iron tests[a]	Results
	General population		
USA[1]	174 507 subjects followed for 8.3 y (2.1–16 y)	TIBC,	TIBC inversely related to risk of lung cancer in women [highest vs lowest quartile: RR: 0.41, 95% CI: 0.23–0.73]
	F: 94 500; M: 80 007	Iron use	Women with recent iron use had a lower risk of lung cancer than those without [RR: 0.60–95% CI: 0.37–0.97]
USA[2]	14 407 subjects followed for >6 y; Age: 24–75 y	TIBC, TS	Low TIBC and high TS associated risk of cancer in men (RR up to 1.37, 95% CI: 0.94–2.01)
	F: 7858F; M: 5434	Iron intake	No association between iron intake and cancer development
Finland[3]	41 276 subjects followed for 14 years. Age 20–74 y	SI, TS, TIBC	TS >60% associated with increased risks of: all sites (RR: 1.43 95% CI: 1.16–1.77)
	F: 22 463; M: 18 813		colon and rectum (RR: 3.04, 95% CI: 1.64–5.62).
USA[4]	8556 subjects followed for > 12 y. Age: 24–75 y	SI, TS, TIBC	High TS associated with increased risks of: cancer occurrence (RR up to 1.81, 95% CI: 1.21–2.71) in both sexes and mortality (RR up to 1.73, 95% CI: 1.03–2.91) in both sexes
	F: 5269F; M: 3287		
USA[5]	38 538 subjects followed for 18 y. Age: 20–84 y	TS	No relationship between TS and risk of epithelial cancer (except for gastric cancer in females)
	F: 28 150; M: 10 388		
The Netherlands[6]	260 subjects followed for 17 y. Age > 65 y.	TIBC, TS	High TIBC associated with low cancer mortality in women only (RR as low as 0.05, 95% CI: 0.007–0.39)
	F: 131; M: 129		
	Patients with chronic liver disease		
Korea[21]	249 patients with chronic liver disease followed 2–17 y. Age: 28–66 y.	SF	Consistently elevated SF levels associated with the development of primary hepatic cancer, especially in men
	F: 69; M: 180		

Note:

[a] TIBC = total iron binding capacity; TS = transferrin saturation; SI = serum iron concentration; SF = serum ferritin concentration.

iron-negative (21%) macroregenerative hepatic nodules[18, 19]. In addition, the relationship of hepatic iron content to age was higher in patients with hepatic cirrhosis and hepatocellular carcinoma than in matched controls with uncomplicated cirrhosis[20]. In a case-control study, Turlin et al.[20] assessed the ratio of hepatic iron concentration to age in patients who developed hepatocellular carcinoma in the absence of cirrhosis, and compared this to the ratio observed in matched non-cirrhotic control subjects who had no hepatocellular carcinoma or who had hepatic metastases (Table 22.2). Patients with hepatocellular carcinoma had higher ratios than controls. Because hepatic iron concentration reflects body iron stores more reliably than serum iron tests[7], this study strengthened the hypothesis formulated by Hann et al.[21] of an association between intrahepatic iron accumulation and the development of hepatocellular carcinoma in non-hemochromatosis patients. Whether *HFE* gene mutations are involved in such cases remains to be determined.

In a study of 162 male patients with chronic hepatic disease related to hepatitis B virus (HBV) infection, patients who had serum ferritin concentrations >300 ng/ml had a 50% chance of developing hepatocellular carcinoma during the follow-up period compared with a 20% risk for patients with lower serum ferritin concentrations (Table 22.1). However, confounding factors such as disease activity, chronic alcoholism, and inflammation[7] that modify serum ferritin concentration irrespective of body iron stores were not taken into account[21]. The prevalence of hepatocellular carcinoma in patients with porphyria cutanea tarda (PCT) varies from 7% to 16% in case-control and follow-up studies[22]. Because hepatic iron overload frequently occurs in PCT patients[23] and the prevalence of the C282Y *HFE* mutation in sporadic PCT is high[24], there may be a role of iron in the development of hepatocellular carcinoma in PCT.

Several autopsy[25], clinical, and epidemiological studies[26–31] have demonstrated the high incidence of primary hepatic cancer, mainly hepatocellular carcinoma, in patients with hemochromatosis (Table 22.3). Primary hepatic cancer accounts for 6.7–14% of deaths in early hemochromatosis series and 27.5%[31], 30%[28], 36.4%[27] and 45%[30] of deaths, respectively, in more recent studies. The relative risk for the development of primary hepatic cancer in hemochromatosis patients with cirrhosis is greater than 200-fold[27, 28]. Iron per se could be involved in the development of hepatocellular carcinoma as suggested by (i) the demonstration that, in hemochromatosis patients, the risk of developing hepatocellular carcinoma is associated with cirrhosis and the amount and duration of iron overload[28, 31, 32]; and (ii) the report of at least six cases of hepatocellular carcinoma that developed in non-cirrhotic but fibrotic hemochromatosis livers[33–35]. That hepatocellular carcinoma risk persists in iron-depleted hemochromatosis patients[28, 31, 32, 35] and is not higher in hemochromatosis-associated cirrhosis than in cirrhosis of other causes[30] does not exclude a carcinogenic role of iron, regardless of its involvement in the development of cirrhosis. Indeed, increased serum levels of non-transferrin-bound iron that is carcinogenic through the production of reactive oxygen species and free radicals may be found (i) in hemochromatosis patients insufficiently treated, i.e., with persistently elevated transferrin saturation values despite low serum ferritin concentrations, and (ii) in cases of hepatic failure, whatever the cause of the hepatic disease[36].

The studies by Amman et al.[37] and Bomford and Williams[26] suggest that hemochromatosis patients are prone to develop extrahepatic cancers. However, these results have not been confirmed by larger cohort studies. Bradbear et al.[27] found no increase in extra-hepatic malignancy in a 7.9-year follow-up study of 208 patients with well-defined hemochromatosis. German[28], Italian[30], and Canadian[38] cohort studies arrived at the same negative result.

In a large study, Nelson et al.[39] found that heterozygosity for hemochromatosis was associated with increased risk for colonic adenoma (in both sexes), colorectal cancer (in males), hematologic malignancy (in males) and gastric cancer (in females) (Table 22.3). Relative risks, however, were relatively low. Only a small proportion of subjects heterozygous for hemochromatosis genes have increased mean values of serum iron tests[40] and a few have slightly elevated hepatic iron stores[41]. Therefore, most hemochromatosis heterozygotes do not have or develop iron overload. However, this does not exclude the putative association between iron and cancer in a subgroup of iron-overloaded heterozygotes, especially those with the polymetabolic iron overload syndrome[42, 43].

Experimental data

In vivo and in vitro studies suggest that iron, especially non-transferrin-bound iron, may be responsible for initiation and promotion of carcinogenesis. Lemurs[44] and birds[45] sometimes develop spontaneous hepatic iron overload and hepatocellular carcinoma. Copper-related chronic hepatic disease in the Long–Evans Cinnamon rat, an animal model for Wilson disease, is frequently complicated by hepatocellular carcinoma after the rats have recovered from initial fulminant hepatitis and subsequently develop fibrosis[46]. This may be related to progressive iron accumulation in these animals[47], because hepatic iron depletion prevents their development of hepatic

Table 22.2. Case-control studies on iron tests[a] and iron intake, and cancer risk

References	Cancer patients and controls	Iron tests[a]	Results
Solomon Islands[8]	105 cancer patients in 2500 subjects followed for 8–12 y Controls matched for sex, hepatitis B status and age	ST, SF	Low ST and high SF associated with increased cancer-related mortality
China[9]	192 cancer male patients in 21 513 males followed for 8 y	TS, SF	High SF associated with death from or development of primary hepatic cancer (RR: 1.41, 95% CI: 1–1.99)
	Controls matched for age and serum HBsAg status		High TS and SF associated with death from all cancers except primary hepatic cancer (RR: 2.88, 95% CI: 1.18–7.03)
USA[10]	145 patients with colonic adenoma and 29 with colon cancer Controls: 159 subjects without neoplastic colonic disease	SF	High SF independently associated with colon adenoma (RR up to 4.3, 95% CI: 2–0.1)
France[20]	hepatocellular carcinomas in cirrhotic($n=24$) or non-cirrhotic ($n=24$) liver Controls matched for age and alcohol consumption	Hepatic iron	Higher hepatic iron concentration to age ratio in patients with hepatocellular carcinoma developed in either cirrhotic or non-cirrhotic liver
USA[11]	467 patients with colorectal polyps Controls matched for sex, age, date and place of endoscopy	SF	SF >289 μg/l associated with increased polyp risk compared to SF <141 μg/l [RR 1.5, 95% CI: 1–2.3]
		Iron intake	Weak U-shaped relation between iron intake and polyps
South Africa[12]	15 patients with hepatocellular carcinoma (M 12, F 3) in 604 autopsy cases	Hepatic iron	Highest hepatic iron grade associated with death from hepatocellular carcinoma [RR: 23.5, 95% CI: 2.1–225]
USA[13]	236 patients with colorectal polyp(s) or cancer ($n=27$) 409 controls without colorectal neoplasia	Iron intake	In women, iron intake was inversely related to the risk of adenoma

Note:

[a] TIBC = total iron binding capacity; TS = transferrin saturation; SI = serum iron concentration; SF = serum ferritin concentration.

Table 22.3. Primary hepatic cancer in hemochromatosis

References	Population	Results
United Kingdom[29]	71 iron-depleted patients (69 M, 2 F; 25–70 y)	13 patients (18%) with cirrhosis developed hepatocellular carcinoma
	Follow-up 1–12 y	6 patients (8.4%) developed extra hepatic malignancies
Australia[27]	208 patients (171 M, 37 F; 16–81 y)	200-fold excess risk of hepatocellular carcinoma compared to general population
	Mean follow-up 7.9 y (0–33 y)	No increased risk of extrahepatic malignancy
Canada[38]	93 patients (59 M, 34 F) 30 with cirrhosis	7 patients with cirrhosis developed hepatocellular carcinoma during follow-up
	Mean follow-up 7.7 (0–33 y)	RR of hepatocellular carcinoma-related death = 350
Italy[36]	212 patients (181M, 31F; 50 ± 11 y)	Annual incidence of hepatocellular carcinoma after 6 y of follow-up: 3.48% (95% CI: 1.36–5.6)
	Mean follow-up 44 months (3–218 mo)	HCC risk reltaed to cirrhosis and co-infection with hepatitis B virus
Germany[28]	251 patients (224 M, 27 F; 46 ± 11 y)	Increased risk of hepatocellular carcinoma-related death (RR: 110.8; 95% CI: 11.4–28.4)
	Mean follow-up 14 ± 7 y	HCC risk related to cirrhosis, amount and duration of iron overload
	Homozygotes – (2) Case-control studies	
Italy[32]	28 patients with HCC	All hepatocellular carcinoma patients had cirrhosis
	Controls: 124 HCC-free patients (of whom 69 cirrhotics)	Factors associated with hepatocellular carcinoma development: cirrhosis, age > 55 y, chronic alcoholism, HBV infection
France[90]	54 patients with primary hepatic cancer (50M, 4F; 63 ± 8 y)	3 hepatocellular carcinoma patients did not have cirrhosis
	Controls matched for age, sex, follow-up duration and iron excess	Factors associated with hepatocellular carcinoma development: cirrhosis, chronic alcoholism, transfusion and tobacco smoking
	Heterozygotes – Follow-up study	
USA[39]	1950 hemochromatosis heterozygotes	Association with colorectal neoplasia and hematologic malignancy in both sexes (+ gastric cancer in females)
	Controls: 1656 unexposed relatives	No association with cancer death or cancers of the lung, breast and cervix

Note:
Controls = general population.

cancer[48]. The development of hepatocellular carcinoma also occurs in β_2-microglobulin knockout mice, an animal model of parenchymal iron overload[49].

Long-term dietary administration of carbonyl iron is believed to induce hepatic or extrahepatic tumors[50]. Moreover, in the same model, *c-myc, c-fos* and *c-Ha-ras* gene expression was not modified[51]. However, chronic iron overload in rats induces oval cell proliferation[52]. Hepatocytic iron-free foci and nuclear changes, including elevated mitotic index and presence of abnormal mitotic figures, have been induced in male BALB/cJ mice whose diets were supplemented with carbonyl iron[53].

Repeated intramuscular[54] or intraperitoneal[55–58] injections of iron compounds (iron dextran[54], ferric nitrilotriacetate[55, 56], ferric saccharate[57], and ferric ethylenediamine-*N*-*N*´-diacetate[58]) induce skin sarcomas[54] and mesotheliomas[57] at the site of injection, and also induce renal cell carcinomas[55, 56, 58]. In contrast, many authors[59] have performed parenteral iron overloading in rats, mice, gerbils, dogs, and primates, but could not induce hepatocellular carcinoma development, even when they succeeded in causing significant hepatic fibrosis[60] and/or nuclear hepatocyte abnormalities[61].

Numerous in vivo studies have demonstrated that tumor cell growth is enhanced by iron supplementation and inhibited by iron deprivation caused by low oral iron intake or chelating agents[15, 62]. This has been consistently shown in various animals, especially rats[63] and mice[64–66] that have a large variety of tumors including colorectal[63] and mammary[67, 68] carcinomas. However, this remains debated with respect to primary hepatic cancer. Indeed, in contrast to the study of Smith et al.[69] who demonstrated that chemically induced hepatic neoplasia in the rat was enhanced by iron, data from Ståal et al.[50] did not support the role of dietary iron supplementation in either initiation or promotion of chemical hepatocarcinogenesis.

Mechanisms whereby iron may be involved in carcinogenesis

Iron may act as a carcinogen through induction of oxidative stress, facilitation of tumor growth, and modification of immune system equilibrium.

Induction of oxidative stress

Iron is the most abundant transition metal in the human body. Redox cycling of iron is closely associated with the production of reactive oxygen species (ROS) and free radicals (FR)[62, 70, 71]. ROS-FR induce tissue damage, especially through lipid peroxidation, and through modifications of protein and DNA molecules[72]. ROS-FR are produced constantly in the human body. This implies the existence of protective mechanisms that include specific and nonspecific reduction systems, protection against lipid peroxidation, sequestration of metals, and DNA repair enzymes[71]. Iron is responsible for overproduction of ROS-FR and induces modifications in protective systems against ROS-FR that are likely to contribute to initiation and promotion of cancer development[62].

Renal adenocarcinoma induced in rats and mice by repeated intraperitoneal injections of the iron-chelate of nitrilotriacetic acid (Fe-NTA) has been used extensively as a model of iron-mediated tissue damage and cancer induction[62, 71]. In that model, the involvement of lipid peroxidation as a critical step of carcinogenesis and the specific role of iron as the initiating factor of lipid peroxidation are well documented[62, 71]. Replacement of iron with a non-transitional metal results in nephrotoxicity but not carcinogenicity[56]. Administration of vitamin E, a scavenger of ROS-FR, decreases the incidence of tumors[73]. Further support for the possible involvement of ROS-FR in Fe-NTA-induced carcinogenesis includes the finding in vivo of 8-hydroxydeoxyguanosine in DNA of rats given Fe-NTA[74], and by the demonstration of an increase of oxidatively modified DNA bases after administration of Fe-NTA in vitro[74, 75]. Most of these modified bases are mutagenic[74, 75]. Iron is present within the nucleus, but whether iron can bind directly to DNA is a matter of speculation[72]. However, Loeb et al.[76] demonstrated that DNA damage and mutagenesis were induced in vitro by oxygen free radicals generated by Fe^{2+}. These effects of iron were prevented by binding of Fe^{2+} with deferoxamine, or by the addition of catalase.

No data on iron, oxidative stress, and hepatocellular carcinoma are available in humans. However, elevated levels of non-transferrin-bound iron[36] and increased lipid peroxidation[77] were demonstrated in the sera of patients with hemochromatosis. Moreover, there is evidence that enhanced lipid peroxidation in the livers of patients with hemochromatosis can be reversed by phlebotomy therapy[78].

In vivo experimental studies indicate that iron does not modify gluthatione peroxidase activity, exerts variable effects on gluthatione levels, reduces vitamin E levels, and inhibits superoxide dismutase activity[62, 70]. In contrast, the expression of gluthatione S-transferase (GST) is significantly induced by Fe-NTA in rat kidney[62]. Because GST is associated with drug resistance, this suggests that iron might have deleterious effects, even when promoting protective systems against ROS-FR. In patients with iron

overload, serum concentrations of tocopherol, retinol, and ascorbate are reduced[77].

Facilitation of tumor cell growth

Every cell needs iron for proliferation and growth. Iron is a mandatory co-factor of ribonucleotidase reductase involved in the first step of DNA synthesis[79]. Iron is supplied to the cell through the transferrin-transferrin receptor pathway. Due to a high proliferation rate, tumor cells require more iron than normal cells. Thus, neoplastic cells express a large number of transferrin receptors and, moreover, may synthesize their own transferrin or transferrin-like proteins[15]. In addition, some tumor cell lines are able to acquire and utilize non-transferrin-bound iron[80, 81]. An iron-rich environment facilitates tumor growth. DNA synthesis is increased in the presence of iron in the model of rat hepatocyte cultures stimulated by epithelial growth factor/pyruvate[82]. This effect is probably related to both cell proliferation and DNA repair of iron-induced DNA damage[82]. Growth of human hepatoma cells is enhanced by iron supplementation and inhibited by deferoxamine[83]. Deferoxamine blocks G_1 phase in human neuroblastoma cells[84] and potentiates the inhibition of human breast and colorectal cancer growth by bispecific antibodies to human transferrin receptor and tumor-associated antigens[85]. Moreover, clinical[86] and pathological[87] studies suggest that iron promotes HBV-related hepatocellular carcinoma by facilitating HBV replication and cancer cell growth. HBV is more likely to infect and replicate in hepatic cells with increased ferritin content. In addition, iron could stimulate ferritin synthesis that would increase intracellular iron and enhance the HBV replication rate. The supply of iron to transformed cells, the iron and virus contents of which are low, could be provided by surrounding non-malignant infected hepatocytes, the iron content of which is much higher. A similar observation was made in iron-overloaded rats[88] and in hemochromatosis patients[89, 90] in whom the growth of pre-neoplastic lesions such as iron-free foci could be ensured by iron delivered by surrounding iron-loaded hepatocytes.

Inhibition of the ability of neoplastic cells to assimilate and utilize iron has been used as a therapeutic process in experimental and clinical studies. Deferoxamine significantly reduced the growth rate of human-derived hepatocellular carcinoma cells injected subcutaneously into athymic nude mice[65]. Antibodies directed against the transferrin receptor, either alone, conjugated to toxins, or added to deferoxamine, inhibited tumor cell growth both in vivo and in vitro[91–93]. Parabactin and vibriobactin, two microbial siderophores of the spermidine catecholamide group, prevent cultured cancer cell lines from entering into S phase, and thus enhance the anti-neoplastic effect of doxorubicin and cytarabine that are especially active in S phase[94]. In humans, attempts have been made to treat cancer patients by iron chelation therapy[95, 96]. Local anti-transferrin receptor immunotoxins have been used in patients with various types of epithelial cancers[97]. Finally, labeled anti-ferritin antibodies can induce remissions in patients with hepatocellular carcinoma[98] or Hodgkin's disease[99] unresponsive to conventional therapy.

Modifications in the immune system

The exact role of iron-induced modifications of immune functions in carcinogenesis remains poorly understood[99]. Due to its critical role in ribonucleotidase reductase activity, transferrin-bound iron is necessary for lymphocyte activation. In the absence of transferrin-bound iron, mitogen-stimulated T-cell proliferation is inhibited[100]. In contrast, non-transferrin-bound-iron may inhibit cell proliferation in vitro, especially that of the CD4 lymphocyte subset[101]. This could explain the reduced CD4:CD8 ratio in thalassemia patients and in some series of hemochromatosis patients[102]. In addition, lymphocyte proliferation in vitro[103] and in vivo[104] is inhibited by ferritin. The tumoricidal function of mice macrophages is markedly decreased by iron salts, iron dextran, carbonyl iron, and iron-containing ferritin[105].

Conclusions

Data from human and experimental studies support the hypothesis that iron is carcinogenic. Non-transferrin-bound iron, a highly reactive form of iron especially present in iron-overload conditions, may act by triggering the production of reactive-oxygen species and free radicals that, in turn, can initiate and promote carcinogenesis. Moreover, transferrin iron, the physiological form of iron, facilitates tumor cell growth. Both forms of iron impair lymphocyte and macrophage functions. This results in two practical implications. First, manipulations of iron metabolism have therapeutic applications in cancer patients in association with conventional therapies. Second, the question of whether continuing iron supplementation of food in developed countries is justified should be studied further.

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23

Clinical spectrum of hepatic disease in hemochromatosis

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Introduction

Due to its direct connections to the intestinal tract, the liver is the first target organ of hemochromatosis[1, 2]. In this respect, it plays a key role in accumulating iron absorbed by the intestine. This storage function has two main consequences: first, it exerts a relative protective effect over other organs[3]; second, longstanding and massive iron excess can induce damage of the liver that may lead to the development of fibrosis, cirrhosis, and hepatocellular carcinoma. The clinical spectrum of hepatic disease in hemochromatosis is therefore particularly broad, ranging from silence to extreme severity. In addition, this expression can be modulated by various coexisting factors and coincidental hepatic disorders. The diagnosis of hepatic disease in persons with hemochromatosis needs a careful differential approach due to the characteristics of newly described hepatic iron overload syndromes. Finally, the response of these hepatic disorders to treatment (i.e., therapeutic phlebotomy) depends on the stage of hepatic damage.

The clinical expression of the hemochromatosis liver

The 'silent' liver

Today, this is often the status of the liver in persons with hemochromatosis. In most patients, particularly those $<$30 years of age, the liver is clinically silent. The clinical examination is negative, there is no detectable hepatomegaly, and hepatic function tests are normal. At this stage, the diagnosis of hepatic iron overload can only be made by magnetic resonance imaging (MRI)[4, 5] and hepatic biopsy[6]. However, performing a hepatic biopsy in the absence of either clinical or biochemical signs suggestive of hepatic disease is becoming less usual in patients suspected of having hemochromatosis, and is reserved for patients in whom the diagnosis of hemochromatosis cannot be established by the detection of homozygosity for the *HFE* mutation C282Y[7, 8].

Expression of hepatic disease in hemochromatosis

In patients who have hepatic disease without cirrhosis, clinical examination usually detects hepatomegaly. This remains one of the most common physical signs at presentation[9], and was present in 70% of patients without cirrhosis in the series of Niederau et al.[10]. The frequency of abdominal pain is variable. Although 39% of German hemochromatosis patients without cirrhosis and approximately 13% of Canadian men and 36% of Canadian females with hemochromatosis had abdominal pain[10, 19], its relation to the liver remains uncertain. Hepatic function test results are either normal or slightly abnormal, consisting of moderate hypertransaminasemia in approximately 49% of patients without cirrhosis. Esophageal varices are not found in hemochromatosis patients without cirrhosis[10]. Hepatic biopsy confirms the presence of major iron overload in persons with non-cirrhotic fibrosis. In addition to hepatic iron concentration, other factors favor the development of hepatic fibrosis in hemochromatosis: male sex, age, alcoholism[11], and viral infection of the liver[12].

Hepatomegaly and fibrosis may be reversible by phlebotomy therapy. In a study by Niederau et al.[10], the degree of non-cirrhotic fibrosis (stages 1 or 2; stage 3 corresponded to cirrhosis) was reduced in 42% (30/71) of patients in whom complete iron removal had been documented by repeat hepatic biopsy. Among them, 10/32

stage 1 cases had complete disappearance of fibrosis. At this pre-cirrhosis stage, life expectancy is considered to be normal[10, 13, 14]. A single study has reported that hemochromatosis patients without cirrhosis may have a five-year survival probability of 72% – a worse prognosis than persons in the general population[15]. However, the number of patients in this study was limited ($n=22$), and the authors stated that iron depletion indicated by normal serum ferritin concentrations could not be achieved in all patients. Further, the risk for the development of hepatocellular carcinoma cannot be excluded; at least six cases of hepatocellular carcinoma have developed in fibrotic (but non-cirrhotic) livers[16–18].

The cirrhotic liver

This stage corresponds to the historical description of uncontrolled hemochromatosis disease of the liver. Like men, women can develop cirrhosis, but their frequency is lower. In a recent series that compared 176 women and 176 men with hemochromatosis who were matched for year of birth, cirrhosis occurred in 21 women (13.8%) and 43 men (26%) (mean difference 11.8 percentage points; confidence interval −3.2 to 20.4 percentage points)[19]. This difference was not due to associated alcoholism. Although excessive alcohol consumption was more common in men than in women in this case series, it was not more common in men with cirrhosis than in women in cirrhosis[19].

The liver is markedly increased in overall volume, and often pseudotumoral in configuration; frequently, there is predominant enlargement of the left lobe. The liver is firm to palpation and its inferior edge is sharp. Hepatomegaly of this etiology is rarely associated with clinical signs of hepatic dysfunction such as hepatocellular insufficiency (indicated by palmar erythema, spider angiomata, and ecchymosis). In a series of Niederau et al., 13% of cirrhosis patients presented with jaundice or peripheral edema, 9% had ascites, and 8% had gynecomastia[10]. The results of hepatic function tests are usually normal or minimally abnormal. The prothrombin time and serum albumin concentration are normal in 70% of cases[10], and there is no hypergammaglobulinemia (serum concentrations of IgG, IgA, IgM are normal) or evidence of cholestasis (serum concentrations of alkaline phosphatase, 5′-nucleotidase, and γ-glutamyl transpeptidase (GGT) are usually normal). The only frequent abnormality is a moderate increase in serum transaminase activity (aspartate and alanine aminotransferases; AST and ALT, respectively) that occurs in 68% of patients[10]. These values are usually $<$ three times the upper reference limit[10]. Hypertransaminasemia can disappear after iron removal[12, 20, 21]; serum aminotransferase concentrations improved in 73% of 183 patients (including patients with and without cirrhosis)[10]. Another biochemical abnormality of hepatic origin is a mild increase in the serum concentration of CA 19.9 antigen ($\leq$ four times the upper limit of normal). This is directly correlated with the amount of hepatic iron excess, hypertransaminasemia, and the degree of fibrosis; this abnormality disappears after iron depletion therapy[21]. In practice, the unexplained finding of increased serum concentrations of CA 19.9 antigen should lead to the evaluation for hepatic iron overload. In a patient with untreated hemochromatosis, further diagnostic procedures should not be performed unless this finding persists after completion of therapeutic phlebotomy.

The prevalence of portal hypertension varies among case series. Some investigators have reported that portal hypertension is uncommon in hemochromatosis[22, 23]. Fracanzani et al. studied a group of 120 hemochromatosis patients with cirrhosis and 120 patients with post-necrotic cirrhosis[24]. At diagnosis, the prevalences of varices (25%), portal vein abnormalities, and splenic enlargement were similar in the two groups. However, the prevalences of hepatitis C virus (HCV) infection (30%), hepatitis B virus (HBV) infection (7%), and alcohol abuse (26%) were higher in these hemochromatosis patients[24]. Further, these investigators reported a beneficial effect of iron depletion therapy on the evolution of portal hypertension in their hemochromatosis patients. After a mean of 6.0 ± 4.3 (mean ± S.D.) years of follow-up, esophageal varices were improved or had resolved in 26% of the patients and in 5% in the control subjects. Bleeding from varices was observed in only one patient with hemochromatosis and in five control subjects. Of 22 patients with hemochromatosis in whom portal hypertension was unmodified or worsened after iron depletion therapy, 16 had co-existent viral hepatitis, further emphasizing the interaction of non-iron-related co-factors in patients in this study[24]. All studies show that cirrhosis is the major clinical factor affecting survival among persons with hemochromatosis[9, 10, 14, 25]. The major complication of hepatic cirrhosis is the development of hepatocellular carcinoma, not the occurrence of hepatic failure.

The cancerous liver

Numerous studies have shown that there is a high incidence of primary hepatic cancer, essentially hepatocellular carcinoma, in patients with hemochromatosis. Primary cancer of the liver accounted for 30%[13], 36.4%[26], and 45%[14] of deaths among hemochromatosis patients in recent studies. Cirrhosis is a key underlying factor in the causa-

tion of primary hepatic cancer; there is a >200-fold increase in relative risk for this complication in hemochromatosis patients with cirrhosis[10, 13]. In large surveys of French and Italian patients with well-defined hemochromatosis and primary hepatic cancer (32 untreated and 22 iron-depleted patients)[18], the clinical and biochemical presentation of the disease was not different from hepatocellular carcinoma complicating alcoholic or HBV-related chronic hepatic disease[27]. Primary hepatic cancer in these patients was characterized by a predominance of men, mean age of 60 years, advanced non-resectable tumor, normal serum concentrations of alpha-fetoprotein (AFP) in 31% of cases, and short median survival (1 year). In patients with cirrhosis, primary hepatic cancer can develop despite adequate iron depletion. In two series, the median interval between iron depletion and the development of primary cancer of the liver was approximately 10 years[10, 18].

In addition to cirrhosis, male sex, and age >50 years, associated factors such as alcoholism, tobacco smoking, and HBV infection may contribute to the development of primary hepatic cancer[14]. The data are inconclusive regarding the effects of HCV infection. Exposure to parenteral blood products was a significant risk factor in the French–Australian series[18]. However, presence of serum anti-HCV antibodies was not significantly increased in 152 Italian patients[12]. The presence of iron-free foci on the initial hepatic biopsy specimen is important, because these lesions were highly prevalent (83%) in the non-cancerous livers of untreated patients who later developed primary hepatic cancer[28]. In practice, hemochromatosis patients >50 years of age are at risk to develop hepatocellular carcinoma whether iron overload has been alleviated or not. These patients should be screened to detect early hepatic cancer, especially if they have histologic evidence of iron-free foci on their initial hepatic biopsy specimen, chronic alcoholism, tobacco smoking, or HBV infection.

The role of co-existing hepatotoxic factors and hepatic diseases

Chronic alcoholism

Alcoholism is an aggravating factor of hemochromatosis expression. Loréal et al. examined 127 homozygous hemochromatosis patients to evaluate the respective roles of iron and non-iron-related factors in the development of liver fibrosis; 27% had cirrhosis, 21% had hepatic fibrosis, and 52% had no fibrosis (pre-fibrosis patients)[11]. Cirrhosis and fibrosis were significantly more frequent in male alcoholics than in the non-alcoholic patients (male alcoholics 83% vs. 34% in the entire control population, 44% in control males; $p<0.001$). The significant relationship between hepatic iron concentration and fibrosis stages was not present in alcoholic patients, in contrast with non-alcoholics ($p<0.01$). Mean values of hepatic iron concentration in alcoholic and non-alcoholic patients were not significantly different (310 ± 159 (mean $\pm$ S.D.) μmol/g dry weight vs. 281 ± 137 μmol/g dry weight, respectively). Of the patients with hepatic iron concentration <500 μmol/g dry weight, 81% of alcoholic patients and 28% of the non-alcoholic patients had hepatic fibrosis or cirrhosis. Mean values of hepatic iron concentration in these groups were not significantly different (270 ± 105 μmol/g dry weight vs. 310 ± 104 μmol/g dry weight). These data were the first to demonstrate the role of alcohol in the development of hepatic fibrosis in hemochromatosis previously suggested by other authors[13, 29–31]. A second study that evaluated the relationship between alcoholism and hemochromatosis was performed in 105 hemochromatosis homozygotes, each of whom had an HLA-identical sibling[32]. Alcohol consumption >80 g/day was found in 15% of hemochromatosis patients. Histological features of alcoholic hepatic disease (Mallory's hyaline bodies, pericentral fibrosis, polymorphonuclear infiltrates, and fatty infiltration) were uncommon in hemochromatosis patients. The prevalence of clinical features was similar between probands with, and without, excess alcohol consumption (except for skin pigmentation that was more common in the alcoholic group). Mean serum AST concentration was higher in the group with excess alcohol consumption. By histologic criteria, a higher prevalence of cirrhosis was observed in alcoholic patients than in persons without excess alcohol consumption (7/16 vs. 9/89, respectively; $p=0.013$). Survival was significantly reduced in alcoholic patients ($p=0.008$), although cause of death was not clearly related to alcohol abuse in most cases (it is likely that the higher prevalence of cirrhosis contributed to reduced survival). Alcohol is also a co-risk factor for the development of primary hepatic cancer in cirrhotic hemochromatosis patients without cirrhosis.

Viral infection of the liver

The prevalence of HBV infection was increased in a series of French hemochromatosis patients (16.6% of anti-HBc positivity; $p=0.05$ vs. controls)[33]. In 78 Italian patients, there were increased prevalences of both HBV and HCV infection; HBsAg was positive in 5% and anti-HCV was detected in 16% of the patients[12]. The damaging effect of

coexisting viral infection on the liver is documented by Piperno et al.[12]. Fourteen of 18 patients with HBV and/or HCV infection had chronic hepatitis. Although most patients had cirrhosis, the amount of mobilizable iron was significantly lower than that of the fibrosis/cirrhosis group without viral infection ($n=31$; $p<0.01$). Moreover, after iron depletion, serum ALT concentrations became normal in the non-infected patients, whereas they improved significantly without becoming normal in the infected hemochromatosis patients. These data suggest that hepatitis viral infection acts synergistically with iron in accelerating hepatic damage.

The adverse influence of viral infection in hemochromatosis patients with hepatic cirrhosis is poorly documented. In the patients with cirrhosis studied by Fracanzani et al., prevalences of HBV and HCV were high, but the co-existence of cirrhosis and viral hepatitis did not markedly influence the prevalence of esophageal varices at diagnosis[24]. The results of an Italian series of patients suggests that viral hepatitis favors the development of hepatocellular carcinoma; 40% of patients with hepatocellular carcinoma were infected with HCV[14]. However, the prevalences of HBsAg (6.2%) and anti-HBc (16.6%) in French hemochromatosis patients with primary hepatic cancer, although higher, were not significantly different than those of hemochromatosis patients without primary hepatic cancer[33]. In a study of German patients, HBsAg was not detected in the sera of any of the 21 patients who had primary hepatic cancer (anti-HBc was present in five patients), and none of the 11 patients tested were positive for anti-HCV[10].

Porphyria cutanea tarda (PCT)

This is the most common form of porphyria, and it is the result of a deficiency of hepatic uroporphyrinogen decarboxylase (URO-D). It also causes skin disease precipitated by excess alcohol consumption, hepatitis B and C virus infections[34, 35], estrogens, and iron overload. Sporadic PCT is more common than familial PCT. In the former, URO-D decarboxylase deficiency is confined to the liver.

Hepatic symptoms are frequent in patients with PCT[36, 37]. Hepatomegaly of variable degree occurs. A moderate increase in serum transaminase or alkaline phosphatase concentration is common. Hepatic lesions appreciable by histologic analysis are either minor (steatosis, portal inflammatory infiltrate, mixed siderosis) or marked (fibrosis, cirrhosis). However, it is not clear whether hepatic signs are the consequence of PCT per se (notably through the hepatic accumulation of uroporphyrin I and heptacarboxylporphyrins that crystallize to form needle-like intra-hepatocytic inclusions) or are due to co-factors such as alcohol consumption or viral hepatitis. Data favoring the role of PCT in hepatic damage are proposed by Stölzel et al.[38], who found that: (i) when all patients of their series with markers of either HCV or HBV infection were excluded, the remaining 88 patients displayed had supranormal ALT and AST concentrations in 51% and 64%, respectively; (ii) significantly higher serum concentrations of these enzymes were found in 39 patients with overt disease and relapse (ALT 59 ± 44 IU/l (mean $\pm$ S.D.); AST 37 ± 21 IU/l) than in 49 patients in remission (ALT 16 ± 8 IU/l; AST 16 ± 7 IU/l; $p<0.001$). The role of alcoholism cannot be excluded. If alcohol consumption did occur, its effect was probably incomplete, because only 35% of anti-HCV-negative patients had an alcohol intake >60 g/day. In patients with overt disease and relapse, the serum transaminase concentrations predominantly affected were those of ALT, not AST.

The relationship between PCT and hemochromatosis can be summarized: (i) hemochromatosis per se does not precipitate PCT[39]; (ii) inheritance of the hemochromatosis gene is an important susceptibility factor for sporadic porphyria cutanea tarda, as indicated by the presence of the C282Y mutation in 18 (44%) patients with PCT and 11 (11%) normal control subjects ($p=0.00003$); 7 (17%) patients were homozygotes for the mutation[40]; and (iii) whether the coexistence of PCT and hemochromatosis aggravates the overall clinical expression of hepatic disease is likely, but awaits further demonstration in light of the recent discovery of a hemochromatosis-associated gene.

Alpha-1-antitrypsin (AAT) deficiency

This disorder is similar to hemochromatosis in several respects. It is a frequent (estimated prevalence 1 in 1700), autosomal recessive metabolic disorder that affects the liver; progression to cirrhosis and hepatocellular carcinoma sometimes occurs. In contrast, the AAT gene is located on chromosome 14, whereas the known hemochromatosis-associated mutations are located on chromosome 6. Some reports have suggested that there is a higher frequency of AAT deficiency in hemochromatosis patients than in normal control subjects. Evidence for this possibility includes: (i) A significant correlation between heterozygous (PiZ) AAT deficiency and hemochromatosis was reported in a series of 15 patients referred to a hepatic transplantation center in the USA[41]; (ii) An increased prevalence of homozygous AAT deficiency was described in 67 Swedish hemochromatosis patients, suggesting that the presence of the rare PiZ allele for AAT deficiency in a

homozygous configuration may contribute to the earlier onset of cirrhosis in these patients[42].

Whether this apparent causal association is real is unclear, because: (i) The validity of the diagnosis of hemochromatosis in the study by Rabinovitz et al.[41] should be reconsidered due to the presently known difficulties of establishing the diagnosis of hemochromatosis in persons with end-stage hepatic cirrhosis (see below); (ii) Neither Eriksson et al.[43] nor Kaserbacher et al.[44] found an association between heterozygous AAT deficiency and hemochromatosis. Elsouki et al.[42] were also unable to establish such an association in their series of patients; and (iii) In a large series of 115 hemochromatosis patients and 290 control subjects, the frequency of AAT deficiency was similar (10% and 9%, respectively)[45]. Patients in whom the two defects co-existed did not have more severe hepatic disease. The prevalence of cirrhosis in hemochromatosis patients with MM phenotype was 53% and 58% in those with non-MM phenotype. The prevalence of hepatocellular carcinoma (that occurred only in cirrhosis patients) was 22% and 28%, respectively. However, the limited number of subjects with a non-MM phenotype does not permit a conclusive evaluation of clinical differences to be made with patients who had hemochromatosis and the MM phenotype.

Approach to differential diagnosis

There are several major aspects of differential diagnosis represented by hematological iron overload, alcoholic disease of the liver, and newly described entities that affect expression of iron and disease in the liver.

Hepatic iron overload of hematological origin

Secondary iron overload of the liver can be due to dyserythropoiesis and repeated erythrocyte transfusions, particularly in congenital hemolytic anemia (e.g., β-thalassemia) and idiopathic refractory sideroblastic anemia (IRSA)[46]. At the end-stage of these disorders, hepatic manifestations may be similar to those of hemochromatosis[47]. However, the vital prognostic factor is often due to the early development of non-hepatic complications (e.g., cardiac failure in β-thalassemia) or to the severity of the underlying hematological condition (e.g., IRSA).

Siderosis and alcoholic disease of the liver

The clinical picture of alcoholic hepatic disease may include glucose intolerance, hypogonadism, and marked increases in serum iron parameters (iron concentration, transferrin saturation, and ferritin concentration)[48, 49]. Thus, affected patients may appear to have 'hemochromatosis.' In the past, it was often customary to perform a hepatic biopsy in these cases to visualize and quantify hepatic iron. In alcoholic siderosis of the liver, iron deposition is mild and occurs primarily within Kupffer cells. Furthermore, the ratio of hepatic iron concentration to age is <1.9[29]; this helps to distinguish alcoholic siderosis from iron overload due to homozygous hemochromatosis. Currently, the diagnostic strategy involves both the estimation of hepatic iron load by MRI and characterizing the results of DNA-based genetic tests for hemochromatosis-associated mutations.

Hepatic siderosis and cirrhosis

Siderosis can occur secondary to cirrhosis. Three types of studies support this recent concept. (i) Variability in the measurement of hepatic iron content from needle biopsy specimens was first reported by Villeneuve et al. in patients with severe cirrhosis[50]; (ii) Examining 447 cirrhotic livers using histologic and biochemical methods, Ludwig et al. found positive iron staining in 145 cases (32.4%), and increased hepatic iron concentration in 91 cases (20.3%), including 38 cases (8.5%) with hepatic iron overload in the hemochromatosis range (defined by hepatic iron index of >1.9)[51]. However, homozygous hemochromatosis seemed to have caused the cirrhosis in only five cases; (iii) Large slices of the explant native livers of 30 patients who underwent liver transplantation for alcoholic or viral hepatitis end-stage cirrhosis were studied by Deugnier et al.[52], with special determination of hepatic iron concentration in the least and most iron-overloaded nodules of each liver[53]. Intranodular parenchymal siderosis was found in 23 cases. The highest hepatic iron indices were suggestive of hemochromatosis (≥ 1.9) in 14 cases. However, the prevalence of HLA-A3 among those patients with an hepatic iron index ≥ 1.9 did not correspond to the prevalence of this marker in persons homozygous for hemochromatosis gene(s). From a clinical point of view, these data show that: (i) the use of hepatic iron index in end-stage cirrhosis may lead to the mistaken diagnosis of hemochromatosis; (ii) Cirrhosis per se is associated with significant hepatic iron overload. These observations emphasize the importance of evaluating patients with uncertain diagnoses for mutations in the *HFE* gene.

Hereditary ceruloplasmin deficiency

This newly described disorder is due to a mutation in the ceruloplasmin gene located on chromosome 3[53]. The

disease therefore differs from Wilson disease which is due to a defect of a copper-transporting P-type ATPase encoded on chromosome 13. Unlike patients with Wilson disease, persons affected with hereditary ceruloplasmin deficiency have no copper accumulation. Hereditary ceruloplasmin deficiency mimics hemochromatosis in that it is familial and is associated with diabetes mellitus, severe hyperferritinemia, and hepatocytic iron overload indistinguishable from that in hemochromatosis[54, 55]. However, hereditary ceruloplasmin deficiency causes neurological symptoms (progressive dementia, extrapyramidal syndrome, cerebral ataxia) due to iron brain deposition, and ocular signs (retinal degeneration). Two biochemical abnormalities characterize this disorder: low serum iron concentrations and transferrin saturation values (seemingly paradoxical in patients with severe iron overload), and low or undetectable serum concentrations of ceruloplasmin. The mechanism(s) that cause the iron overload remain(s) to be defined, but there may be a defect in iron release from tissue stores. Altogether, this entity illustrates the close relationship between the metabolism of copper and iron[56, 57].

Dysmetabolic hepatosiderosis

This newly described syndrome defines patients who present with hyperferritinemia associated with normal serum transferrin saturation and normal serum iron concentration in the absence of any other known cause of hyperferritinemia (e.g., inflammation, alcoholism, or cytolysis)[58]. Hepatic iron overload, usually moderate, is always present. In one-third of the cases, the iron excess is severe and the hepatic iron index is >1.9, i.e., in the range classically associated with hemochromatosis. This syndrome is not linked to the HLA-A locus, indicated by the significantly lower frequency of HLA-A3 antigen in these patients than in persons homozygous or heterozygous for hemochromatosis gene(s) ($p<0.0001$ and $p<0.0002$, respectively)[58]. Moreover, five HLA-A-identical siblings of patients had normal serum ferritin concentrations. Most patients (95%) had one or more of the following: obesity, hyperlipidemia, abnormal glucose metabolism, and increased arterial blood pressure. This suggests that there may be a link between increased hepatic iron stores and various common metabolic disorders. However, the exact nature and significance of this possible association remain to be clarified.

The characteristics of this new iron overload syndrome underline the necessity to measure both transferrin saturation and serum ferritin concentration when testing for iron overload. It also implies that an hepatic iron index cut-off value of 1.9 does not discriminate between the different iron-overload conditions, just as 'idiopathic' iron overload does not always correspond to hemochromatosis. From a therapeutic viewpoint, iron overload is usually moderate in these patients and the adverse influence of iron overload on their general health remains questionable. However, considering the various experimental, epidemiological, and clinical data that suggest a role of increased iron stores in human carcinogenesis, it seems justified to treat iron excess in patients with dysmetabolic hepatosiderosis, whatever its degree.

Therapeutic aspects

The specific effects of therapeutic phlebotomy on the expression of hepatic disease will be considered in this section. It is generally acknowledged that the application of therapeutic phlebotomy before the appearance of signs of hepatic disease will prevent hepatic complications. Iron depletion therapy has significant beneficial effects in patients who have symptoms of hepatic disease. Regression and sometimes disappearance of hepatomegaly occur, as does improvement in hepatic function, measured by regression (and often normalization) of serum transaminase concentrations. Even portal hypertension can be significantly improved by iron depletion therapy. With regard to non-cirrhotic fibrosis, the possibility of improvement with iron depletion therapy is well documented. Once cirrhosis has developed, three main points should be kept in mind: (i) The overall prognosis is far better than with other types of cirrhosis; for German[13], Canadian[25], and Italian[14] series, survival at 5 and 10 years were 92% and 75%, 72% and 62%, and 75 and 47%, respectively. In a recent report, life expectancy of hemochromatosis patients with cirrhosis was 10–20 years longer than that reported for other forms of hepatic cirrhosis, particularly alcoholic cirrhosis[10]; (ii) reversibility of cirrhosis probably does not occur. There have been occasional reports of disappearance of cirrhosis[17], but the most likely explanation is a sampling problem, especially due to the development of macronodular cirrhosis. Intriguing data have, however, been reported by Müting et al.[59] who documented reversal of cirrhosis by repeat laparoscopy in 12 patients[19]; (iii) the risk for developing primary hepatic cancer persists despite iron depletion, pointing out the necessity to screen patients regularly by evaluating the appearance of the liver by ultrasonography and measuring the serum concentration of AFP every 6 months.

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The arthropathy of hemochromatosis

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Introduction

Arthropathy is one of the common and early clinical manifestations of hemochromatosis. Described in 1964, hemochromatosis arthropathy was described as a form of arthritis with distinctive synovial membrane changes and characteristic joint distribution and radiographic findings[1]. It has been increasingly recognized as a clinical feature of hemochromatosis that sometimes leads to early diagnosis and treatment before irreversible iron-associated injury occurs to the liver and other organs.

Pathogenesis

Morphologic studies of joints affected with hemochromatosis arthropathy reveal iron deposits in the synovium and cartilage and degenerative changes in the articular cartilage. Iron deposition is generally most prominent in the synovial lining layer (Fig. 24.1). Proliferation of the synovial lining cells is mild or moderate, and characteristically there is little deeper inflammation (Fig. 24.1). By electron microscopy (EM), aggregates of ferritin are visualized predominantly in the synovial type B (synthetic) cells and, to a lesser extent. in the synovial type A (phagocytic) cells[2, 3] (Fig. 24.2). Nearly all iron detected by EM and light microscopy is intracellular, and most cells that contain iron appear intact. Ferritin granules occur diffusely throughout the cytoplasm and form aggregates between the cisterns of rough endoplasmic reticulum. When iron loading is severe, macrophages also contain deposits. However, the presence of stainable iron in joint tissues is also found in rheumatoid arthritis, osteoarthritis[4], pigmented villonodular synovitis[5, 6], hemophilia[7], and hemarthrosis[8]. In these latter disorders, iron deposition tends to occur more in deep macrophages, although increased iron may also occur in type B cells. In patients with hemochromatosis, iron deposition in chondrocytes of the articular cartilage is a common but not invariable finding, and ferritin particles occur in intact and degenerating chondrocytes (Fig. 24.3). Most ferritin is intracellular, but small amounts are sometimes found in the interstitium. Crystals are observed in the joint tissues of at least 30% of patients with hemochromatosis (and probably more frequently when sensitive tests such as EM are used). Apatite or calcium pyrophosphate dehydrate (CPPD) crystals or both are found in injured cartilage, but do not have a consistent morphologic relationship with iron deposits. Apatite crystals have been found in cartilage in which no stainable iron is identified. Elemental analysis shows that no ferritin is associated with apatite or CPPD crystals, and that there is no measurable calcium in the ferritin deposits (Fig. 24.3). Crystals can be found on the cartilage surface and in matrix deposits (Fig. 24.4).

The mechanism by which iron participates in joint damage is unknown. It has been proposed that iron contributes to the degeneration of cartilage or the deposition of CPPD and apatite crystals by a variety of mechanisms. The presence of iron in synthetic synovial lining cells and chondrocytes could alter the formation of proteoglycans and collagen, and ferric iron can oxidize ascorbic acid and impair hydroxylation of proline, leading to deficient collagen formation[9]. Free iron can catalyze the formation of toxic hydroxyl radicals from superoxide and hydrogen peroxide, and thus contribute to joint damage. This mechanism has been proposed to occur in rheumatoid arthritis, wherein increased superoxide production occurs and iron is common in macrophages in areas of greatest damage[10]. Collagenase production is increased in synovial fibroblasts incubated with iron[11]. Because iron inhibits pyrophosphatase, increased iron in joint tissues could favor the deposition of CPPD crystals[12]. However, CPPD and apatite crystals are not always present in the joints of patients with hemochromatosis arthropathy, and therefore they are not the only factors that cause cartilage destruction. EM

studies with elemental analysis reveal that iron is not bound to crystals, but may occur in nearby cells. Some studies of gouty arthropathy suggest that iron may coat monosodium urate crystals and that this increases the inflammatory activity of the crystals in vitro[13], although this possible pathogenetic mechanism has not yet been studied with CPPD crystals in hemochromatosis arthropathy. There appear to be no studies of proteases, cytokines, superoxide, or other potentially important molecules in hemochromatosis joints.

Clinical features

General features

Arthropathy is one of the most common clinical features in patients with hemochromatosis at the time of diagnosis[14]. As a presenting symptom, arthralgia or arthritis are detected in 28–81% of patients in large series[15–18]. When rigorous clinical and radiologic criteria are used, the incidence of hemochromatosis arthropathy is approximately 50%[19–22]. Arthritis of hemochromatosis is frequent in men who have a mean age of diagnosis of 60 years. Patients with hemochromatosis without clinical or radiologic evidence of arthropathy tend to be younger, and young patients with hemochromatosis have a lower frequency of arthritis. In young patients with hemochromatosis arthropathy, there is an equal sex distribution[23]. Cases of young women presenting with secondary amenorrhea and arthritis have been reported[16,24]. The prevalence of arthropathy among women with hemochromatosis is 6.1–40%, and few are diagnosed before menopause[19]. However, a comparative study of the clinical features found prevalences of arthritis of 44.9% and 35.2% for women and men, respectively[25]. There is a high frequency of HLA-A3 and HLA-B7 antigens in patients with arthritis and hemochromatosis[20]. These antigens are also commonly associated with hemochromatosis in Caucasians. There is no evidence that a different genetic pattern of HLA phenotypes occurs when hemochromatosis arthritis is present. Likewise, with the recent demonstration of the *HFE* gene and two mutations related to hemochromatosis[20a], there are as yet no identified differences in genes of patients who develop arthropathy vs. those who do not.

Clinical presentation

The arthropathy of hemochromatosis presents in different patterns. Patients may be asymptomatic despite radiographic signs of joint disease, or only complain of arthralgias. The usual presentation is that of chronic, indolent arthritis with stiffness and enlargement of joints and very mild signs of inflammation. Despite the fact that initial findings are often subtle, destruction of large joints, especially hips and knees, often occurs in patients with advanced disease. Less commonly, patients experience acute inflammatory episodes of arthritis, some but not all of which are related to CPPD crystals[20,26] Rarely, patients with hemochromatosis develop *Yersinia* septicemia that can involve joints with septic arthritis; joints with prosthetic replacements seem especially prone to such infections[27,28].

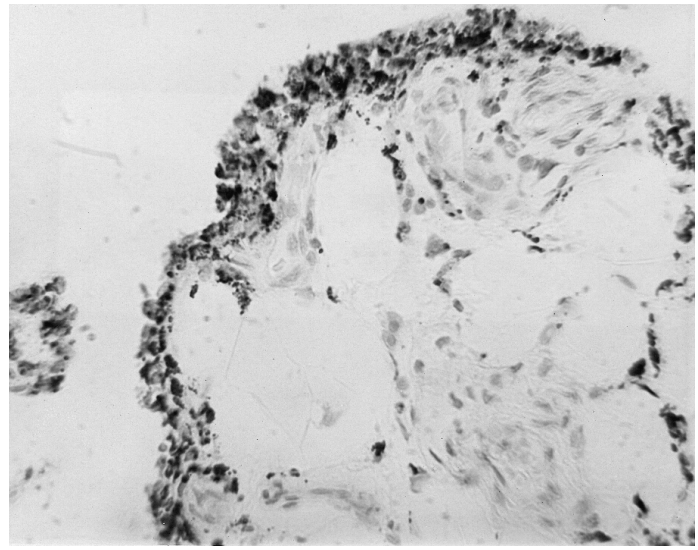

Fig. 24.1. Light microscopy of the synovial membrane of a patient with hemochromatosis. Dark iron particles are present in lining cells; inflammatory cells are absent.

One of the most helpful clues to the clinical diagnosis of hemochromatosis arthropathy is its distribution. Symmetric, polyarticular involvement of the metacarpophalangeal (MCP) joints, proximal interphalangeal joints, and wrists, and destructive arthritis of the hips and knees is the usual pattern. Distal interphalangeal joints are involved less frequently than in typical primary osteoarthritis, and MCP joints are affected more often. Shoulders, not usually affected in persons with primary osteoarthrosis, can be involved in hemochromatosis arthropathy. Elbows are rarely affected in persons with hemochromatosis, but any diarthrodial joint can be involved.

Radiologic abnormalities

The roentgenographic findings of bones and joints in hemochromatosis arthropathy are distinctive. Approximately 50% of patients have radiographic abnormalities[29,

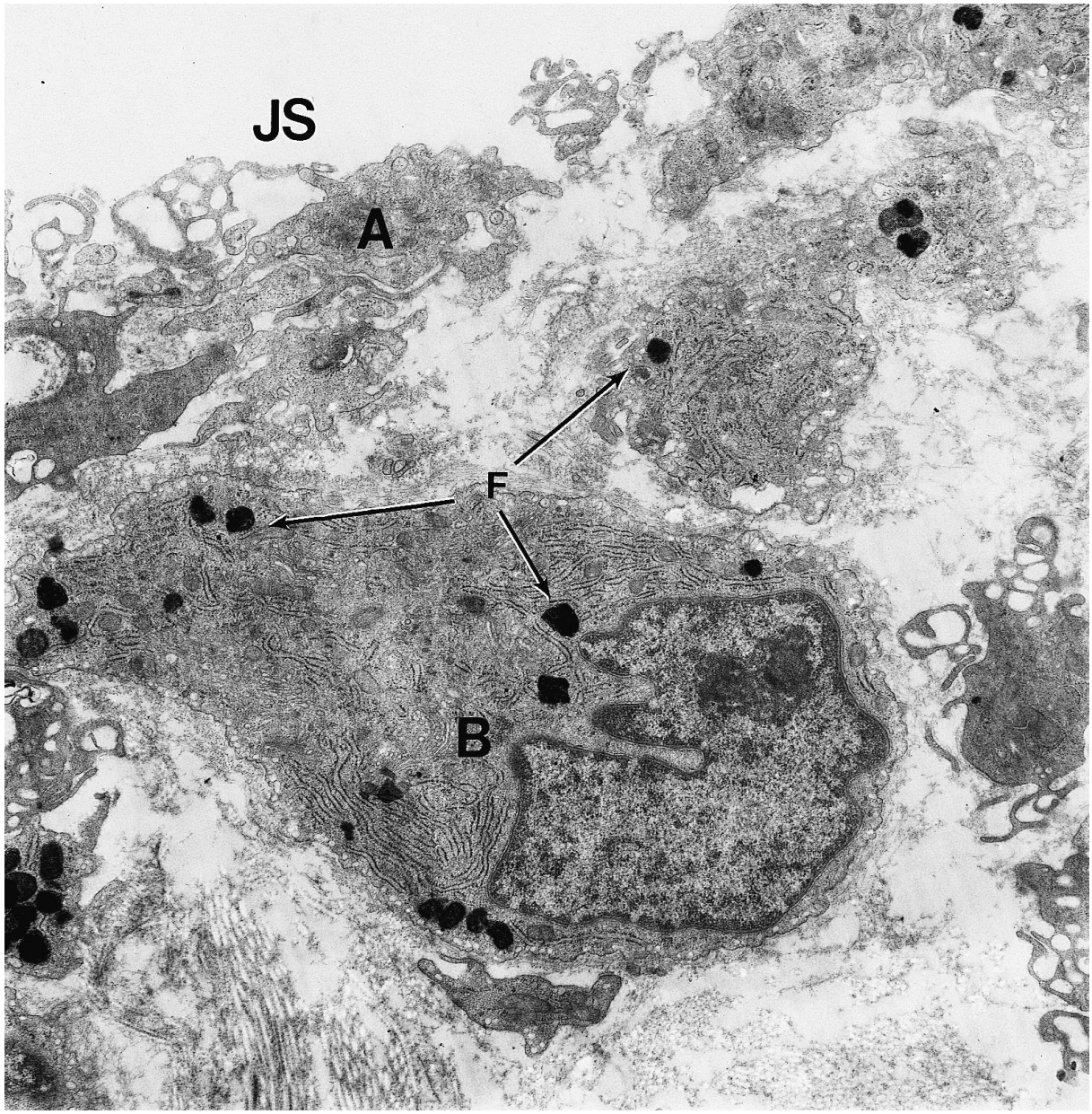

Fig. 24.2. Electron microscopy of a synovial type B (B) cells with ferritin (F) aggregates in the cytoplasm. The more superficial type A (A) cells generally contain no iron. Joint space (JS).

[30] consisting mainly of progressive degenerative arthritis of distinctive distribution. Approximately 30% have also have chondrocalcinosis[31], even in the absence of clinical or radiological evidence of other arthritis. Generalized osteoporosis in persons with hemochromatosis was described after the first report of hemochromatosis arthropathy[1]; the occurrence of this abnormality has been confirmed[29, 32].

The earliest radiographic lesions are subchondral cysts with sclerotic margins at the metacarpal heads, particularly the second and third MCP joints[29, 31, 33, 34] (Fig. 24.5). The first, fourth, and fifth MCP and interphalangeal joints are involved less frequently; when involved, these joints are affected to a lesser extent than the second and third MCP joints. With progression of joint damage, there is joint space narrowing, usually accompanied by an asymmetric loss of cartilage that is often adjacent to a subchondral

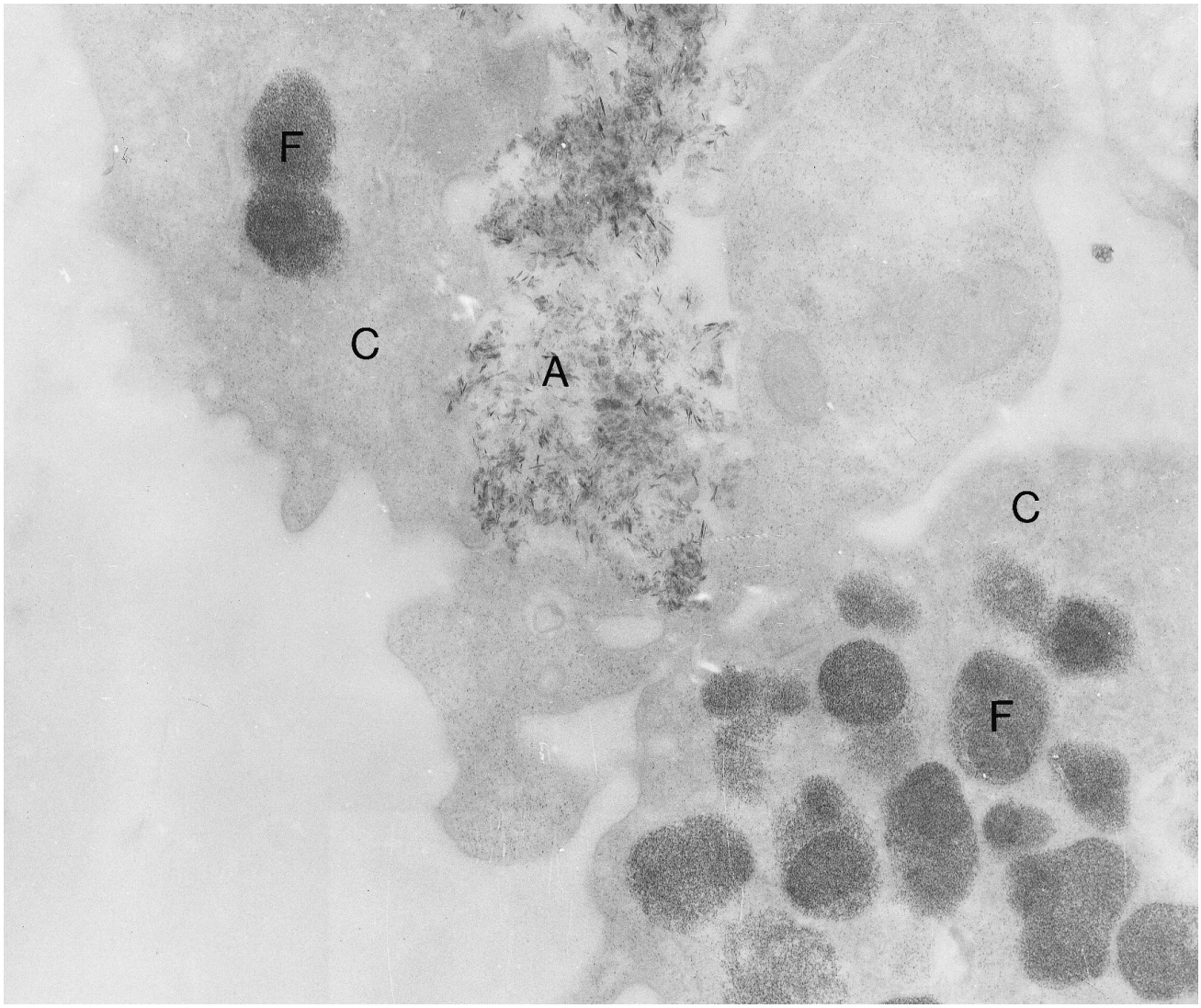

Fig. 24.3. Electron microscopy of two chondrocytes (C) with aggregates of ferritin (F) and apatite crystals (A) between the cells. No iron was detected among the crystals in the matrix, and no crystals were directly associated with the iron (× 44 000).

cyst[29, 34] (Fig. 24.6). Osteophytes, sometimes hook-like in configuration, are often present at severely affected MCP joints (Fig. 24.6). Generalized osteoporosis of the affected hands is another common finding. In the carpal bones, multiple cysts, joint space narrowing, and sclerotic changes are prominent. Chondrocalcinosis of the triangular cartilage of the wrist occurs in approximately 50% of cases[29] (Fig. 24.7). Radiocarpal joints may be involved, but radio-ulnar joints are usually not affected. The large joints most frequently affected are the hips and the knees (Fig. 24.8), the destruction of which is often is severe enough to require prosthetic replacement. Typical radiologic findings in the hip are cartilage narrowing and marginal osteophytes, often indistinguishable from those of idiopathic osteoarthritis[29]. Diffuse cartilage loss and protrusio-acetabuli may result from crystal-associated inflammation (Fig. 24.8). Subarticular cysts may be present in femoral heads, and calcification of the fibrocartilaginous articular labrum has been described. In the knees, subchondral cysts and sclerosis are uncommon[35], but chondrocalcinosis is most common in this location (Fig. 24.9). Shoulders and elbows are affected less frequently than hips and knees, but may develop cystic changes and chondrocalcinosis. Visible intervertebral disk calcification at one or more levels and calcification of the ligamentum flava is present more frequently in patients with hemochromatosis than in normal

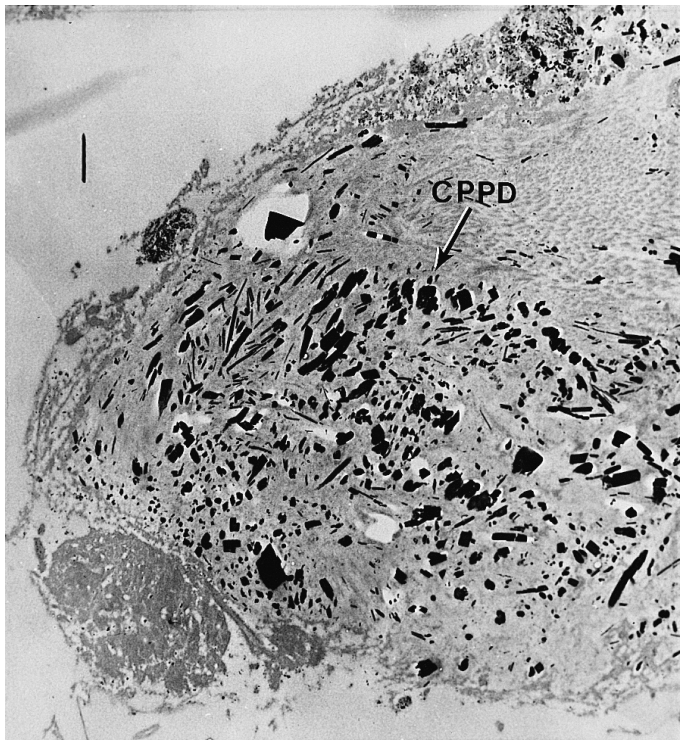

Fig. 24.4. Profuse calcium pyrophosphate crystal deposition (CPPD) in the cartilage of a patient with hemochromatosis. On elemental analysis, these crystals had a calcium to phosphorus ratio of 1:1typical of CPPD, and contained no iron (×10000).

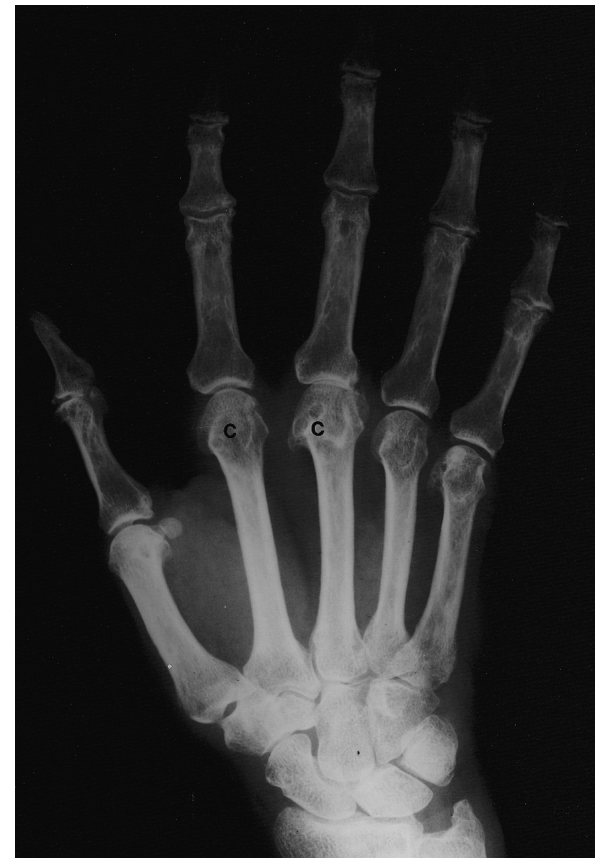

Fig. 24.5. Mild lesions in the hand of a patient with hemochromatosis. Typical subchondral cysts (C) with sclerotic margins are present, especially in the second and third metacarpophalangeal joints. There is osteopenia of the digits. There are no abnormalities of the proximal and distal interphalangeal joints.

subjects. The distribution of the calcification appears more often in the annulus than in the nucleus[36]. Osteophytes can also occur in the spine. Although these superficially resemble those of ankylosing spondylitis, they are more like those of diffuse idiopathic skeletal hyperostosis; however, this is an infrequent feature[36].

Differential diagnosis

The arthritis of hemochromatosis is often considered to be a very uncommon form of joint disease, and therefore it is frequently misdiagnosed and confused with other arthropathies such as rheumatoid arthritis, osteoarthritis, idiopathic pseudogout, and the Missouri metacarpal syndrome related to repetitive joint trauma.

Rheumatoid arthritis

In hemochromatosis arthropathy, the polyarticular and symmetric involvement of the MCP joints and of the wrists can give the clinical appearance of rheumatoid arthritis. The arthritis in hemochromatosis is typically associated with firm bony enlargement, whereas inflammation and synovial thickening are more characteristic of rheumatoid arthritis. Bursitis, tendinitis, and subcutaneous nodules (which unfortunately were not histologically examined) have been described in hemochromatosis patients with arthropathy. A positive rheumatoid factor has also been occasionally reported in patients with hemochromatosis, but this is unusual[37, 38]. The juxtaarticular osteoporosis, synovial swelling, marginal erosions, and ulnar deviation of rheumatoid arthritis are not seen in the arthropathy of hemochromatosis, although rheumatoid arthritis has occurred as an apparently coincidental disorder in persons with hemochromatosis.

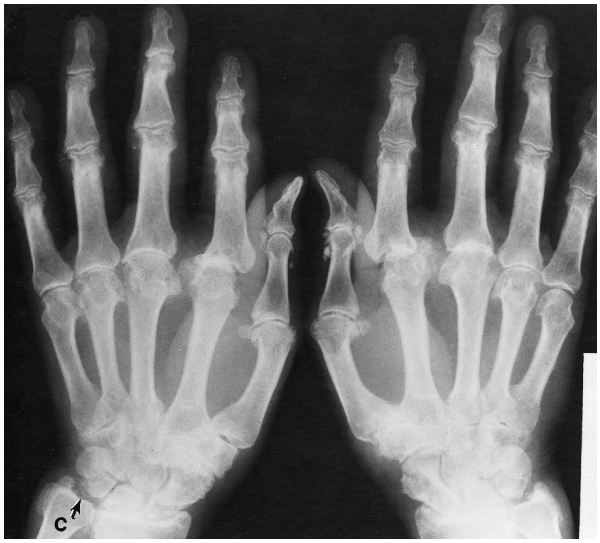

Fig. 24.6. Advanced lesions in the hands of a patient with hemochromatosis. There is chondrocalcinosis (c) of the tringular wrist ligament, narrowing of the second and fourth metocarpophalangeal joint spaces, bony enlargement of the metacarpal heads with the occurrence of hook-like osteophytes, and a subchondral cyst. There is sclerosis of the radiocarpal joint, narrowing of the joint space, and hypertrophic osteophyte formation at the proximal and distal interphalangeal joints.

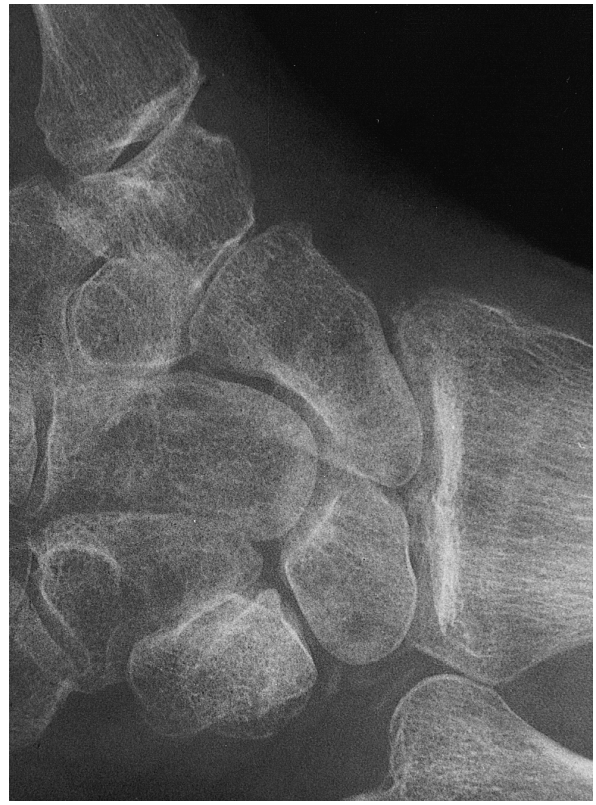

Fig. 24.7. Close-up view of chondrocalcinosis of the triangular wrist ligament in a patient with hemochromatosis arthropathy.

Osteoarthritis

In many cases, the only way to clinically differentiate osteoarthritis joint disease from that of hemochromatosis is the characteristic distribution of the latter. The changes of the hips can be indistinguishable, although osteophyte formation is milder and chondrocalcinosis more prevalent in hemochromatosis[34]. The sparing of the distal interphalangeal joints and selective involvement of the MCP joints is characteristic of hemochromatosis.

CPPD disease

Approximately 30% of patients with hemochromatosis have chondrocalcinosis and some patients may present with acute inflammatory arthritis attacks due to CPPD crystals. Because the differentiation of hemochromatosis arthropathy from idiopathic CPPD crystal deposition disease can difficult, it is advisable to test for hemochromatosis when CPPD disease is diagnosed, especially in younger patients and in Caucasians. Features shared by hemochromatosis arthropathy and idiopathic CPPD disease are involvement of MCP joints, midcarpal, and radiocarpal wrist compartments, the occurrence of subchondral cystic lesions, and uniform loss of articular space. Scapholunate dissociation, however, is more prominent in CPPD crystal deposition disease[26]. Hemochromatosis arthropathy has a predilection for affecting the second and third MCP joints; in 30–50% of patients, the carpal bones are involved. Beak-like osteophytes of metacarpal heads, with flattening or mild collapse of the osseous surface, are particularly characteristic of hemochromatosis arthropathy.

Osteoporosis

Hemochromatosis should be considered in the differential diagnosis of osteoporosis, especially in men. Diffuse bone demineralization occurs in some persons with hemochromatosis, and the androgen deficiency secondary to hypogonadism in hemochromatosis[39] may contribute to osteoporosis. Acute iron overload in pigs can produce iron deposits in osteoblasts and trabeculae, resulting in a decrease in the number and activity of osteoblasts[40]. In humans, bone histomorphometry shows a decrease in trabecular bone volume associated with a decrease in cortical bone thickness. Osteoclast and osteoblast activities are

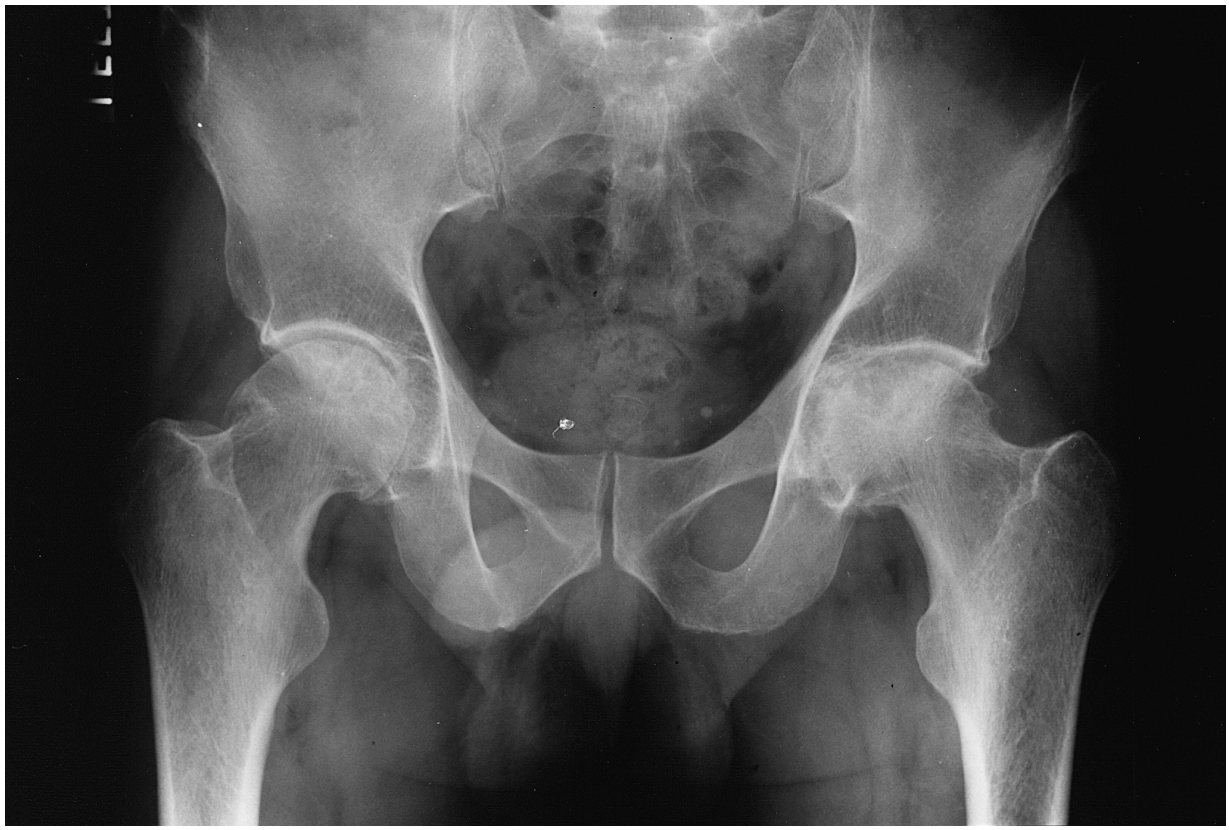

Fig. 24.8. Radiographic changes of the hips of a patient with hemochromatosis. There is joint narrowing due to cartilage destruction, cyst formation, and irregular flattening of the left femoral head. The medial protrusion of the left femoral head in this patient is not the usual pattern present in idiopathic osteoarthritis.

reduced without any sign of osteomalacia, and iron has been found at the interface between mineralized trabecular bone and bone marrow[39]. In two young male patients with hemochromatosis, one of them eugonadal, spontaneous osteoporotic fractures were presenting signs of hemochromatosis[33, 41]. These observations suggest that there is more than one mechanism involved in the pathogenesis of osteopenia of hemochromatosis, and that osteoporosis may be a more frequent clinical presentation than recognized.

Missouri metacarpal syndrome

This syndrome is attributed to heavy manual labor, and has been described as another cause of degenerative-like arthritis that preferentially affects the MCP joints. Tight gripping while driving tractors was suggested as an etiologic factor[42].

Treatment

The survival of patients with hemochromatosis treated with phlebotomies before the development of hepatic cirrhosis is similar to that of the general population[19]. As a clue to early diagnosis in some patients, hemochromatosis arthropathy can play an important role in successful treatment.

However, the high prevalence of joint-associated complaints and their unremitting nature is detrimental to quality of life[43]. Further, although therapeutic phlebotomy may prevent hepatic and anterior pituitary injury due to iron overload in hemochromatosis, this treatment does not invariably improve the arthropathy. In many patients, arthropathy worsens despite therapeutic phlebotomy. Iron may persist in the synovia and cartilage of patients who have received apparently adequate phlebotomies[24]. Although some patients have enjoyed improvement in their joint symptoms after phlebotomy therapy, one cannot predict those patients who will respond favorably.

Other patients have become worse, and there are a number of reports of initial joint symptoms occurring during therapeutic phlebotomy.

The treatment of hemochromatosis arthropathy is mainly directed to the control of symptoms. Acetaminophen or nonsteroidal anti-inflammatory drugs alleviate the symptoms of arthritis in some patients. Before administering these drugs, a careful evaluation of hepatic and renal function is necessary. Older patients have more side-effects with the use of non-steroidal anti-inflammatory drugs. Exercise, physiotherapy, and application of heat to the affected joint can also relieve symptoms. Acute episodes of pseudogout must be diagnosed with demonstration of CPPD crystals in synovial fluid removed by joint aspiration, and treated with a potent non-steroidal anti-inflammatory drug such as indomethacin, or with the administration of colchicine or intra-articular glucocorticoids. Because CPPD or apatite crystals are common in persons with hemochromatosis arthropathy, chronic low-dose colchicine (0.5–1 mg/d) sometimes suppresses lowgrade, crystal-induced inflammation. Experimental measures used in treatment of osteoarthritis merit consideration, including therapy with tetracyclines and protease inhibitors. Intra-articular hyaluronic acid therapy may be more effective in treating osteoarthritis joints with crystals than without crystals[44]. When there is progressive joint deterioration, surgical intervention must be considered. Prosthetic hip and knee arthroplasties are frequently needed; in general, these procedures are successful and the life expectancy of the protheses is the same as that in other indications. Smaller joints can also be replaced surgically. Successful, short-term arthroscopic treatment of the MCP joints has been reported in hemochromatosis patients. Hemochromatosis patients with prosthetic joint replacements can develop sepsis with *Yersinia enterocolitica*[27, 45–47].

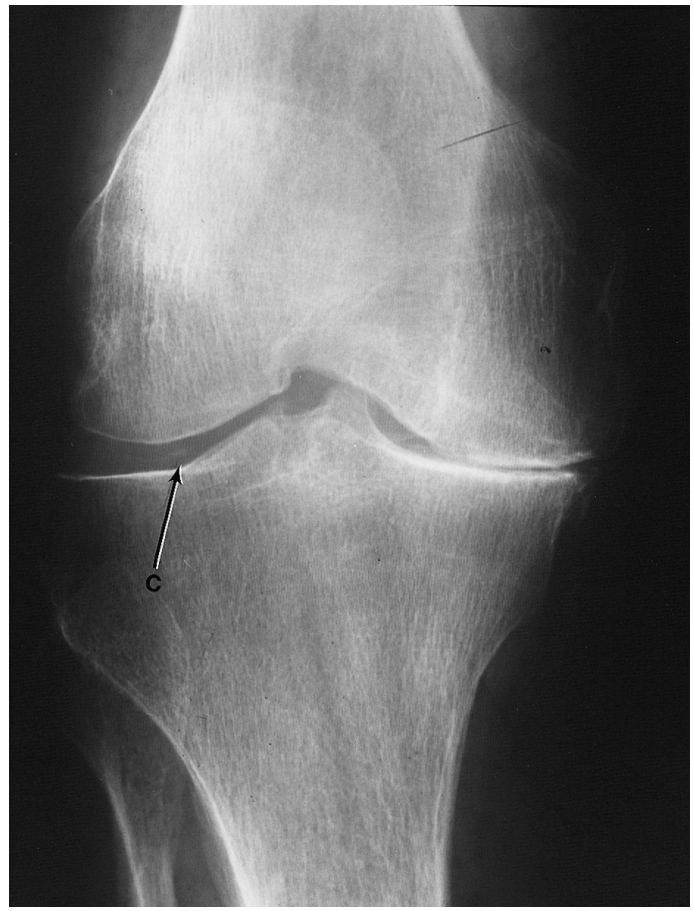

Fig. 24.9. Frontal projection of the knee of a hemochromatosis patient with chondrocalcinosis (c) of the lateral meniscus. There is also medial joint space narrowing and hypertrophic osteophyte formation.

Conclusions

Although phlebotomy therapy is often effective in preventing or alleviating many complications of iron overload in hemochromatosis, innovative approaches for treating the hemochromatosis arthropathy are needed. Advances in therapy may come from a better understanding of the mechanisms involved in pathogenesis of the joint disease. There is a high incidence of arthropathy in family members of hemochromatosis probands who do not apparently have hemochromatosis[15, 46, 47]. Therefore, the prevention of joint disease by phlebotomy treatment of clinically and radiographically normal relatives of hemochromatosis probands who are also hemochromatosis homozygotes may be effective, but this has not been examined. Few studies of the effects of hemochromatosis and iron overload on the phenotypic expression of non-hemochromatosis arthropathy have been reported. Therefore, it remains possible that hereditary or environmental factors other than inheritance of hemochromatosis alleles and the development of iron overload are also important in the pathogenesis of hemochromatosis arthropathy.

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25

Diabetes mellitus and hemochromatosis

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Introduction

The association of diabetes mellitus, hepatic cirrhosis, pigment deposits in the liver, and a bronze skin color in hemochromatosis was recognized over a century ago[1–3]. Diabetes mellitus was the most frequent term in the early descriptions that reflected the various beliefs as to whether the disease was primarily pancreatic, hepatic, or hematological in origin, or whether it was a separate disease entity[1–5].

Pathophysiological concepts of diabetes mellitus and impaired glucose tolerance in hemochromatosis

Although the close association between diabetes mellitus and hemochromatosis is well established, its cause remained uncertain until recently. Hepatic cirrhosis, damage to pancreatic beta-cells, and genetic predisposition have been proposed as the major factors causing impaired glucose tolerance and diabetes mellitus in hemochromatosis[6–11]. Recently we have re-evaluated our own clinical studies[12] to characterize the association of diabetes mellitus and hemochromatosis further, to establish its causes, and to assess the contribution of diabetes mellitus to the complications and mortality of hemochromatosis.

Prevalence of diabetes mellitus and impairment of glucose tolerance in hemochromatosis

Early studies (Table 25.1) reported a prevalence of diabetes mellitus in hemochromatosis of almost 80%[13–16]. In more recent studies, the prevalence is reported to be 50–60%[7, 9, 11, 12, 17–20]. In a series of 474 French patients with hemochromatosis, 191 (40%) had diabetes mellitus at the time of diagnosis[21, 38]. The prevalence and stage of diabetes was related to the degree of iron deposition in the pancreas. Patients with diabetes had twice as much mobilizable iron as did non-diabetic patients[21]. The decreased prevalence of hemochromatosis-associated diabetes during the last two decades is probably due to the fact that an increasing number of patients are diagnosed in early stages when glucose metabolism is still normal or only slightly abnormal. The decrease of the prevalence of diabetes mellitus in hemochromatosis is more apparent during the period 1947–1969 than during the period 1970–1981 (Table 25.2). In our series of 251 patients with hemochromatosis, 120 patients (47.8%) had diabetes mellitus at the time of diagnosis; approximately one-half of

Table 25.1. Prevalence of diabetes mellitus in primary hemochromatosis

References	Number of patients (*n*)	Prevalence of diabetes (% of patients)
13	311[a]	82
14	30	87
15	707[a]	78
16	1500[a]	76
18	115	63
20	33	63
6	96	69
7	40	70
9	74	62
11	163	55
21, 38	474	40
12	251	48
22	85	21

Note:

[a] indicates review from previous literature.

Table 25.2. Changes in clinical features and mobilizable iron during three different time periods of follow-up

	1947–69 ($n=84$)	1970–81 ($n=84$)	1982–91 ($n=83$)	total ($n=251$)	$\chi^2=$	$p=$
Liver cirrhosis	79.8%	48.8%	41.0%	56.6%	28.7	0.59×10^{-6}
	67/84	41/84	34/83	142/251		
Diabetes mellitus	73.8%	39.2%	30.1%	47.7%	35.6	0.18×10^{-7}
	62/84	33/84	25/83	120/251		
Arthalgia	39.3%	45.2%	48.2%	44.2%	1.34	0.49
	33/84	38/84	40/83	111/251		
Diagnosis of asymptomatic patients	4.8%	22.6%	30.1%	19.1%	18.4	0.10×10^{-3}
	4/84	19/84	25/83	48/251		
Mobilizable iron (g)	25.5	20.7	17.1	21.2	(t-test)	<0.01
	$\pm$ 1.8	$\pm$ 1.7	$\pm$ 1.4	$\pm$ 1.1		

Table 25.3. Laboratory findings at diagnosis in subgroups of patients with hemochromatosis

		Patient subgroups		
	All patients ($n=251$)	Cirrhotic ($n=142$)	Non-cirrhotic ($n=109$)	p value by z-test
Glucose metabolism		Percent of group (number of patients)		
Glucose tolerance test[a]				
Normal ($n=98$)	39 (98)	20 (28)	73 (80)	0.001
Abnormal ($n=33$)	13 (33)	13 (18)	14 (15)	NS[b]
Diabetes mellitus[c] ($n=120$)	48 (120)	72 (102)	17 (18)	0.001
Non-insulin dependent ($n=51$)	20 (51)	28 (39)	11 (12)	0.01
Insulin dependent ($n=69$)	27 (69)	44 (63)	6 (6)	0.001

Notes:

[a] Abnormal glucose tolerance was defined as a plasma glucose increase >200 mg/dl 2 h after oral intake of 75 g glucose.

[b] NS denotes not significant at the 0.05 level of significance.

[c] Diabetes mellitus was defined according to WHO criteria: a fasting or postprandial plasma glucose >120 mg/dl or 200 mg/dl, respectively.

these diabetic patients were insulin-dependent at the time of diagnosis ($n=69$) (Table 25.3). Diabetes was absent in 131 of the 251 patients (52.2%) at the time of diagnosis; more than three-quarters of these non-diabetic patients had normal glucose tolerance (Table 25.3). The absence of diabetes was often associated with an early non-cirrhotic stage of hemochromatosis; 91 of the 131 non-diabetic patients (69.5%) did not have cirrhosis at the time of diagnosis (Table 25.4). In contrast, diabetes mellitus was often associated with hepatic cirrhosis; the prevalence of diabetes in patients with hepatic cirrhosis was 72% (102 of 142) (Table 25.4). Thus, the prevalence of patients who had already developed cirrhosis in our series is similar to that reported in earlier studies in which most patients were diagnosed in the cirrhotic stage[13–16]. In contrast, diabetes was diagnosed in only 18 of 120 of patients (15%) who did not have cirrhosis at the time of diagnosis (Table 25.4). Among French patients, there was a decreasing prevalence from 25% to 12.7% of insulin-dependent diabetes and from 28.5% to 21.8% of non-insulin dependent diabetes when the diagnosis of hemochromatosis was made before severe complications of iron overload occurred[22]. In recent cohort studies that included mostly pre-cirrhotic patients, the prevalence of diabetes was only 21.1%[21].

Table 25.4. Relationship between hepatic cirrhosis and diabetes mellitus[a]

	Diabetes		
Cirrhosis	Present	Absent	Total
Present	102 (71.8%)	40 (28.2%)	142 (56.6%)
Absent	18 (16.5%)	91 (83.5%)	109 (43.3%)
Total	120 (47.8%)	131 (52.2%)	251 (100%)

Note:

[a] These data represent observations in 251 patients with hemochromatosis at the time of diagnosis. Chi-square analysis of these figures revealed: $\chi^2 = 73.4$; $p < 0.9 \times 10^{-15}$.

Prevalence of hemochromatosis in diabetic patients

The prevalence of unrecognized hemochromatosis in diabetic patients has been estimated in Europe and elsewhere. Screening revealed a prevalence of 5–8 unrecognized cases per 1000 in Europe[23–25] and 9.6 unrecognized cases per 1000 in Australia[26]. Therefore, persons with diabetes should always be evaluated for the possible presence of iron overload.

Pathophysiology of diabetes mellitus in hemochromatosis

Liver disease and insulin resistance

Diabetes mellitus and impaired glucose tolerance are common features in several chronic liver diseases[27–30]. Because insulin resistance is a major cause of impaired glucose metabolism in chronic liver disease, it is conceivable that it might also be responsible for the impaired glucose metabolism associated with hemochromatosis.

Early alterations of glucose metabolism have been evaluated in ten male non-cirrhotic patients with primary hemochromatosis, all of whom had normal circulating glucose concentrations after an oral administration of 100 g of glucose ([10], Fig. 25.1). However, insulin concentrations after the oral glucose load increased significantly in the patients compared with matched healthy control subjects (Fig. 25.1). Thus, patients with hemochromatosis had hyperinsulinemia and hence insulin resistance without impaired glucose tolerance in the non-cirrhotic stage. In contrast to increased insulin concentrations, circulating concentrations of C-peptide were normal in non-cirrhotic patients with hemochromatosis, indicating normal insulin secretion by the pancreatic beta-cells in this early non-cirrhotic stage of the disease. Circulating glucagon concentrations in response to the oral glucose load were also normal. The latter results are in accordance with two previous studies that demonstrated normal glucagon concentrations after arginine stimulation of patients with cirrhotic and non-cirrhotic hemochromatosis[31, 32].

The observation of normal blood glucagon concentrations in cirrhotic patients is further substantiated by histological studies showing that the pancreatic iron deposits in hemochromatosis occur almost exclusively in the beta-cells and not in the alpha-cells of the pancreatic islets. Iron deposits in gastric and duodenal mucosa can be demonstrated in most patients with hemochromatosis during the non-cirrhotic stage. Thus, impairment of the enteroinsulinar axis involving the release of gastric inhibitory polypeptide (GIP)[33] might also contribute to alterations of glucose metabolism in hemochromatotic patients. However, the circulating concentration of GIP in response to oral glucose was not significantly different from that of healthy control subjects. Thus, insulin secretion by the pancreatic beta-cell (C-peptide), glucagon secretion by the pancreatic alpha-cell, and the enteroinsulinar axis (GIP) are not impaired in non-cirrhotic patients with hemochromatosis. The increase in circulating insulin concentrations is likely to be due to a decrease in diminished hepatic extraction of insulin. Iron accumulation in hepatocytes may cause impaired hepatic degradation of insulin.

There is a close correlation between the progression in the alteration of glucose metabolism and the development of hepatic cirrhosis. It is likely that a part of diabetes is due to the development of hepatic cirrhosis. However, the high frequency of diabetes, often insulin-dependent, in patients with cirrhosis also reflects the advanced iron overload in the beta-cells of the pancreas that causes both complications.

Impaired beta-cell function due to iron deposition

Insulin secretion by the pancreatic beta-cell is normal in the non- or pre-cirrhotic stage of hemochromatosis[10]. With the progression of iron overload and the degeneration or destruction of the beta-cell and the development of hepatic cirrhosis, pancreatic insulin secretion becomes impaired[19]. In end-stage hemochromatosis, insulin deficiency is associated with severe reduction in the mass of immunoreactive beta-cells[34]. In contrast, the secretion of other pancreatic islet hormones is normal[10]. The frequency of insulin-dependent diabetes in patients with cirrhosis due to hemochromatosis is significantly greater

than that in patients with cirrhosis of other origin, indicating that beta-cells damage and insulin resistance caused by liver cell damage must both occur to cause diabetes.

With impaired insulin secretion by damaged beta cells, there is obligatory insulin resistance observed in iron overload disease. This is due to an insulin receptor/post-receptor defect in the iron-loaded liver, that is, in contrast to the destruction of the beta-cells, partially reversible after completion of phlebotomy therapy[11, 12, 35–37].

Genetic factors

In addition to insulin resistance and impaired beta-cell function, genetic factors have been postulated to be responsible for the frequent association of diabetes mellitus and hemochromatosis. This hypothesis was based on studies reporting that the prevalence of diabetes mellitus among relatives of hemochromatotic patients was markedly higher than expected for a normal control population[17]. This was interpreted as an increased genetic predisposition of diabetes mellitus in hemochromatosis per se. Subsequent studies revealed that the prevalence of diabetes mellitus was significantly higher among relatives of hemochromatotic patients with diabetes compared to those without diabetes[18, 19]. The detailed genetic analysis, however, suggested that maturity-onset diabetes mellitus was inherited in families with hemochromatosis at about the same rate as in non-hemochromatotic families[6, 38].

Studies in recent years did not confirm the hypothesis that diabetes mellitus in hemochromatosis is mainly due to specific genetic factors, but showed that diabetes originates from secondary beta-cell damage caused by iron overload. There was no correlation between family history and the prevalence of diabetes mellitus or glucose intolerance[20]. There was also no evidence that diabetes mellitus or glucose intolerance are markedly increased among non-hemochromatotic relatives of patients with hemochromatosis[39, 40].

The pathophysiological concept of increased iron deposition as the main factor of disturbed glucose metabolism is also supported by the results of experimental animal studies in which glucose intolerance and diabetes mellitus were produced by intraperitoneal or oral iron administration. The morphological alterations of experimental iron overload are similar to human hemochromatosis[34, 38]. Early administration of iron chelators or venesection prevented or improved the manifestations of diabetes. This is in accordance with clinical observations that the manifestation and progression of diabetes in hemochromatosis (and secondary overload) is related to the degree of the iron deposition (mobilizable iron) in the pancreatic beta-cells.

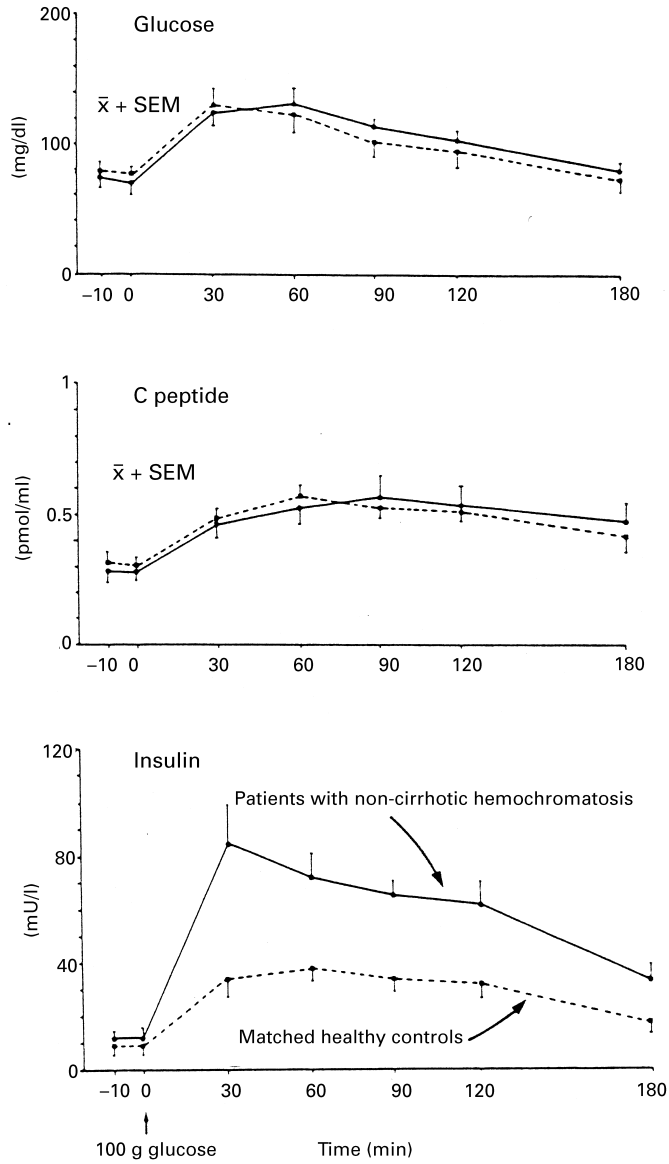

Fig. 25.1. Plasma insulin, C-peptide and glucose concentrations after an oral glucose load (100 g) in 10 non-cirrhotic patients with hemochromatosis and 10 age- and sex-matched healthy control subjects. Data are expressed as mean ± SD. Only the insulin response was enhanced in patients with hemochromatosis when compared with the control, whereas glucose and C-peptide values were similar between patients and controls (adapted from Niederau[11]).

Secondary iron overload due to erythrocyte transfusions to treat chronic hemolytic anemia is a model disorder study for the association of iron overload and diabetes mellitus in the absence of genetic factors. The prevalence of both diabetes mellitus and impaired glucose tolerance is markedly increased in patients with iron overload due to thalassemia

major: 31 of 167 patients with thalassemia major had diabetes mellitus (19%) and 45 of 88 patients with thalassemia major who had an oral glucose tolerance test showed impaired glucose tolerance (51%)[41–51]. Although these frequencies appear to be somewhat lower than those reported for the association of diabetes mellitus and hemochromatosis, this difference is probably due to the fact that hemochromatosis develops over the much longer period of 30–50 years, whereas most secondary cases of iron overload are seen in children or adolescents. Indeed, insulin-dependent diabetes has been reported to develop only in patients with thalassemia who are more than 12 years of age[51]. At this age, however, impaired glucose tolerance is observed in most patients[51]. This time-dependent progression of alterations in glucose metabolism in secondary iron overload is further substantiated by a longitudinal study observing patients who received multiple transfusions during a 5-year period[49]. During this period, glucose tolerance progressively deteriorated and diabetes mellitus developed in an increasing number of patients.

Excessive dietary intake of iron in combination with increased alcohol consumption may cause secondary iron overload such as that reported in several African tribes such as the Bantu. The prevalence of diabetes in this type of dietary iron overload is as high as 50%[52]. In addition, impaired glucose tolerance was reported in ~70% of patients with dietary iron overload[53]. The incidence of diabetes was reported to be 10 times higher in Bantu with iron overload compared to those without iron overload[41].

The studies of secondary iron overload due to thalassemia or due to long-standing dietary iron intake, therefore, support the concept that impaired glucose tolerance is the predominant complication of accompanying hepatic disease in early stages of iron overload. Iron overload for more that one decade may cause diabetes mellitus and insulin dependency that is likely to be caused by damage of the pancreatic beta-cells due to iron deposition rather than by some unknown genetic factor.

Macrovascular and microvascular complications of diabetes mellitus in patients with hemochromatosis

Early studies had claimed that diabetes mellitus in hemochromatosis had less microvascular and macrovascular complications as compared to primary diabetes mellitus[13, 54–56]. It has, therefore, been speculated that diabetic complication are due to genetic factors and not due to hyperglycemia itself. The work of Dymock et al. challenged this hypothesis by showing a high incidence of vascular complications in hemochromatotic patients[18, 19]. We evaluated 54 hemochromatotic patients suffering from insulin-dependent diabetes who were followed for 11.6 ± 5.1 years (mean $\pm$ SD; range 1–29 years), and found a high frequency of macrovascular and microvascular complications (Fig. 25.2). Retinopathy, coronary artery disease, neuropathy, and arterial hypertension were observed in 20–32% of the insulin-dependent patients; nephropathy and peripheral artery disease were detected in 15–16% of insulin-dependent patients with hemochromatosis (unpublished data). One or more of these microvascular or macrovascular complications occurred in $>60\%$ of patients with insulin-dependent diabetes and hemochromatosis. The incidence of vascular complications in insulin-dependent hemochromatotic patients is, therefore, similar to that reported for patients with primary insulin-dependent diabetes of similar duration[57–59].

Effect of phlebotomy treatment on diabetes mellitus and impaired glucose tolerance

Treatment by repetitive phlebotomy resulted in depletion of excessive iron in 185 of 251 patients with hemochromatosis; the remaining 66 patients either died before iron depletion could be achieved or iron removal had not been achieved at the time of analysis of the data. The analysis of patients in whom complete iron depletion could be achieved showed that therapy did not eliminate insulin dependency (Table 25.5). However, in 19 of the 46 (41%) insulin-dependent patients in whom iron depletion had been achieved, the insulin dose could be reduced by more than four units daily for at least half a year. A smaller reduction over during a shorter time was not regarded as a 'significant' reduction due to treatment, but was interpreted as a variation due to other factors. The mean insulin dose in the total group of the 40 patients was 42.2 ± 3.2 units/day (mean $\pm$ SD). The mean insulin dose in the group of patients in whom the dose could be reduced by more than 4 units/day was 46.4 ± 4.2 units/day at entry and 37.8 ± 3.7 units/day after depletion of excessive iron (mean reduction = 8.6 units/day). In 27 of the 46 (59%) insulin-dependent patients, removal of iron did not significantly change the insulin dose required (normal variation defined as a change of $+4$ units/day).

In contrast to patients with insulin-dependent diabetes mellitus, less advanced alterations of glucose metabolism, such as non-insulin-dependent diabetes and impaired glucose tolerance, improved in about half of the patients in whom complete iron depletion was achieved (Table 25.5). Similar to insulin-dependent diabetes, hepatic cirrhosis

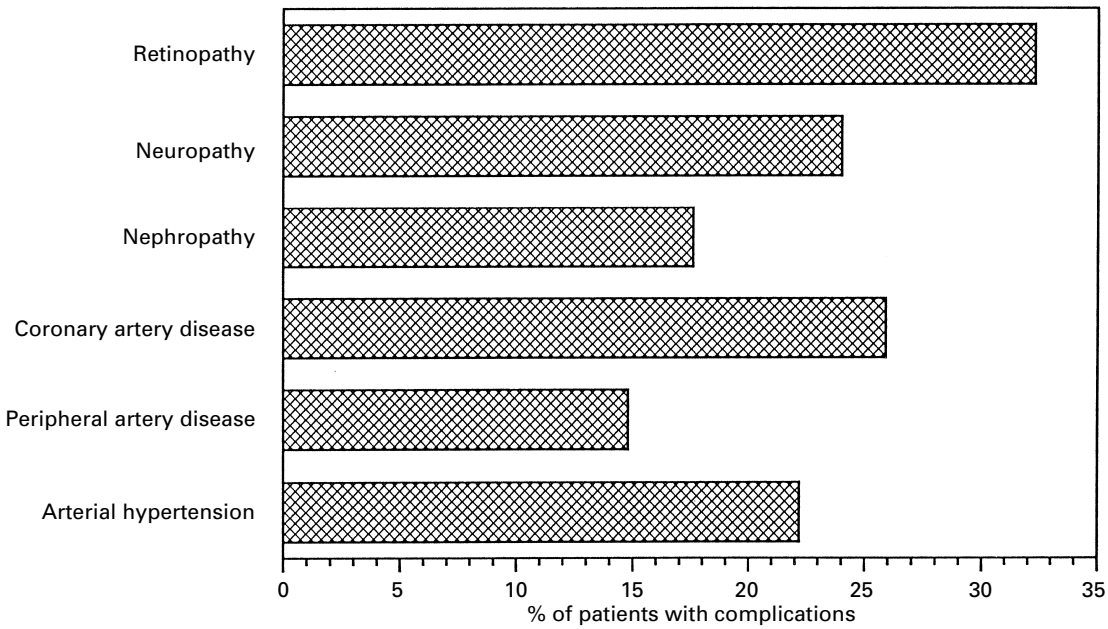

Fig. 25.2. Macro- and microvascular disease in patients with insulin-dependent diabetes mellitus due to hemochromatosis (mean duration of diabetes 11.6 ± 5.4 years).

could not be reversed by removal of iron[12]. However, less advanced stages like hepatic fibrosis, and the elevated concentrations of hepatic enzymes and abnormalities in hepatic function responded well to removal of iron[12]. Experimental data have shown that insulin resistance due to hepatic disease causes the early alteration of glucose metabolism in hemochromatosis[10]. Thus, the improvement of less advanced alterations of glucose metabolism seen after removal of iron are likely to reflect the improvement of hepatic disease, and thus probably improvement of insulin resistance. The lack of response of insulin-dependent patients with hemochromatosis to removal of iron suggests that the insulin deficiency is caused by irreversible destruction of pancreatic beta-cells due to long-standing massive deposition of iron. Indeed, histological studies reveal that pancreatic beta-cells may be heavily loaded with iron, whereas the alpha-cells are usually much less affected.

Results comparing the frequency of diabetes mellitus and impaired glucose tolerance after depletion of excessive iron versus the time at the end of the study did not show any significant further alterations during an interval of approximately nine years. There was no further deterioration nor improvement of glucose metabolism in the absence of gross iron overload.

Degree of iron overload and severity of alterations of glucose metabolism

The correlation between the amount of mobilizable iron that reflects the degree and duration of iron overload, and the progression of alterations of glucose metabolism are displayed in Fig. 25.2 and in Table 25.6. The amount of mobilizable iron was calculated from the amount of blood removed by phlebotomy during the initial period of treatment until iron depletion had been proven by hepatic biopsy. The group of 183 patients in whom complete iron depletion could be achieved had 21.2 ± 1.1 g (mean ± SD) of mobilizable iron. This amount is typical of excess iron stored in patients with hemochromatosis, and is 8–10 times that of healthy subjects. Analysis of mobilizable iron in patients grouped according to the severity of the alteration of their glucose metabolism showed a stepwise increase in mobilizable iron. This relationship was apparent as the alterations of glucose metabolism progressed from impaired glucose tolerance to non-insulin-dependent diabetes, and finally, to insulin-dependent diabetes (data not shown in detail). Statistically, hemochromatotic patients without diabetes mellitus had significantly less mobilizable iron than patients who had already developed non-insulin-dependent diabetes or insulin-dependent diabetes (Table 25.6). Thus, the severity of iron overload appears to reflect the degree of alteration in glucose metabolism.

Table 25.5. Changes in clinical features during initial treatment in 182 hemochromatosis patients with biopsy-proven iron depletion[a]

Feature	At time of diagnosis	After iron depletion[b]		
		Improved	Unchanged	Worsened
	Percent of group (number of patients)			
Diabetes mellitus ($n=81$)	44 (81)	41 (33)	53 (43)	6 (5)
Insulin-dependent	25 (46)	41 (19)[c]	50 (23)	10 (4)
Non-insulin dependent	19 (35)	40 (14)	57 (20)	3 (1)
Glucose tolerance ($n=101$)	56 (101)	10 (10)	87 (88)	3 (3)
Impaired	15 (27)	37 (10)	56 (15)	7 (2)
Normal	40 (74)	—	99 (73)	1 (1)

Notes:

[a] Sufficient follow-up information could not be obtained in 2 of the 185 patients with biopsy-proven iron depletion.

[b] The daily insulin dose could be reduced in 19 of the 46 insulin-dependent patients, but insulin dependency could not be abolished in any patient.

[c] Data obtained from a 6-month period after the end of the initial phlebotomy period.

Table 25.6. Iron (in grams[a]) removed by phlebotomies in 185 hemochromatosis patients with biopsy-documented iron removal

All patients ($n=185$)	21.2 ± 1.1		
	Present	Absent	p value
Liver cirrhosis	25.7 ± 1.7	14.8 ± 1.5	≤ 0.001
Diabetes mellitus	26.3 ± 1.7	16.5 ± 1.5	≤ 0.001
Arthropathy	21.4 ± 1.7	20.9 ± 1.8	> 0.2
Outcome	Survived ($n=150$)	Died ($n=35$)	
	19.4 ± 1.7	29.1 ± 2.6	≤ 0.01

[a] The amount of iron removed was calculated by assuming that 1 liter of blood contains ~500 mg of elemental iron. Data are displayed as shown as grams of iron (mean ± S.E.) for patients in whom iron depletion could be documented by repeated hepatic biopsy.

Diabetes mellitus and survival in hemochromatosis

In a retrospective study, survival and causes of death were analyzed among 251 patients with hemochromatosis diagnosed between 1947 and 1991[12]. The mean follow-up was 10.5 ± 5.5 years (mean ± SD). Cumulative survival was 93% at 5 years, 77% at 10 years, 62% at 15 years, and 55% at 20 years (Fig. 25.3). Survival was significantly reduced in patients who had diabetes mellitus at the outset of the study in comparison to patients without diabetes mellitus ($p < 0.002$ by logrank test[60] (Fig. 25.3). The survival curve for the non-diabetic patients was virtually identical to that of the expected survival in a matched normal population (Fig. 25.3). Survival was also significantly reduced in the presence of hepatic cirrhosis and in patients with high iron stores that could not be depleted during the first 18 months of phlebotomy treatment[11]. However, the patients with diabetes, most of whom also had hepatic cirrhosis, had the worst prognosis of all subgroups analyzed[11]. These results show that hepatic cirrhosis and diabetes mellitus are late sequelae of iron overload, and are associated with a shortened life expectancy. Hemochromatosis should be diagnosed before cirrhosis and diabetes have developed, in which case patients treated by 'prophylactic' phlebotomy appear to have a normal life expectancy[11, 12].

Diabetes mellitus as a cause of death

69 of our 251 patients with hemochromatosis died during the study period (1947–1991)[12]. Only four hemochroma-

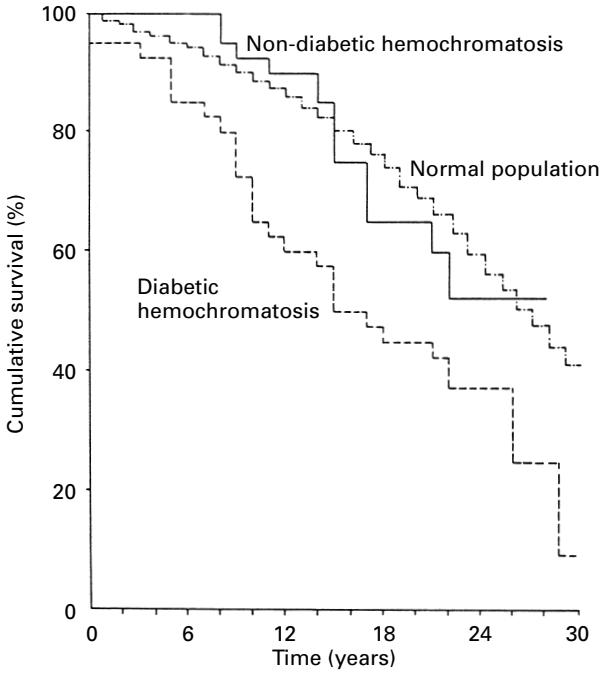

Fig. 25.3. Cumulative survival in 120 diabetic and 131 non-diabetic patients with hemochromatosis (H). Survival was significantly reduced in diabetic patients when compared with non-diabetic patients ($p \leq 0.01$; log-rank test). The survival of non-diabetic patients was similar to that of a sex- and age-matched normal control subjects (broken line), whereas diabetic patients had reduced survival (expected survival rates lying outside the confidence intervals are not shown).

totic patients died as a direct consequence of diabetes mellitus; two of these died from ketoacidotic coma, one died from hypoglycemia induced by inadequate insulin therapy, and one died of renal failure due to diabetic nephropathy. Although the absolute number of patients who died as a direct consequence of diabetes mellitus is small, patients with hemochromatosis have a 14-fold greater risk of death from diabetes mellitus than persons in a sex- and age-matched normal population. In addition, other causes of death may indirectly be linked with the accompanying diabetes, e.g., myocardial infarction, peritonitis due to proctocolitis and diverticulitis, and defective wound healing after trauma.

Recent investigators attributed the reduced life expectancy of hemochromatotic patients with diabetes primarily to the complications of massive iron overload of liver and heart, and not to the diabetes complications *per se*[21,61]. In our recent cohort study, however, diabetes was an independent risk factor in the multivariate analysis[12]. That diabetes was not a significant risk factor in the Canadian study[21] might be due to the low prevalence of diabetes (only 21.1%) in the latter cohort.

Conclusions

Diabetes mellitus in hemochromatosis appears to be caused by iron deposition in the pancreas and in the liver that leads to damage of these organs. Hepatic iron accumulation is primarily responsible for hyperinsulinemia and insulin resistance in early stages of iron overload. In advanced stages, impaired insulin secretion is caused by selective deposition of excess iron in pancreatic beta-cells that are irreversibly damaged by iron overload. Today, there is no substantial evidence that diabetes in patients with hemochromatosis is due to a genetic factor in addition to the heritable defect that leads to iron overload.

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26

Non-diabetic endocrinopathy in hemochromatosis

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Introduction

The earliest reports of hemochromatosis recognized involvement of the endocrine system in this condition; diabetes mellitus was the first recorded endocrine disorder[1]. With wider recognition of hemochromatosis, involvement of other endocrine glands was suspected and early reports emphasized the occurrence of hypogonadism[2, 3]. Diagnosis at that time was based solely on clinical features, though in some instances was supported by histological data obtained at autopsy. In 1935, Sheldon described in detail the occurrence of sexual hypoplasia presenting as 'loss of hair and impotence with atrophic testes and the body showing feminine characteristics.' He suggested that this clinical syndrome was the most frequent manifestation of hemochromatosis, after the classical triad of diabetes mellitus, hepatomegaly, and pigmentation[2]. Since then, other endocrinopathies have been reported in association with hemochromatosis, though none with the frequency of hypogonadism.

In the foregoing account, the non-diabetic endocrinopathies associated with hemochromatosis are discussed. There are profound differences in the relative frequency with which the various glands are affected. This may simply be a function of the severity and duration of iron deposition. However, one might question this hypothesis, bearing in mind the severity of iron deposition seen in certain glands, notably the thyroid and parathyroids, whose function seems rarely to be affected. However, there may be a variability in the vulnerability of different secretory cells to the toxic effects of iron. The reason for this is not apparent, nor is it clear why iron deposition should be more severe in some glands than in others. At present, methods for quantifying iron in endocrine cells is relatively imprecise, and improved methods may be informative.

The prevalence of dysfunction of individual glands varies strikingly in different studies, and there are a number of possible explanations for this observation. For example, in several studies, diagnosis of hemochromatosis was made relatively late and so iron deposition was substantial; this increases the risk of organ damage. Referral bias may be a factor, because specialists in gastroenterology, hepatology, or diabetology are more likely to attract complex or advanced cases and reports from such physicians and their centers are likely to record a higher prevalence of endocrinopathy. Several studies fail to clarify the hepatic status of patients and, in particular, whether hepatic cirrhosis (which itself can be associated with endocrine abnormalities) was present. Some reports do not document the iron status of the patients at the time of investigation. Because alcohol intake can affect sexual function and sex hormone production, this aspect of the medical history is important but is often not regularly recorded. Finally, centers with a particular interest in hemochromatosis that pursue active screening programs are more likely to have large numbers of patients with lesser degrees of iron deposition and consequently a lower prevalence of organ damage.

Iron deposition in the anterior pituitary gland

Deposition of iron in the anterior pituitary gland occurs frequently in patients with hemochromatosis[2]. In an autopsy series of 211 cases, McDonald et al. observed iron deposition in the anterior pituitary gland in 86%[4]. Although the consensus view has been that iron is predominantly deposited in the gonadotroph cells, there is evidence of iron deposition in other secretory cells within the gland, though generally to a lesser degree. However, there is little information available that gives details of iron deposition in specifically identified secretory cells. Using a tetrachrome stain, Peillon et al. observed the greatest

Table 26.1. Impaired responses to dynamic stimuli in hemochromatosis patients[a]

Gonadotrophins	Growth hormone	Prolactin	TSH	References
2/12	0/10	—	1/11	11
10/12	2/3	—	0/12	21
10/10	—	—	—	18
17/36	3/11	Normal mean values	2/19	12
7/12	—	9/12	0/12	20
7/14	0/14 (mean values)	Normal mean values	Normal mean values	19
7/11	Decreased mean values	Decreased mean values[b]	Normal mean values	22

Notes:

[a] These data are tabulated from reports of at least ten subjects in whom basal sex hormone values were recorded; data from cases in which stimuli appeared to be inadequate are excluded. The data represent the numbers of subjects with impaired responses to dynamic stimuli observed, and the total numbers of patients evaluated.

[b] There is uncertainty whether hypoglycemia was achieved during testing in these subjects.

degree of iron deposition within the gonadotrophs[5], an observation supported by the more specific studies of Bergeron et al. who used immunocytochemical techniques in six patients with iron overload, two of whom had hemochromatosis[6]. These observations are consistent with the clinical experience that impaired function of the gonadotrophs and consequent hypogonadism is the most commonly encountered non-diabetic endocrinopathy, and that the pattern of anterior pituitary dysfunction that occurs in hemochromatosis largely parallels that seen in other pathological states affecting that gland.

Hypothalamic–pituitary–gonadal axis

Anterior pituitary

Clinical features of hypogonadism in hemochromatosis have long been recognized. The earliest reports in which hormonal assays were used suggested that hypogonadism was extremely common, but the relatively insensitive nature of the assays and the limited data create difficulty in interpreting the findings[7–9]. With the availability of more specific and sensitive hormonal assays and the availability of gonadotrophin-releasing hormone (GnRH), the pituitary-gonadal axis was studied in greater detail. Many studies involved relatively small numbers of patients and have been helpful in evaluating the pathophysiology of the problem, but have been of little value in assessing the prevalence of the disorder. Tournaire et al.[10] found impaired gonadotrophin responses to GnRH in each of five patients tested; Walsh et al. found low testosterone values and impaired gonadotrophin responses to GnRH in two of 12 patients[11]. In the largest studies involving dynamic testing, Charbonnel found that 47% of 36 patients (most of whom had been iron-depleted by therapeutic phlebotomy) had subnormal serum testosterone concentrations with low or normal basal gonadotrophin concentrations that did not respond to GnRH administration[12]. Other publications of single cases or small numbers of patients in which the pituitary–gonadal axis was fully evaluated reported similar findings, i.e., when hypogonadism is present, serum testosterone concentrations are subnormal and basal gonadotrophin concentrations are usually low or within the 'normal range' (rather than being appropriately elevated), and responses to GnRH administration are impaired[13–17]. These observations are consistent with gonadotrophin dysfunction, although they do not exclude a hypothalamic defect (Table 26.1).

Hypothalamus

A hypothalamic defect may be at least partly responsible for hypogonadism in hemochromatosis. Williams et al. reported a patient with transfusional iron overload in whom basal serum testosterone, gonadotrophin, and thyroxine concentrations were subnormal, but in whom thyroid-stimulating hormone (TSH) values were normal[23]. After thyrotropin-releasing hormone (TRH) stimulation, there was a delayed and exaggerated TSH response in this patient; luteinizing hormone (LH) responded normally to GnRH administration. Simonski reported a man with hypogonadism and low serum testosterone concentrations in whom normal LH values responded briskly to GnRH; clomiphene administration failed to elicit a response. These findings are consistent with a hypothalamic defect[24]. In

seven untreated men with hemochromatosis, Piperno et al. observed relatively low iron stores, no hepatic injury, and no clinical evidence of hypogonadism[25]. Mean serum testosterone and gonadotrophin concentrations were lower than in normal control subjects. Serum testosterone concentrations responded normally to human chorionic gonadotrophin (HCG) administration, and LH responses to GnRH administration were normal or exaggerated. The authors suggested that the findings are consistent with a hypothalamic defect that possibly develops at an early stage of iron overload, with the subsequent occurrence of pituitary and testicular failure as iron overload increases[25]. Although a clomiphene test was not performed in these patients, these remarkable findings in patients at such an early stage of iron overload need to be confirmed. In contrast, Duranteau et al. administered pulse doses of GnRH over 15–30 days to seven untreated men with iron overload (five had hemochromatosis) who had hypogonadism and low testosterone and LH values. This had no effect on their hormone responses, suggesting that they had a pituitary rather than a hypothalamic defect.[26]. If the hypothesis of Piperno et al.[25] is correct, however, subjects with more severe iron overload may have thalamic and pituitary dysfunction. Further investigation should include postmortem histopathologic studies of the hypothalamus, quantification of hypothalamic iron stores using MRI, and evaluation of hypothalamic function in patients with gonadotrophin deficiency and other pituitary defects.

The report of Kelly et al.[27] is of value in assessing the current prevalence of hypogonadism. It noted that just five of 41 males and none of 23 females were hypogonadal. Such observations are more likely to reflect present-day experience and are the result of earlier diagnosis, often the result of family screening and greater awareness of the disorder.

Most data are derived from the study of men, and less information is available on women with hemochromatosis[13, 18, 20, 21, 28–31]. A few cases of gonadotrophin deficiency have been recorded in pre- and post-menopausal women, although the largest study (23 women) found no evidence of abnormal pituitary–gonadal function[27]. Thus it appears that when hypogonadism does occur in women, it follows the pattern described in the majority of affected males and is due to gonadotrophin deficiency.

Gonads

Testes

Pathological changes in the testes in hemochromatosis are well-described. Testicular atrophy may be obvious in patients with hypogonadism, although significant functional impairment may be present when testicular size is normal[16]. The most frequently observed morphological change noted is sclerosis or atrophy of the seminiferous tubules; decreased numbers or absence of Leydig cells have also been reported. Iron deposition in the testes is found predominantly in the walls of the blood vessels and in some cases is confined to that area. Deposits also occur occasionally in the seminiferous tubules and, less frequently, in the Leydig cells. Thus significant deposition of iron outside the blood vessels is relatively uncommon and, when present, is usually mild, even in patients with established hypogonadism[2, 3, 6].

In the majority of patients with hypogonadism, the defect is a central rather than a peripheral one, an observation supported by the finding that serum or plasma testosterone concentrations may increase in some cases after HCG therapy[24, 27, 32]. That the serum testosterone concentration does not increase in all cases after HCG administration may reflect a more advanced degree of testicular atrophy due to longstanding lack of stimulation by gonadotrophins[21]. It may also indicate the occasional concurrence of central and gonadal defects. However, exceptional cases of primary gonadal failure have been reported[27].

Ovaries

There is a paucity of histological data on the ovary in hemochromatosis. Sheldon, from a very limited number of cases, concluded that there was no marked deposition of iron in the ovaries or uterus[2]. Farina et al. reported the case of a young woman with hypogonadotrophic hypogonadism in whom an ovarian biopsy was performed after iron depletion; no iron was detected, but no other information was given[29]. Hypogonadism in females is invariably due to gonadotrophin deficiency. Meyer et al. report the case of a young woman who developed secondary amenorrhea after excessive iron stores had been depleted by therapeutic phlebotomy. Her gonadotrophin responses to GnRH were impaired, but there was a positive estradiol response to human menopausal gonadotrophin (HMG), indicating preservation of ovarian function[30].

Clinical features

The clinical features of hypogonadism in patients with hemochromatosis are not especially different to those of hypogonadism due to other causes. The clinical syndrome is considerably better recognized in males in whom reduced body hair, altered fat distribution, gynecomastia, and testicular atrophy of variable degrees are the predom-

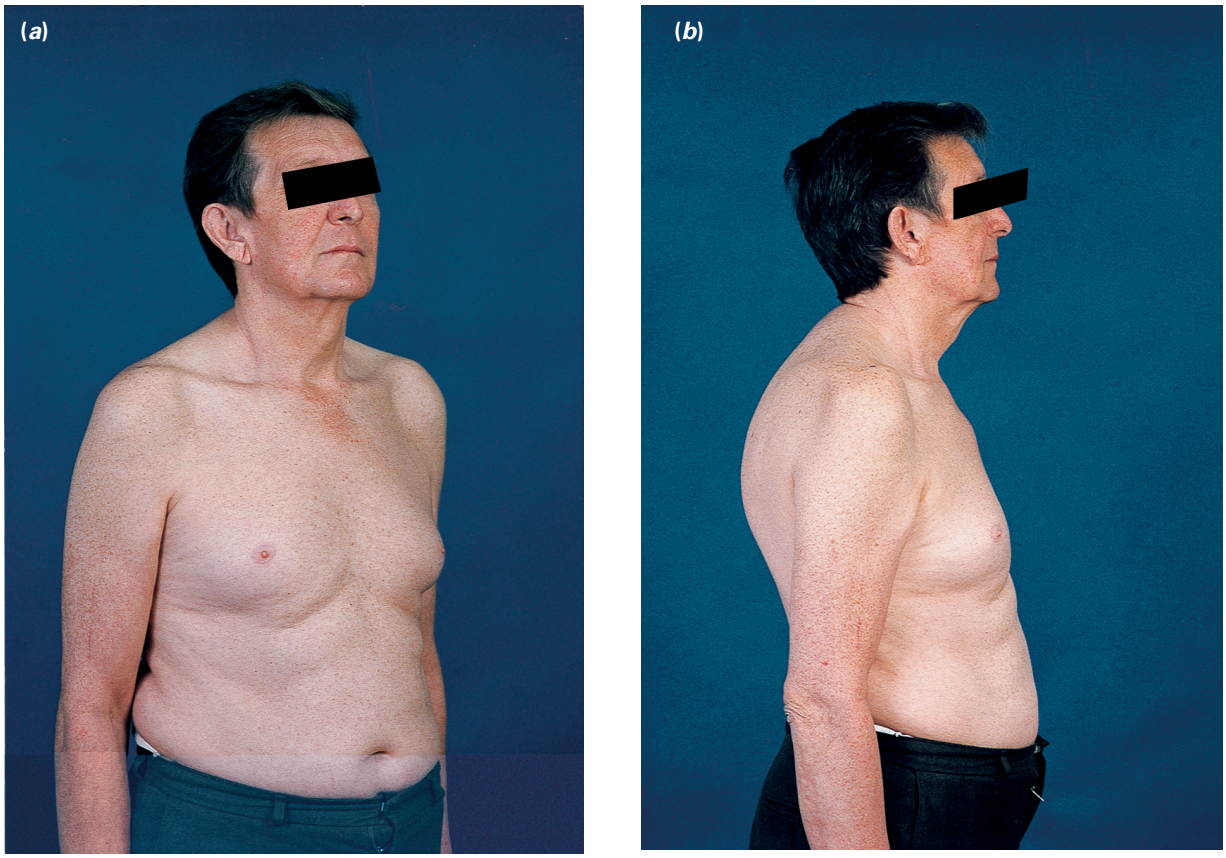

Fig. 26.1. Features of hypogonadism (*a*) and kyphosis due to osteoporotic vertebral collapse (*b*) in a man with hemochromatosis and gonadotrophin deficiency.

inant signs. The more common symptoms include reduced shaving, loss of libido, failure of ejaculation, and impotence, though in our experience a significant proportion of males with hypogonadism do not volunteer these symptoms. In young women, loss of libido, amenorrhea, and infertility are the main symptoms; loss of libido may be the only symptom in post-menopausal women. It might be anticipated that impaired gonadotrophin secretion is more common among older (post-menopausal) women. If it does occur, particularly as an isolated pituitary defect, it may be overlooked unless routine assessment of anterior pituitary function is performed because there may be little else to indicate gonadotrophin deficiency. Youth does not appear to protect against hypogonadism; gonadotrophin deficiency can occur in men and women in the second and third decades of life[13, 28, 29, 31]. Although hemochromatosis must be an extremely rare cause of hypogonadism in early life, it is one that could be overlooked easily.

There is discordance between the clinical features of hypogonadism and impaired endocrine function in some males with hemochromatosis. Walsh et al.[11] observed features suggestive of hypogonadism in nine of twelve men, but in only two cases were the plasma testosterone concentrations reduced; similar observations have been reported by others[33]. There are several possible explanations for these observations. Impotence is often difficult to evaluate and its potential causes are many. The fatigue and debility observed in many patients with hemochromatosis are often underestimated and may be a potential cause of sexual dysfunction, a suggestion supported by the observation of Barton et al. who found that iron depletion in two men with iron overload, impotence, and normal testosterone values resulted in correction of the fatigue and resolution of impotence[34]. Sexual dysfunction (in the absence of hormonal insufficiency) is a relatively common complication of diabetes mellitus, a frequent complication of hemochromatosis. Other factors such as drug therapy, alcoholism, hepatic cirrhosis, increasing age, vascular disease, and psychological factors may likewise contribute

to or cause impotence. Furthermore, testicular atrophy, a sign that strongly suggests hypogonadism, has occasionally occurred in patients with hemochromatosis in the presence of normal plasma testosterone concentrations[27]. Testicular volume is determined mainly by the seminiferous tubules and it seems likely that tubular damage may occur in the testes in occasional cases in the absence of significant Leydig cell destruction. This possibility could be evaluated further by semen analysis in affected patients.

A reliable diagnosis of hypogonadism cannot be based solely on clinical features and must be supported by appropriate hormonal measurements. This should be performed routinely in all new patients with hemochromatosis; minimal investigation requires measurement of gonadotrophins and testosterone concentration in males and gonadotrophins in non-menstruating females. The need for further investigation is dictated by the results, and may include dynamic testing with GnRH and, in occasional cases, testing the hypothalamus using either clomiphene or prolonged GnRH stimulation.

Treatment

In males with hypogonadism, testosterone replacement is the appropriate therapy. Although this can be administered by transdermal application, experience in hemochromatosis has so far been with parenteral therapy, usually using testosterone enanthate given by intramuscular injection. This treatment may restore libido and potency, and patients often report that they have improved muscle strength and a greater sense of well-being. These benefits become apparent shortly after commencement of treatment, may last for two to three weeks after each injection, and do not appear to diminish with long-term treatment. No significant adverse effects were observed in patients who were carefully monitored for periods as long as 96 months[35]. Occasionally, impotence in men with low testosterone levels does not improve or does so only temporarily. In these cases, one must conclude that other factors complicate the problem, and the presence of sexual dysfunction in men with normal testosterone levels is not likely to be reversed by replacement therapy.

Patients with hypogonadotrophic hypogonadism are usually infertile. In clinical practice, this is not a major problem because most patients do not seek fertility. However, in cases in which infertility persists despite iron depletion therapy, fertility can sometimes be restored using HCG and HMG, though such successes have been recorded rarely[31, 36]. In young females with hypogonadism, cyclical estrogen and progesterone replacement therapy is appropriate. If correction of infertility due to gonadotrophin deficiency is desired, gonadotrophin therapy should be tried, in addition to intensive phlebotomy to achieve iron depletion. Restoration of fertility in a 26-year-old woman with secondary amenorrhea was accomplished using such treatment[29].

The results of iron depletion therapy

For many years it was believed that iron depletion had no beneficial effect on the hypogonadism associated with hemochromatosis. However, Kelly et al. documented the results of phlebotomy therapy in three patients with hypogonadotrophic hypogonadism. In one of these cases and in one other case report[37] hormone values became normal and sexual function improved. Similar findings were noted in a case of primary hypogonadism[27]. Siemons and Mahler reported the case of an 37-year-old man who had impotence, azoospermia, hypogonadotrophic hypogonadism, and hepatic cirrhosis. A testicular biopsy specimen showed atrophy of the seminiferous tubules and absence of Leydig cells; no significant iron deposits were visualized. After intensive iron depletion therapy, his serum gonadotrophin and testosterone concentrations returned to normal, his sexual function recovered, and he subsequently fathered a child[38]. In contrast, Cundy et al.[39] found that correction of iron overload in six impotent men with hypogonadotrophic hypogonadism resulted in improved sexual function in only one (whose serum testosterone concentrations and sperm count remained subnormal nonetheless). In a detailed study, Lufkin et al. found that hypogonadotrophic hypogonadism was not corrected by iron depletion therapy in any of seven men[22].

Thus hypogonadism may be reversed by iron depletion therapy in some but not all cases, and the likelihood of success seems to be greater in younger subjects. In patients who are treated with testosterone supplements during therapeutic phlebotomy, hormone replacement therapy should be withdrawn when body iron stores have been normalized; pituitary–gonadal function should be reassessed thereafter.

Growth hormone

In the anterior pituitary gland, iron deposition in somatotroph cells is less marked than in the gonadotrophs. In early investigations of 35 cases of hemochromatosis, GH responses were impaired in 13 patients, several of whom had testicular atrophy. However, evaluation of the findings is difficult because the criteria for diagnosing hypoglyce-

mia were less stringent than would currently be applied[8, 9]. Lufkin et al. observed reduced mean serum GH concentrations after hypoglycemia in 11 untreated patients, seven of whom had hypogonadism (no individual results were given). After restoration of normal body iron stores, no improvement in growth hormone responses was observed[22]. In four other studies in which 27 patients were investigated using various stimuli, GH responses were impaired in six, five of whom had hypogonadism[12, 13, 16, 21]. In contrast, GH responses to hypoglycemia were normal in fourteen other subjects[11, 40].

It is not possible to determine the prevalence of GH deficiency in hemochromatosis based on available reports. However, its occurrence is usually associated with hypogonadism. Impaired responses may reflect iron deposition within somatotroph cells. However, in some cases the impaired responses may be influenced by the co-existence of hypogonadism, because GH responses to hypoglycemia are impaired when hypogonadism is present[41].

There is no reported experience in administration of growth hormone to patients with hemochromatosis. At present, the merits of growth hormone replacement therapy in adults with growth hormone deficiency of any cause are uncertain, although it seems likely that clearer guidelines will emerge. In many studies of pituitary function, assessment of somatotroph function was not undertaken. If growth hormone treatment were found to be beneficial, then clinicians who treat patients with hemochromatosis must determine the growth hormone status of their patients carefully. If growth hormone replacement is indicated in patients with hemochromatosis who have co-existing hypogonadism, it will be desirable to confirm that growth hormone deficiency persists after sex hormone replacement therapy has been initiated. Because it is possible that growth hormone deficiency may be corrected by iron depletion therapy, the growth hormone status of such patients should be reevaluated when iron overload has been corrected.

Pituitary–thyroidal axis

Thyrotroph cells

Although iron deposition can occur in thyrotrophs, it appears to occur in only a minority of the cells and the deposits appear substantially less than those found in the gonadotrophs[6]. Thus, adequately documented cases of impaired thyrotroph function have been recorded in a very small number of cases. Based on low protein-bound iodine levels and the thyroid response to thyroid-stimulating hormone (TSH) administration, earlier investigators found that secondary hypothyroidism occurred in three of 48 cases[8, 9], at least two of whom had gonadotrophin deficiency. There have been other case reports of TSH deficiency co-existing with gonadotrophin deficiency[17, 37, 38]. Several detailed studies of the pituitary–thyroidal axis have been reported in which no abnormality was found, even in patients with other pituitary defects. For example, in each of 24 patients (16 of whom had hypogonadism), the pituitary–thyroid axis was intact[20, 21]. In 49 patients, Edwards et al. found no evidence of thyrotroph dysfunction[42]. Thus, detectable impairment of thyrotroph function is extremely rare, even in those with other pituitary abnormalities; when it does occur, it is rarely an isolated pituitary abnormality.

Thyroid gland

In striking contrast to the thyrotrophs, iron deposition occurs in the thyroid gland in a majority of patients with hemochromatosis and to a substantial degree[2, 4]. However, primary thyroid dysfunction occurs infrequently. From a total of 26 cases, Bezwoda et al.[21] and Varela et al.[19] observed two cases of primary hypothyroidism, both of whom had hypogonadism; whether these patients had anti-thyroid antibodies was not reported. Edwards et al. reported that three of 34 men (8.8%) but none of 15 women with hemochromatosis had primary hypothyroidism[42]. All affected patients had clinical features of hypothyroidism and elevated serum titers of antimicrosomal antibodies. Histological examination of the thyroid, undertaken at autopsy in one case, showed lymphocytic infiltration and near-total replacement of thyroid tissue with fibrous connective tissue. Residual follicular cells were atrophic and infiltrated with iron. In one case, thyroid function was normal when hemochromatosis was diagnosed, but clinically apparent hypothyroidism occurred only after iron depletion[42]. Konno et al. reported the case of a woman with primary hypothyroidism who also had elevated antimicrosomal antibodies; histological examination of a thyroid biopsy specimen showed features similar to those reported by Edwards et al.[43].

Primary hypothyroidism is a relatively common disorder that occurs with significantly greater frequency among females. Thus, it may occur coincidentally in persons who also have hemochromatosis. However, a prevalence of primary hypothyroidism of 8.8% among males with hemochromatosis raises the question of whether hemochromatosis predisposed to its occurrence. Edwards

et al. postulated that iron deposition could lead to tissue damage that stimulated autoantibody production, and that the higher prevalence of primary hypothyroidism among males might be due to the greater severity of their iron overload[42]. If this is correct, one might expect to find more frequent reports of primary hypothyroidism in patients with hemochromatosis. Furthermore, one might also expect to find that patients with primary hypothyroidism and iron overload due to other causes such as repeated transfusion might have anti-thyroid antibodies. However, there is little evidence to suggest that this is the case. Nonetheless, further investigation of the possibility that immunological mechanisms triggered by iron deposition may play a role in the causation of thyroid disease in hemochromatosis is needed.

Hyperthyroidism has been recorded among a few patients with hemochromatosis, though in most the diagnosis has not been confirmed by laboratory evaluation. There are reports of two adequately documented cases, but the coexistence of both disorders was probably fortuitous[42, 44].

Clinical features

The presentation of hypothyroidism that occurs in association with hemochromatosis is usually typical. However, another endocrine disorder is present in most cases and, most frequently, hypogonadism dominates the clinical picture (and perhaps the perception of the clinician). Consequently, hypothyroidism may be overlooked. Because non-specific symptoms such as fatigue and lethargy are common not only to hemochromatosis and a variety of other endocrine disorders, thyroid function must be assessed objectively in all cases of hemochromatosis when the latter condition is first diagnosed. Measurement of basal TSH and FT4 concentrations is adequate in most cases. When the results suggest primary hypothyroidism, anti-thyroid antibodies should be measured. In cases in which the results suggest a central defect, dynamic testing with TRH may help differentiate between a pituitary or hypothalamic defect. In the latter circumstance, the TSH response may be delayed.

Management

Regardless of its pathogenesis, the treatment of hypothyroidism in hemochromatosis is similar to that for other causes of hypothyroidism. Maintaining the serum TSH concentrations within the normal range is the target in primary hypothyroidism. In cases attributable to thyrotrophin deficiency, total T4 (or preferably FT4) should be measured and the concentrations should be kept within the mid-normal range. Careful consideration must be given to the patient's cardiac status. In patients who have cardiomyopathy or other cardiac disease, therapy should be initiated cautiously with thyroxine, 25 μg daily. This should be increased in increments of 25 μg daily at intervals of at least three or four weeks; careful monitoring of cardiac status and thyroid function tests should be performed. The pituitary–adrenal axis should also be assessed, especially in patients with secondary hypothyroidism, to ensure that there is no evidence of glucocorticoid deficiency. If this is present, it is better to initiate steroid replacement therapy before instituting thyroxine replacement. If hypothyroidism is diagnosed when iron overload is present, thyroid function should be re-evaluated after iron depletion therapy is completed.

Parathyroid glands

The parathyroid glands are almost invariably a site of iron deposition, and often to a considerable degree. However, parathyroid function appears to be affected rarely. A few patients have been reported in whom hypocalcaemia occurred and was thought to be due to hypoparathyroidism due to iron toxicity. Similar observations have occasionally been made in subjects with transfusional iron overload[45, 46].

Pituitary–adrenal axis

Iron deposits have been noted in a minority of the corticotroph cells in patients with hemochromatosis, similar to those observed in other types of iron overload. In contrast, iron deposition in the adrenal cortex is almost invariable. Sheldon reported that iron was present in all 49 cases for which adequate data were available. The deposits were predominantly in the zona glomerulosa (involved in 100% of cases). In the zona fasciculata, the deposits were less frequent and less pronounced, an observation confirmed in a small number of cases[6]. Whether significant impairment of the pituitary–adrenal axis occurs in hemochromatosis is controversial. In one large study, it was suggested that adrenocortical insufficiency of some degree was present in $>80\%$ of cases[47]. These conclusions were based on clinical features and urinary measurement of 17-OH corticosteroids and dihydroepiandrosterone (DHA) in the basal state, or after metyrapone administration or an 8-hour infusion of adrenocorticotropic hormone (ACTH). The authors also concluded that the defect was due to corticotroph

dysfunction and rarely involved the adrenal cortex in most cases[47]. Impaired cortisol responses to hypoglycemia have been reported in other cases[8, 9], but whether adequate hypoglycemia was achieved remains uncertain and so a clear conclusion cannot be drawn.

Several studies have investigated the pituitary–adrenal axis in patients with hemochromatosis, many of whom also had hypogonadism. Various stimuli have been used including insulin-induced hypoglycemia, metyrapone, or corticotrophin-releasing factor (CRF). In two studies involving a total of 25 patients, only mean values were reported and these were normal; no mention was made of any individual abnormality[19, 22]. In other studies involving a total of 46 patients in which individual results are documented, the possibility of an impaired adrenocortical response arose in only two instances[11, 13, 16, 17, 21, 40, 48]. In one of these, the response to metyrapone was blunted but hypoglycemia induced a normal response[16]. In the other case, the response to hypoglycemia was 24.9 μg/dl (normal >25 μg/dl)[21]. In most of these cases, gonadotrophin deficiency was present and the patients were in various stages of iron depletion therapy (Fig. 26.2).

Other studies have evaluated the pituitary–adrenal axis using the short Synacthen® (tetracosactrin) test. In a total of 37 subjects, some of whom were undergoing therapeutic phlebotomy and the majority of whom had hypogonadism, responses were normal in all patients (Fig. 26.3)[20, 32, 48].

Thus using generally accepted criteria and current laboratory methodology, it appears that no unequivocal case of corticotrophin or cortisol deficiency has been documented among patients with hemochromatosis. Accordingly, it is reasonable to postulate that the pituitary–adrenal axis is normally maintained in hemochromatosis due to the pattern of iron distribution within the adrenal gland and the relatively mild iron deposition found in the corticotrophs and the zona fasciculata.

Iron deposition in the zona glomerulosa is severe, and is of potential pathophysiologic importance. Because this area of the adrenal cortex is responsible for aldosterone production, it might be anticipated that mineralocorticoid deficiency would be a well-established feature of hemochromatosis. Surprisingly, only one case has thus far been reported. A patient who had gonadotrophin and thyrotrophin deficiency who was disabled by faintness and sweating associated with postural hypotension was found to have hypoaldosteronism, but had no evidence of glucocorticoid deficiency (Fig. 26.4). In a subsequent systematic study of mineralocorticoid status in 11 patients with hemochromatosis, no instance of aldosterone deficiency was detected[49]. However, evidence of mineralocorticoid deficiency has thus far been sought in extremely few patients with hemochromatosis, and this area merits further investigation.

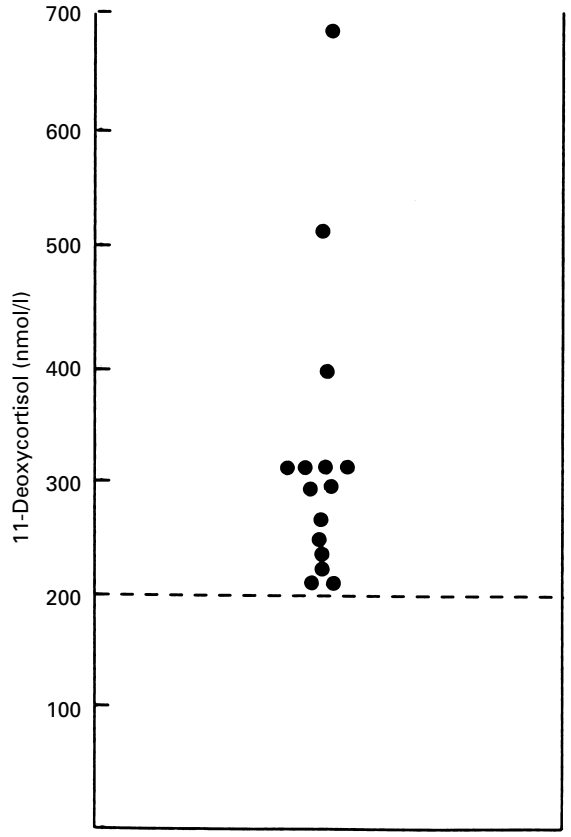

Fig. 26.2. 11-Deoxycortisol responses in 15 hemochromatosis patients after an overnight metyrapone test. The normal response is >200 nmol/l. (Reproduced with permission of *Clinical Endocrinology.*)

Clinical features

Glucocorticoid deficiency has not been clearly identified in patients with hemochromatosis. If it were to occur, its clinical manifestations are not likely to be unusual. However, the diagnosis could be delayed in patients with hemochromatosis in whom fatigue or pigmentation are prominent. Thus, a high index of clinical suspicion is required and thorough evaluations should include blood pressure measurements in erect and supine positions; postural hypotension might also suggest the possible occurrence of aldosterone deficiency. The diagnosis of adrenocortical deficiency cannot be established solely on the basis of clinical features, and in all suspected cases

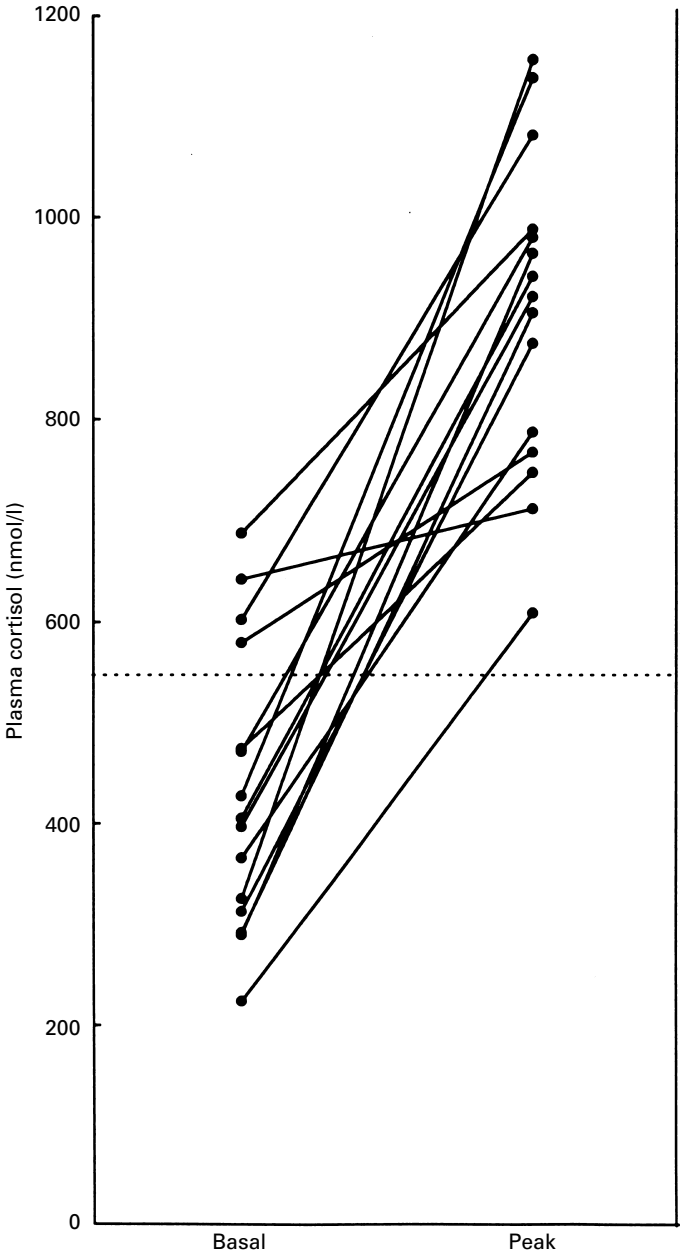

Fig. 26.3. Plasma cortisol values in the basal state and peak values obtained after tetracosactrin administration in patients with hemochromatosis. (Reproduced with permission of *Clinical Endocrinology.*) A peak cortisol value of ≥ 550 nmols/l is normal (hatched line).

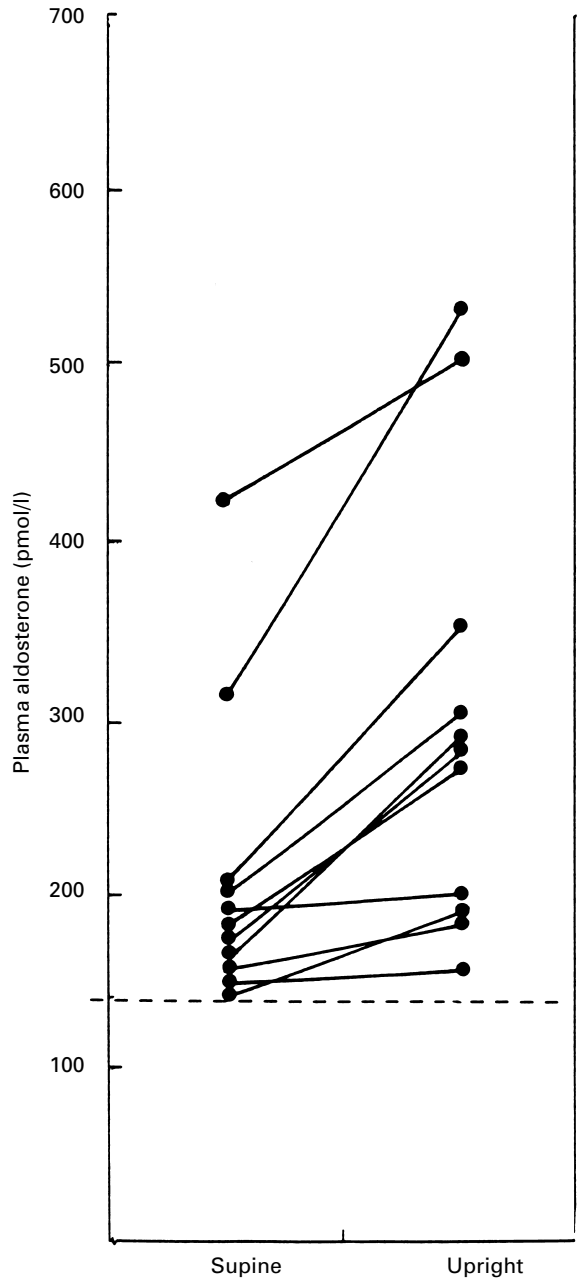

Fig. 26.4. Plasma aldosterone concentrations in the supine basal state and after 3 hours in the upright position in patients with hemochromatosis; normal standing range is 140–1400 pmol/l (Reproduced with permission of *Clinical Endocrinology.*)

appropriate biochemical investigations must be undertaken to support clinical suspicion.

At present, it is difficult to justify routine assessment of the hypothalamic–pituitary–adrenal axis in patients with hemochromatosis. However, the matter merits further investigation in cases in which there is clinical suspicion of adrenocortical deficiency, or in which there is established dysfunction of any of the secretory cells of the anterior pituitary gland. A simple procedure, easily done in the outpatient setting, is the short Synacthen® test. However, an

abnormal response does not define whether the site of the defect is at the hypothalamic, pituitary, or adrenal level. A normal response is found in some patients who have acute pituitary failure, but whether a normal response will occur in chronic situations such as hemochromatosis remains controversial[50]. In cases in which the result is abnormal, further investigation should be undertaken, including simultaneous measurement of basal cortisol and ACTH concentrations, and performing an overnight metyrapone test, an insulin stress test, or CRF stimulation.

Management

If adrenocortical deficiency due primarily to an adrenal defect is detected, hydrocortisone and fludrocortisone therapy are required. If the defect is central, the latter is not necessary. In aldosterone deficiency, fludrocortisone is the treatment of choice; the dose must be titrated against the satisfactory correction of postural hypotension and the avoidance of edema and electrolyte abnormalities. Although measurement of plasma renin concentrations may be helpful in assessing dosage, it is not necessary.

Prolactin

After gonadotrophs, the lactotrophs are most involved by iron deposition in hemochromatosis or by iron overload of other causes[6]. The earliest studies of prolactin (PRL) in patients with hemochromatosis were confined to basal measurements that were normal[11]. Using dynamic testing, impaired responses to TRH were observed although TSH responses were normal; hypogonadism was present in most cases[16, 20, 40]. In eleven men with untreated hemochromatosis, most of whom had hypogonadism, Lufkin found that the mean PRL responses to TRH were impaired and did not improve following normalization of body iron stores[22]. As with gonadotrophins, impaired PRL responses may be found at a relatively early age, as in three subjects $\leq$20 years of age, each of whom had hypogonadism[31]. Impaired responses may also be observed when iron overload is relatively mild[25]. In contrast, no evidence of impaired PRL responses were observed in any of 33 patients, almost half of whom had hypogonadism and some of whom had already completed therapeutic phlebotomy[12, 19]. There is no obvious explanation for these apparently contradictory findings.

Thus, lactrotroph function may be impaired in some patients with hemochromatosis, and this often occurs in association with gonadotrophin deficiency. Although iron toxicity is likely to be responsible for these abnormalities, whether the defect is at hypothalamic or pituitary level, or both, another possible explanation remains. Walton et al. suggested that impaired PRL responses might be related to the co-existence of subnormal circulating concentrations of sex hormones due to gonadotrophin deficiency[20]. PRL responses to TRH are reduced in patients with isolated gonadotrophin deficiency; the administration of estrogen and testosterone to hypogonadotrophic women and men, respectively, is associated with an increase in basal values and responses to TRH stimulation[51]. However, this cannot be the complete explanation, because not all patients with subnormal PRL values have hypogonadism. There is no convincing evidence that impaired PRL responses are corrected by iron depletion therapy.

Posterior pituitary gland

Information on the effects of hemochromatosis on the posterior pituitary gland is sparse. However, there is no evidence of iron deposition in the posterior pituitary gland, nor is there evidence to indicate that vasopressin secretion is disturbed in patients with hemochromatosis.

Conclusions

Hypogonadism is the most frequent non-diabetic endocrinopathy associated with hemochromatosis and is almost invariably due to gonadotrophin deficiency, reflecting the central origin of the problem. Its reported prevalence varies from 15% to 100% in different studies. Gonadotrophin deficiency is often an isolated abnormality, but may occur in association with other pituitary abnormalities. Although less extensively studied, impaired GH and prolactin responses appear to be the next most common findings, although they rarely occur without other endocrine abnormalities. TSH deficiency is exceptional, and it is questionable whether ACTH deficiency has ever been adequately documented in patients with hemochromatosis. Peripheral gland involvement is rare; when it occurs, it may not always be due to hemochromatosis. There is a need for further functional studies of the hypothalamus, especially in the early stages of hemochromatosis. Additional histological and immunocytologic studies of the anterior pituitary gland are needed. The continued development of non-invasive techniques for quantification of intracellular iron may help to explain the varying responses of different endocrine cells to iron overload. The findings of earlier endocrinologic studies in hemochromatosis are not likely to be representative of current experience with hemochromatosis, because this

disorder is now diagnosed much earlier in its natural history than in the past. Further careful prospective investigation of the endocrine status of adequate numbers of unselected patients with hemochromatosis are necessary to make a valid assessment of the extent of endocrine dysfunction in this disorder.

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Cutaneous manifestations of hemochromatosis

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Introduction

Skin manifestations of hemochromatosis have been described by many authors[1–5], and studied comprehensively in a group of 100 patients by Chevrant-Breton et al.[6]. These manifestations are usually overlooked by the patient, and sometimes by the physician. This chapter describes the clinical and histologic findings of skin abnormalities associated with hemochromatosis, complications of iron overload, and related disorders.

Skin pigmentation

Clinical aspects

Hyperpigmentation is one of the most characteristic signs of hemochromatosis, first named 'bronzed diabetes.' With diabetes mellitus and hepatic cirrhosis, this major symptom belonged to the classical triad of hemochromatosis. Today, this triad is exceptional and occurs in less than 10% of patients in recent studies[7]. The frequency of pigmentation has changed progressively due to earlier diagnosis of hemochromatosis in many patients made possible by routine iron screening or family studies[7–11]. The incidence of hyperpigmentation and other skin manifestations has therefore fallen from 98% ([6]; Fig. 27.1) to 72% in symptomatic cases[5], to 5% in a series of Bacon et al.[11]. Hyperpigmentation is absent in the children of homozygous subjects[12].

Hyperpigmentation is usually diffuse and generalized, but is prominent in sun-exposed areas. It involves the external genitalia of one-third of patients, and flexion folds, scars, buccal mucosae, and perilimbic conjunctivae in one-fifth of patients[6]. The color of the pigmentation is often metallic gray (50% of cases), brown (20% of cases), or of mixed hue (30% of cases). It may be absent in persons with red hair[6]. Progression of the increased pigmentation occurs very slowly, and is usually unnoticed by the patient or family members. Typically, it appears in the second decade. Because the pigmentation is often considered to be a 'familial trait,' many affected persons do not seek medical attention. Hyperpigmentation often worsens during exacerbations of iron overload due to hemochromatosis, and regresses slowly but certainly after iron depletion therapy.

Histopathologic aspects

Microanatomical aspects of cutaneous manifestations of hemochromatosis have been described in some short case series[2, 13]; two larger series of more than 50 cases each allow more conclusions to be formulated[5, 6]. Melanosis demonstrated by Fontana-Masson's stain was observed in 52% of non-treated patients and in 59% of treated patients described by Chevrant-Breton et al.[6], and in 58% of the patients described by Milman et al.[5]. Melanin pigment is seen in the basal layer of the epidermis, or occurs diffusely throughout the epidermis; there is no dermal pigment incontinence. Hemosiderin visualized with Perls' acid ferrocyanide or Tirmann technique is present in more than 60% of cases studied[5, 6]. Siderosis in the skin is observed more frequently in untreated patients (87%) than in treated patients (37%)[6]. Iron-containing pigment is located within and around eccrine sweat glands. This seems to be a specific location for iron deposition, because similar findings are not observed in specimens from a control group[6]. In one ultrastructural study, siderotic pigment was localized in the clear cells as irregular-shaped membrane-bound bodies containing iron, the identity of which was verified by energy dispersive X-ray microanalysis[14]. However, similar deposits have also been described in one case of secondary iron

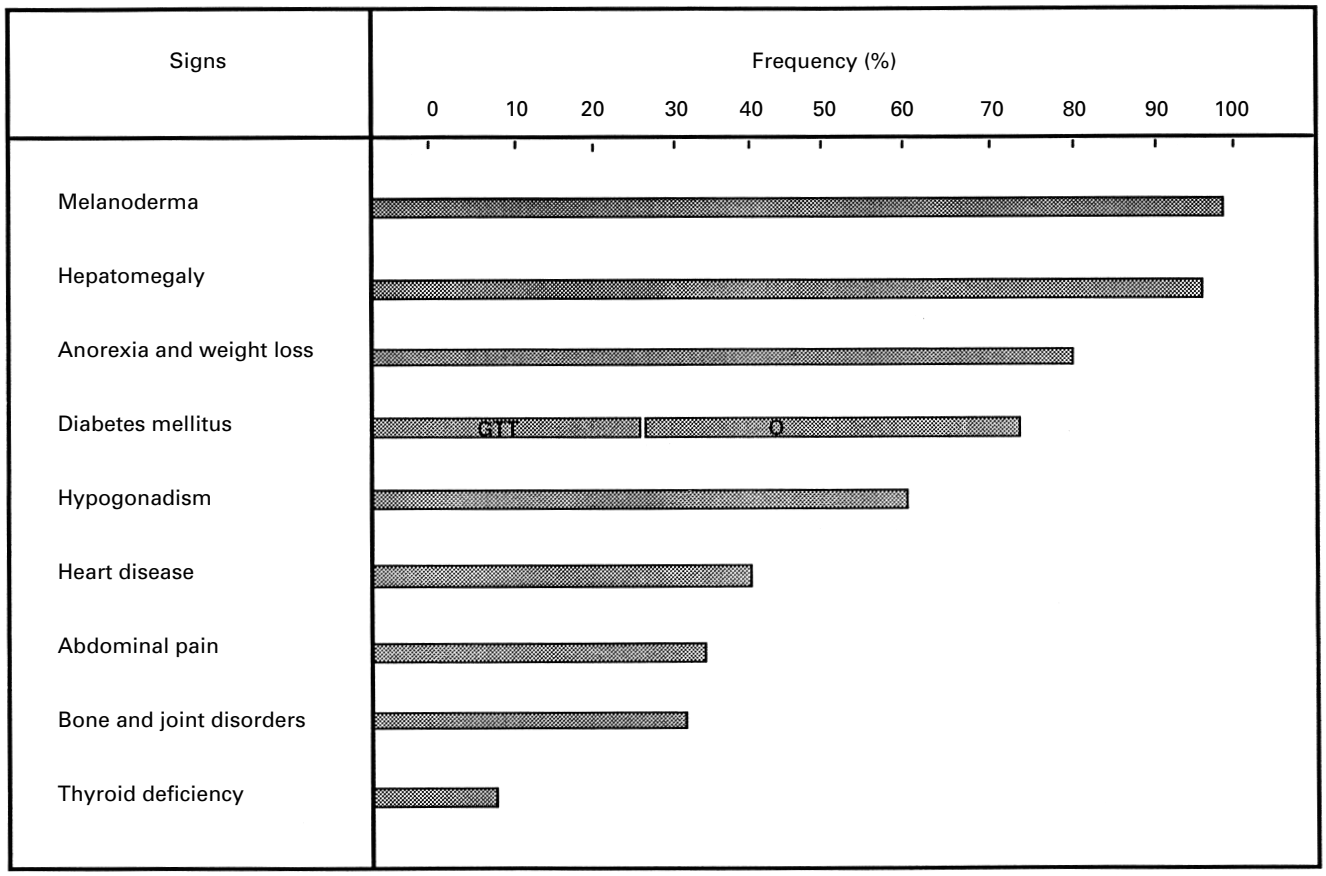

Fig. 27.1. Frequency of skin pigmentation and systemic symptoms in hemochromatosis. GTT = abnormal glucose tolerance test; O = overt diabetes mellitus.

overload (transfusion iron overload)[14]. Deposits of iron also occur around capillaries within dermal macrophages or free in the dermis. In exceptional cases, iron deposits are found in the basal layer or in sebaceous glands[6].

Correlation of hyperpigmentation with other symptoms of hemochromatosis

Because hyperpigmentation is usually correlated with severe iron overload, it is a late symptom associated with a bad prognosis, and is the only symptom that is more frequent in persons with hemochromatosis who are alcoholics[15] or women[16]. Fargion et al.[8] reported that pigmentation was more frequent in patients with hepatic cirrhosis (45%) than in patients without cirrhosis (15%). However, Niederau et al.[9] found no difference in the frequency of hyperpigmentation (or hair loss) in two similar groups. Hyperpigmentation is directly and significantly correlated with hepatic iron concentration ($P<0.001$)[10]. Skin siderosis has also been positively correlated with bone marrow siderosis and diabetes mellitus[5].

Pathophysiology of pigmentation of hemochromatosis

Correlation of the color of hyperpigmentation and the type of pigment is not clear, but a grayish shade of hyperpigmentation usually corresponds to siderosis of skin structures. Hypermelanosis is not attributable to adrenal insufficiency[6] and/or hypersecretion of melanocyte-stimulating hormone in persons with hemochromatosis. A melanogenic action of vitamin C deficiency (frequently found in hemochromatosis) could explain some cases of hyperpigmentation, but this is unproven. The relationship between iron and melanin pigment has been recently highlighted in normal subjects. Iron content of human skin is greater in sun-exposed sites[17]. Ultraviolet irradiation of transferrin releases iron in a potentially toxic form[18] that

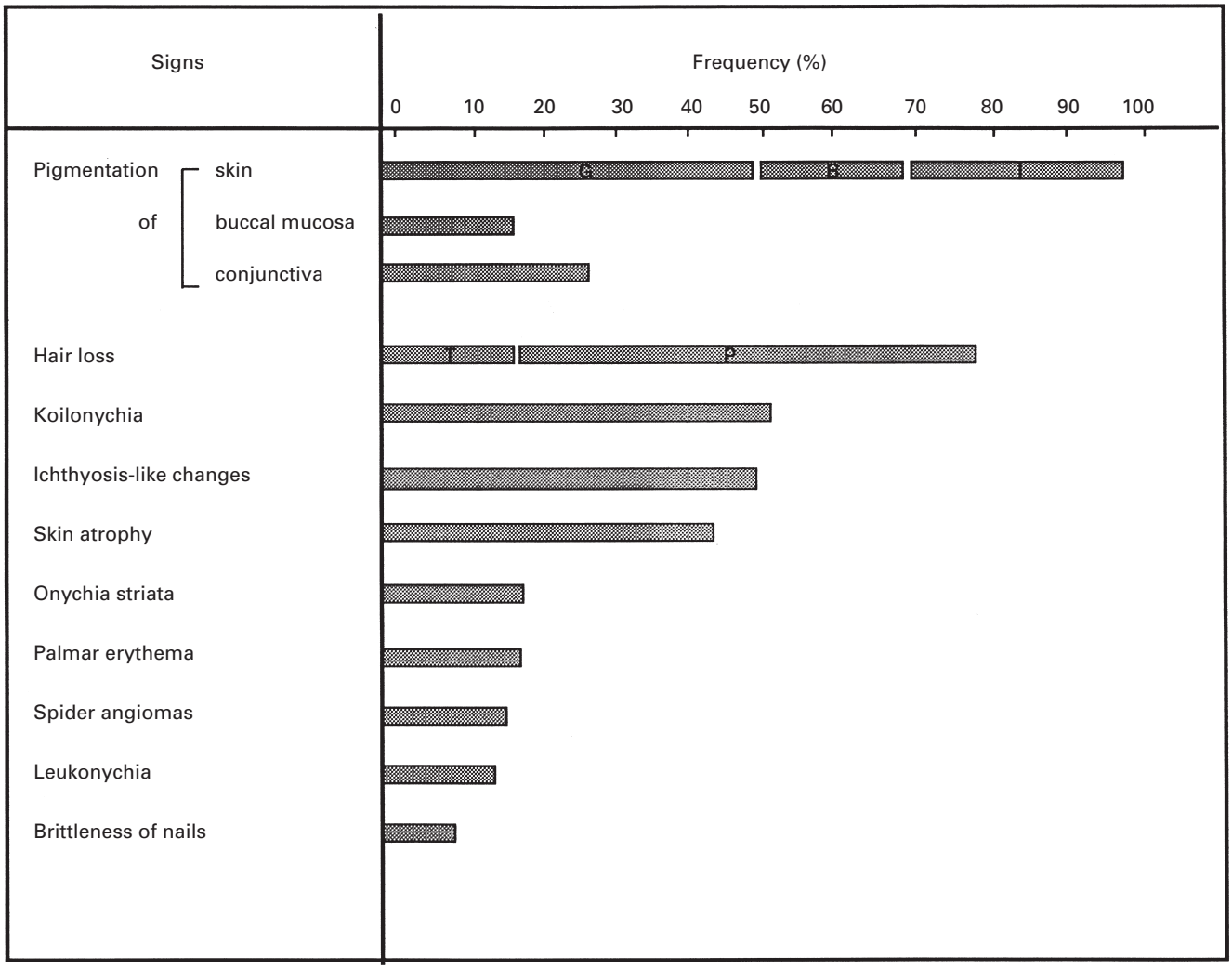

Fig. 27.2. Frequency of skin, hair, and nail signs in hemochromatosis. G = grey; B = brown; I = intermediate shade; T = total; P = partial.

can then cause skin photodamage by way of the participation of iron in oxygen radical production. The combination of an iron chelator (2–furildioxime) and a sunscreen are synergistic in providing topical photoprotection[19]. These observations suggest that iron overload plays a role in injury of sun-exposed skin in persons with hemochromatosis.

Differential diagnosis of skin hyperpigmentation in hemochromatosis

When present, hyperpigmentation must be differentiated by clinical and histopathologic studies from other causes of diffuse melanoderma such as Addisonian, metabolic, and drug-induced hyperpigmentation[20]. Persons with iron overload secondary to non-hemochromatosis causes can present with a similar clinical and histological picture, but the etiology of the secondary iron overload is usually apparent. Intense melanoderma sometimes occurs in persons with postalcoholic or biliary cirrhosis. Skin siderosis must be interpreted with caution when a biopsy specimen is taken from an area of previous trauma or hemorrhage (such as the legs), because dermal macrophage siderosis is frequent in these areas. Physiologic iron deposits can also be found in apocrine glands situated on axillary, genital, inguinal, or mid-facial zone.

Other cutaneous symptoms of hemochromatosis

In addition to hyperpigmentation, other cutaneous manifestations of hemochromatosis and iron overload have been described, primarily in a single report[6] (Fig. 27.2).

Fig. 27.3. Cutaneous pigmentation in hemochromatosis: (*a*) greyish hue; (*b*) brown hue.

Cutaneous atrophy

Atrophic skin is usually thin, smooth, 'velvety,' and shiny. Skin with these characteristics occurs frequently among persons with hemochromatosis (42% of cases), especially in the pretibial area. It can assume a scleroderma-like appearance, somewhat similar to that of persons with African iron overload. Skin with these characteristics is often diffusely distributed in an affected individual, and is frequently associated with ichthyosis and koilonychia. Histologic evaluation shows predominantly epidermal (62%) and also dermal atrophy (25%) with some fibrosis and loss of elastic fibers that are coarsely granular and are therefore somewhat different from changes associated with skin ageing alone (Fig. 27.3(*a*) (*b*)).

Ichthyosis-like state

This occurs in 56% of patients; symptoms are marked in 20%, moderate in 28%, and limited in 8%. The skin changes vary from simple, diffuse xerosis to generalized ichthyosis of the vulgaris type (14%). Histologic changes include orthokeratosis with reduction or absence of stratum granulosum like that seen in ichthyosis vulgaris (Fig. 27.4).

Hair loss

Total hair loss occurred in 12% of patients with hemochromatosis and partial loss in 62%. Scalp hair is usually gray or brown, fine and silky, but not shiny. Hair loss has been correlated with hypogonadism defined by decreased excretion of 17-ketosteroids, and with hepatic insufficiency or cirrhosis. Direct toxicity of iron could also be responsible for this manifestation, because hair contains iron[21], participates to iron excretion[22], and contains increased amounts of iron in persons with iron overload[23].

Nail abnormalities

Koilonychia or platonychia is frequent, particularly on the first three digits. Marked spooning of the nails is present in

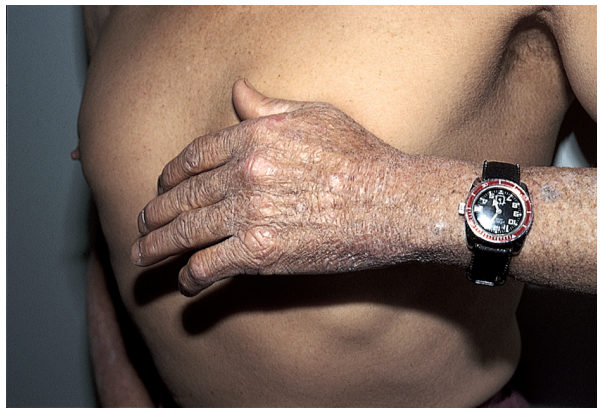

Fig. 27.4. Skin atrophy and ichthyosis in hemochromatosis.

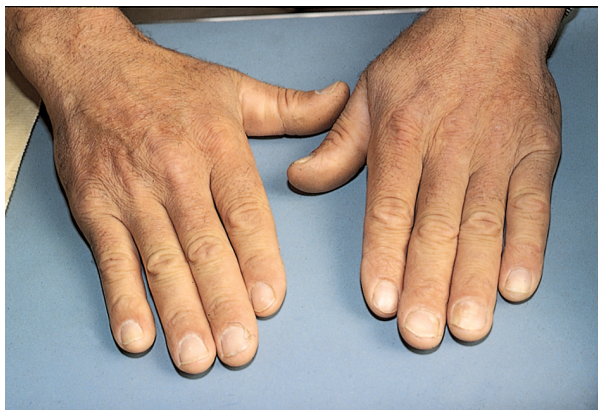

Fig. 27.5. Koilonychia and leukonychia in hemochromatosis.

25% of cases. Leukonychia, more often apparent than of the 'true' type, is present in >10% of patients, and is usually associated with brittleness and longitudinal striation of the nails (Fig. 27.5). However, these nail signs are not specific[24]. Paradoxically, koilonychia is often present in iron deficiency (and in many other disorders, including alcoholic cirrhosis).

Vascular spiders and palmar erythema

Spider angiomas are present in 9–11% of patients in older series[4, 6], and palmar erythema in 15%[6]. These signs are no longer mentioned in recent series, and are probably attributable to hepatic cirrhosis (not specifically with hemochromatosis or iron overload).

Miscellaneous cutaneous symptoms

Skin and visceral malignancy may be more frequent in persons with hemochromatosis than in persons in the general population[25]. Pruritus was the presenting symptom of hemochromatosis in two patients in whom central nervous system involvement and direct iron toxicity in peripheral nerve fibers was suggested as the possible cause of pruritus[26]. Necrolytic migratory erythema was described in one patient with hemochromatosis (but without glucagonoma)[27].

Porphyria cutanea tarda (PCT) and hemochromatosis

Most patients with PCT have evidence of abnormal iron metabolism, including siderosis of periportal hepatocytes. A few patients with PCT appear to be hemochromatosis homozygotes, and a substantial minority of patients with PCT are heterozygous for the C282Y hemochromatosis-associated mutation. These observations suggest that hemochromatosis mutations and iron overload contribute significantly to the clinical and laboratory findings observed in persons with PCT.

Skin manifestations of porphyria cutanea tarda

Clinical manifestations include cutaneous photosensitivity blisters and erosions on sun-exposed skin (face and dorsum of the hands) that heal with scars, milia, extreme skin fragility, and often malar hypertrichosis. Other uncommon symptoms are sclerodermatous changes of the face and upper trunk, diffuse melanoderma, alopecia, and chronic ulcers. The diagnosis of PCT can be established by obtaining a skin biopsy specimen that reveals bullae, and by the biochemical demonstration of porphyrinuria. Deficiency of the enzyme uroporphyrinogen decarboxylase (URO-D) occurs in all tissues in persons with familial PCT, and in the livers of persons with sporadic PCT.

Relationship between PCT and hemochromatosis

The frequency of overt clinical PCT in persons with hemochromatosis, rarely mentioned in large series of hemochromatosis patients, appears to range from 2–3%[4, 6]. In a recent study, the incidence of PCT detected by biochemical means in a group of hemochromatosis patients was 23%[28]. Ferrous iron inhibits URO-D activity in PCT. Iron overload is frequent but usually moderate in PCT, and iron removal by therapeutic phlebotomy usually results in complete remission. The causes of hepatic iron overload in PCT are still unknown. Some precipitating factors of PCT are now well delineated, and include alcohol ingestion, drugs,

and viral infections (including hepatitis A, B, and C, and human immunodeficiency virus infections).

Recent genetic studies suggested that there is not a consistent association of PCT and HLA-linked hemochromatosis[29]. However, the hemochromatosis-associated mutation C282Y is relatively frequent in patients from northern European countries who have sporadic or familial PCT[30–32]. C282Y homozygosity could induce a familial form of PCT at an early age[33]. Santos et al.[34] suggests that first-degree relatives of PCT patients should be screened for hemochromatosis, although these conclusions are not shared by Italian investigators. However, there is greater genetic heterogeneity in Italian PCT (and hemochromatosis) cases, and the C282Y mutation is less frequent in southern Italy than elsewhere in western Europe[35].

Conclusions

Skin manifestations of hemochromatosis are common, often provide an indication of the degree of iron overload, and sometimes suggest the presence of complications of iron overload and related disorders. Although many of the cutaneous abnormalities observed in persons with hemochromatosis are not diagnostic for this disorder, greater awareness of the clinical, histologic, biochemical, and genetic features of these abnormalities can facilitate the differential diagnosis and treatment of persons with hemochromatosis and other iron overload disorders.

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Cardiac abnormalities in hemochromatosis

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Introduction

Hemochromatosis is a systemic disease that may involve nearly every organ of the body. Since the availability of insulin therapy for diabetes, congestive heart failure rather than diabetes is the leading cause of death in hemochromatosis[1]. Patients who have hemochromatosis with cardiac involvement almost always have involvement in other organs, including the liver, joints, pancreas, spleen and pituitary gland. The severity of myocardial involvement varies widely and only roughly parallels that in other organs.

The cardiac complications of hemochromatosis usually develop late in the course of the disease, but a significant number of patients present initially with cardiovascular complications. Cardiac involvement usually leads to a mixed dilated/restrictive cardiomyopathic presentation with both systolic and diastolic dysfunction, often associated with arrhythmias[2, 3]. It is important for the care of the patient that the appropriate diagnosis be made and definitive treatment be promptly started.

The purpose of this chapter is to review the pathogenesis, pathophysiology, clinical features, diagnostic approaches, treatment and prevention of the cardiovascular complications of hemochromatosis.

Pathogenesis

In hemochromatosis the localization of iron in different tissues is determined by a combination of factors including the stage of the disease, the total amount of iron overload and the genetic susceptibility of certain organs to accumulate iron. Excessive tissue iron can be stored in the reticuloendothelial system until a threshold level is exceeded[4]. When this happens, other solid organs including the liver, heart, pancreas, spleen and pituitary gland, can also act as storage sites for the excessive iron. Animal model studies suggest that the source of excessive iron may partially determine the degree of cardiac iron deposition[5]. Iron from transfusional sources is more likely than ingested iron to accumulate in the heart.

During cardiac iron accumulation, iron preferentially deposits in the myocytes of the epicardium, only later to involve the transmural wall thickness. Cardiac iron overload initially results in increased perinuclear iron deposits, followed by deposition throughout the cell. Iron deposition is more extensive in the ventricles than in the atria, and the cardiac conduction system is frequently involved. The severity of myocardial dysfunction is proportional to the quantity of myocardial iron deposition[6–8]. As myocardial iron deposition increases, this results in an increase in the thickness of the left ventricular wall. This may lead to reduced left ventricular compliance, a decline in left ventricular systolic function, and ventricular dilatation.

The mechanical changes occurring as a result of myocardial hemochromatosis are aggravated by the cytotoxic effect of iron within the myocytes[9]. Iron within these cells dramatically accelerates the production of hydroxyl ions, an extremely reactive oxygen free-radical species that can disrupt the lipid bilayer of the cell, the lysosomes, other organelle membranes, and can lead to leakage, dysfunction and cell death[10, 11].

Free iron may combine with superoxide dismutase, the endogenous intracellular free radical scavenger, and paradoxically may turn it into a free radical producer[12]. If myocardial ischemia is present, the resulting intracellular acidosis can also release massive amounts of free iron from ferritin stores[13]. Thus there may exist an autocatalytic process that becomes accelerated late in the development of cardiomyopathy.

The mechanical changes in the heart (left ventricular

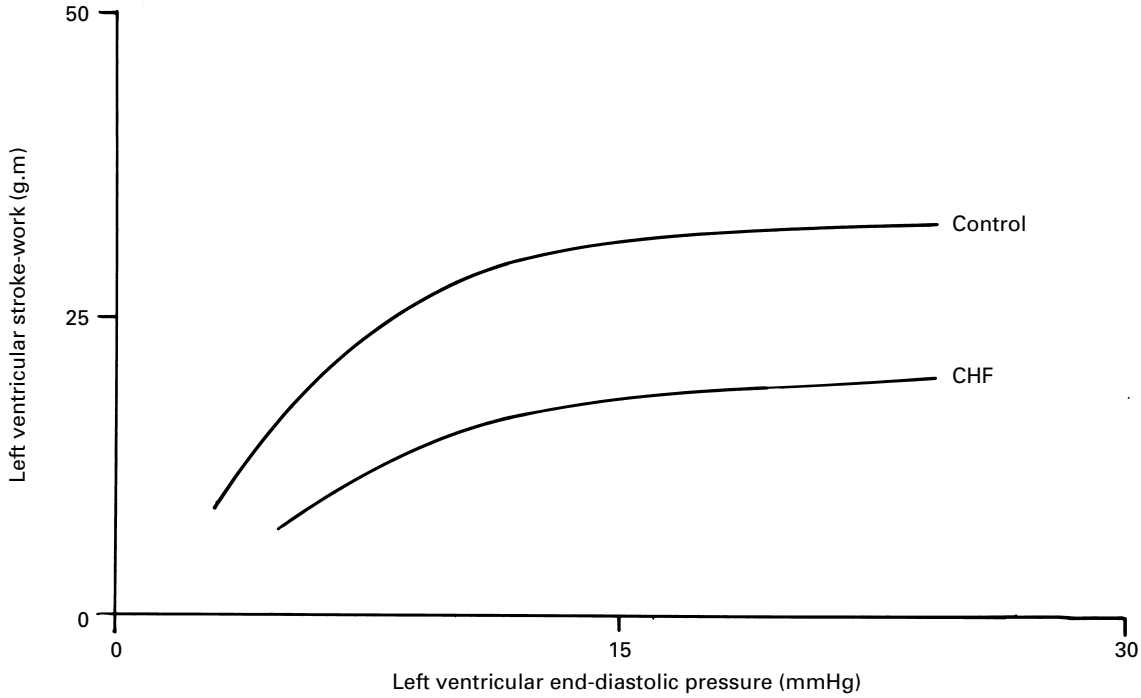


Fig. 28.1. The Frank–Starling mechanism is altered in the congestive heart failure of cardiac hemochromatosis, as in other types of heart failure. The failing ventricle becomes unable to respond normally with an increase in left ventricular stroke-work in the presence of an increase in pre-load (left ventricular end-diastolic pressure).

thickening) plus the cytotoxic effects of iron deposits can result in the combination of diastolic and systolic dysfunction as well as the development of arrhythmias. Very low levels of iron likely do not interfere with cardiac function, since iron in ferritin molecules is relatively inert, due to tight binding of iron to the protein shell. However, there is a suggestion that at low levels, iron can interfere with the contractile apparatus and manifest as diastolic dysfunction[14]. The mechanism of this process is not clear but the behavior of hearts with modestly increased iron resembles a state of elevated resting cardiac levels of calcium. This can lead to inadequate relaxation, spontaneous early depolarizations and subsequent failure of systolic contraction.

The cardiovascular conduction system appears to be involved frequently with hemochromatosis, but the sinus node appears to be relatively spared[15]. In a recent pathologic study performed on 14 men with hemochromatosis, stainable iron was graded histologically in the conduction system, the atria, and 10 sites in the ventricles[7]. Iron was found to be exclusively sarcoplasmic; no iron was observed in the interstitium. The amount of stainable iron for the same anatomic site varied among hearts and among different anatomic sites within the same heart. Ten hearts had stainable iron in all ventricular sites. The heart from one of the three patients who had undergone therapeutic phlebotomy had no visible iron at any site. Seven hearts had iron in the atria but at a lesser grade than in the ventricles. Six hearts had modest focal iron deposition in the atrio-ventricular conduction system. None of the 14 hearts had stainable iron in the sinus node. Among hearts from 11 patients who had received no prior phlebotomy, three had no stainable iron in the right ventricular septal subendocardium, suggesting that sampling error may be a problem in evaluation of hemochromatosis by endomyocardial biopsy. In this study, the sarcoplasmic location of the iron indicated that cardiac involvement in hemochromatosis represents a storage disease and not an infiltrative process[7].

Pathophysiology

Myocardial involvement

Two forms of cardiomyopathy may develop in hemochromatosis, dilated or restrictive. Dilated cardiomyopathy

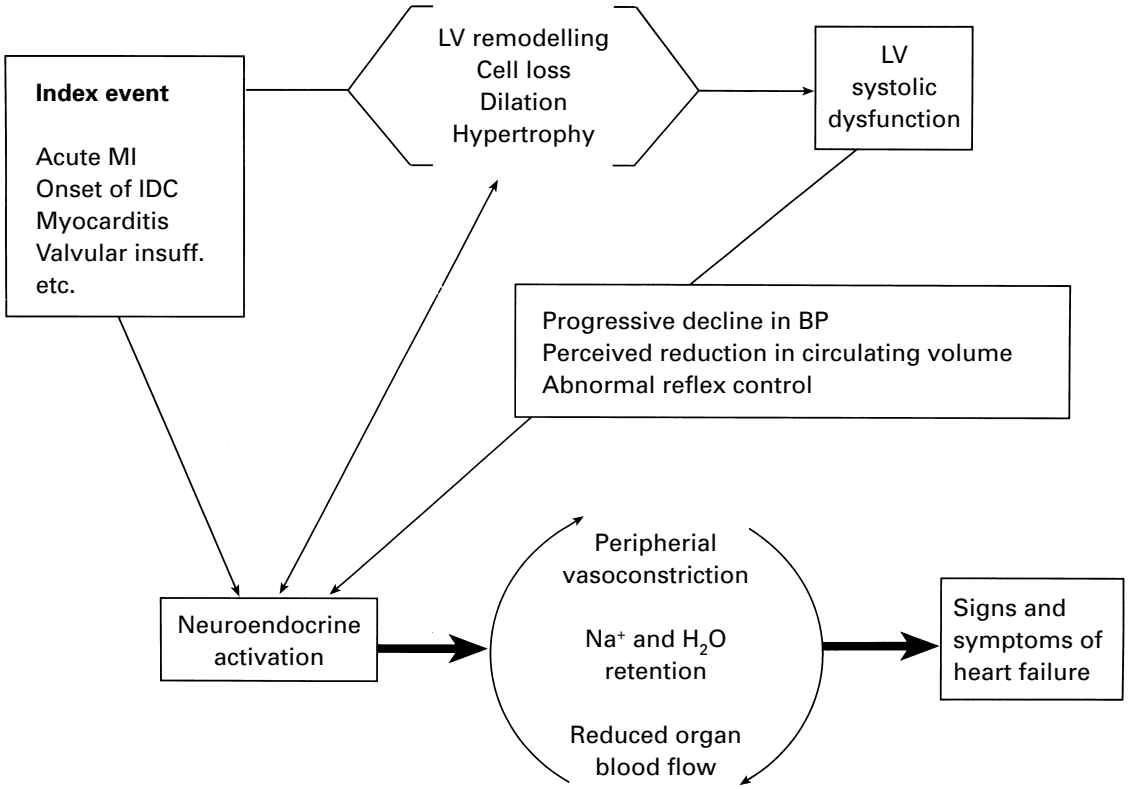


Fig. 28.2. After an initial cardiac injury (index event), neuroendocrine activation occurs, leading to a progressive change in the size, shape, and function of the left ventricle. If the underlying pathologic process is not corrected, eventually signs and symptoms of overt heart failure occur.

occurs more commonly than the restrictive type, but in some patients a restrictive cardiomyopathy is the principal cardiac abnormality.

In dilated cardiomyopathy (Fig. 28.1), increased ventricular internal dimensions are associated with decreased systolic function. Decreased systolic function is due to decreased left ventricular contractility. In restrictive cardiomyopathy, ventricular dimensions are normal and wall thickness is normal or increased. The principal abnormality in restrictive cardiomyopathy is impaired ventricular filling while systolic function initially remains normal.

In patients with cardiac hemochromatosis who have restrictive physiology, as iron accumulation continues, the resultant decrease in left ventricular contractility results in systolic dysfunction. In any circumstance in which cardiac systolic function is diminished, a number of adaptive functional, structural and humoral changes occur, all designed to compensate for the inadequate mechanical function of the heart[16]. Most important among these are: (i) the Frank–Starling mechanism in which an increased pre-load helps to sustain good cardiac performance; (ii) myocardial hypertrophy with or without chamber dilatation, that may temporarily increase the total work capacity of the cardiac pump; (iii) increased release of catecholamines by the adrenergic nervous system, which raises myocardial contractility, activates the renin–angiotensin/aldosterone system, and other neurohumoral adjustments that act to preserve arterial pressure and perfusion to vital organs (Fig. 28.2)[17].

The capacity of the adaptive mechanisms described above to sustain cardiac performance is finite and may have conflicting consequences. The clinical syndrome of heart failure results from the interaction of cardiac dysfunction, hemodynamic overload and secondary compensatory changes that cause functional and humoral effects[18]. Myocardial dysfunction leads to decreased cardiac output and increased ventricular filling pressures, and the compensatory mechanisms result in an increased blood volume, vasoconstriction and increased impedence to ventricular ejection.

If this vicious cycle of pathophysiological events is not broken, the patient will experience deterioration of cardiac function to the point of severe congestive heart failure and death. The decrease in left ventricular function can be

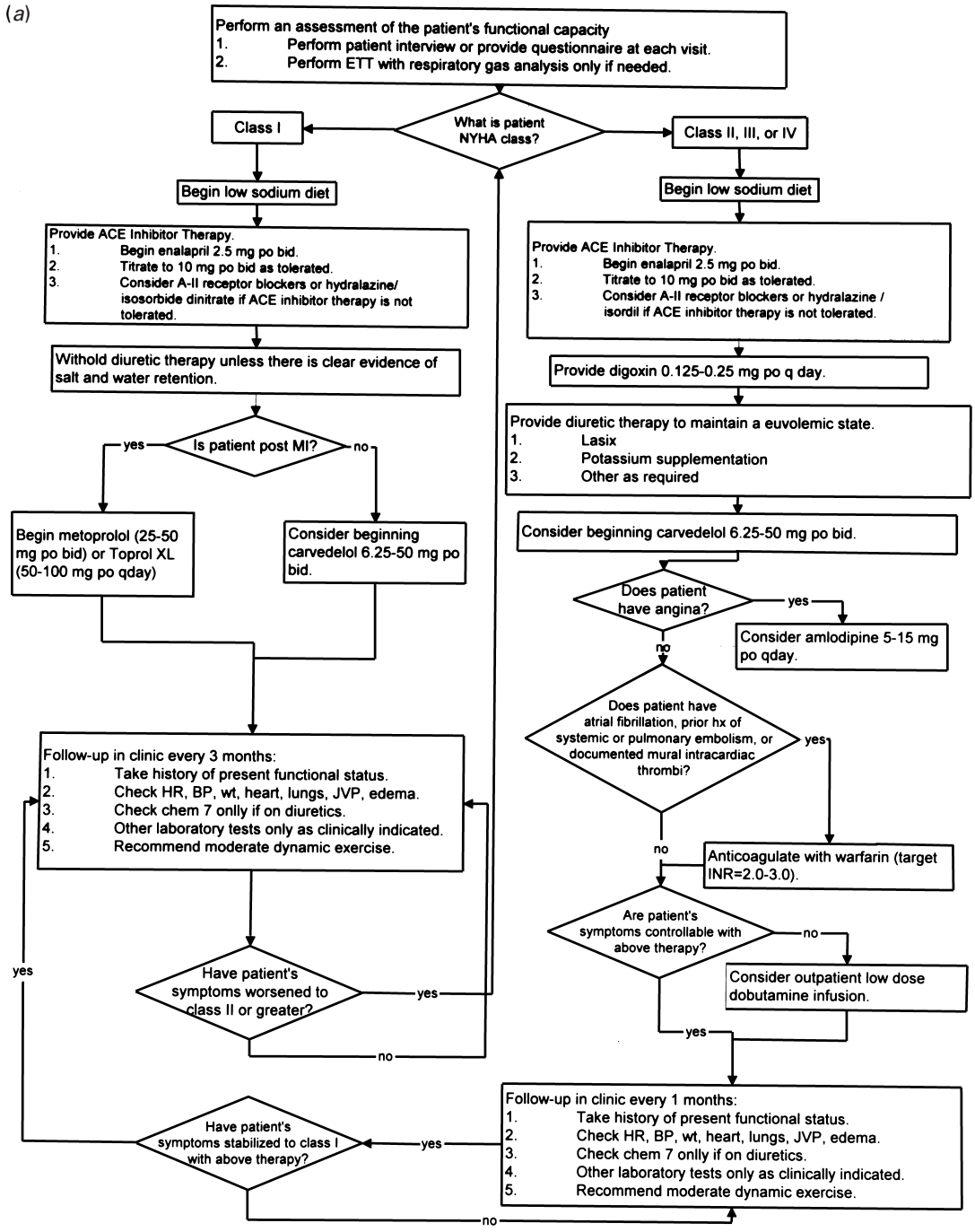


Fig. 28.3. An algorithm that can be employed for the systematic management of the congestive heart failure that is due to systolic (*a*) or diastolic (*b*) dysfunction in cardiac hemochromatosis.

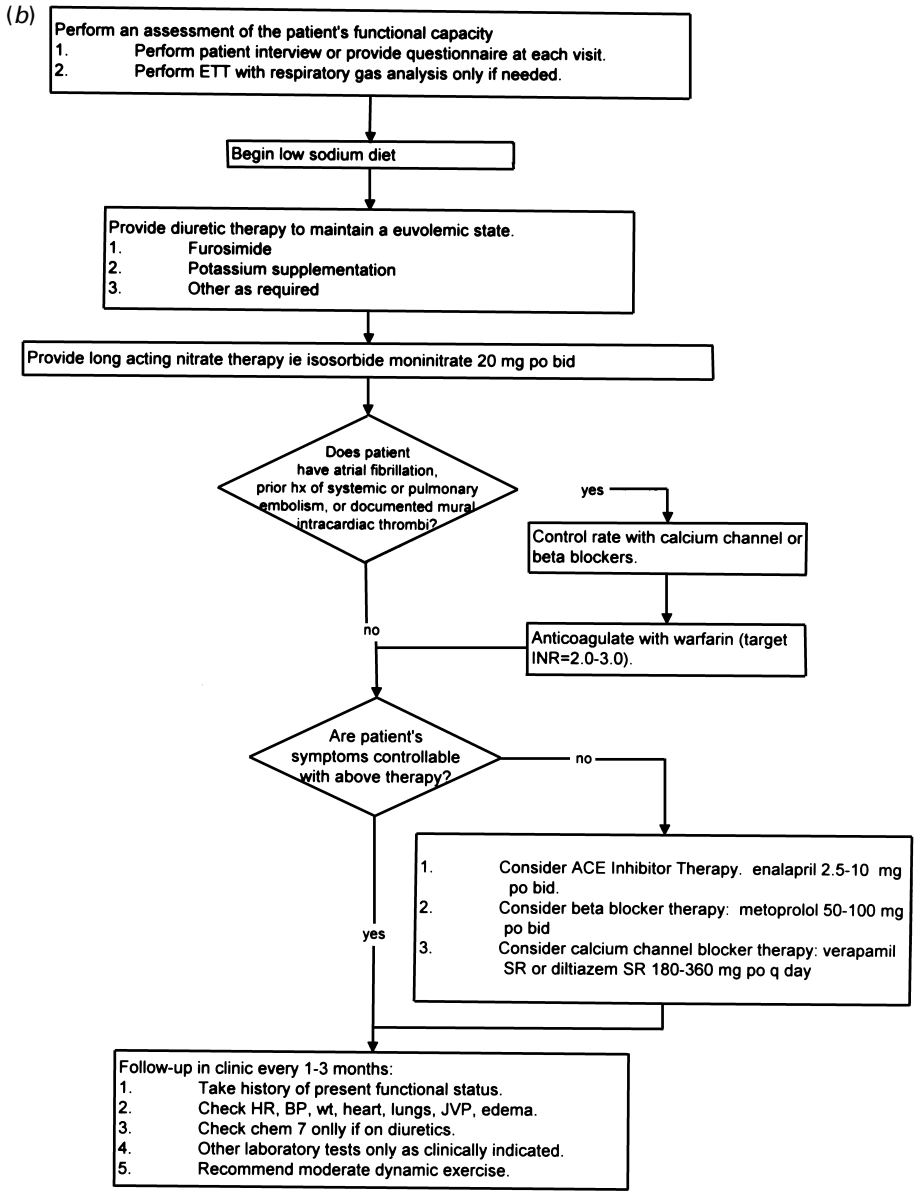


Fig. 28.3. (*cont.*)

compounded by supraventricular or ventricular tachycardias that result in decreased diastolic filling time, followed by progressive inefficiencies of cardiac function. Atrial fibrillation can result in decreased left ventricular function through the loss of atrial systole, which normally contributes 20% of the cardiac output.

There are three reports of patients with hemochromatosis who had right-sided heart failure compatible with restrictive cardiomyopathy or constrictive pericarditis[19–21]. The left ventricle was unable to dilate during diastole. All three patients had been asymptomatic prior to cardiac decompensation that caused death within three weeks. All three were found to have normal pericardia at the time of thoracotomy.

In general, diastolic dysfunction occurs early in cardiac iron overload, while systolic dysfunction usually occurs after greater iron accumulation is present (Fig. 28.3).

Conduction defects

In hemochromatosis, myocardial iron deposits are usually found within the sarcoplasmic reticulum of myocytes of the subepicardial region, followed by the subendocardial region. Iron stores are more extensive in ventricular than atrial myocardium, and are least common in the mid-myocardial wall[7]. Iron accumulation in the cardiac conducting system is common. This causes degeneration and fibrosis of the atrio-ventricular node and the His–Purkinge system, which causes atrial and ventricular arrhythmias and various types of heart block[7, 22]. While iron deposition has not been observed in the sinus node, sinus nodal dysfunction has been observed in some hemochromatosis patients who had the sick sinus syndrome[3].

Clinical features

The clinical manifestations of cardiac hemochromatosis vary, depending on the extent of myocardial involvement. Early on, patients may remain asymptomatic despite echocardiographic and other evidence of myocardial involvement. Cardiac manifestations become significant in about a third of patients with hemochromatosis[23], and a similar percentage die of cardiac complications. The clinical manifestations of cardiac hemochromatosis can be segregated into three categories, including arrhythmias, congestive failure due to systolic dysfunction, and congestive failure due to diastolic dysfunction.

Hemochromatosis and cardiac arrhythmias

Atrial and ventricular arrhythmias and heart block are common in patients with cardiac hemochromatosis due to myocardial dysfunction and iron deposition in the atrio-ventricular node and conduction system[15, 22]. Presenting symptoms may be simple palpitations or frank syncope. Palpitations are common, occurring in up to 37% of patients in some series[1]. An irregular heart beat may be due to atrial fibrillation, premature atrial contractions, premature ventricular contractions, or sinus arrhythmia. A regular, rapid heart beat may be due to paroxysmal supra-ventricular tachycardia, sustained or non-sustained ventricular tachycardia, or atrial flutter. Palpitations may be associated with light-headedness, chest discomfort, diaphoresis and shortness of breath.

Some patients may experience symptoms of pre-syncope or frank syncope associated with bradyarrhythmias, including inappropriate sinus bradycardia, second degree Mobitz type II heart block or third degree atrio-ventricular block. Some patients may experience light-headedness before passing out, but at other times experience no warning prior to syncope–Stokes–Adams attacks. Patients with sick sinus syndrome may have intermittent palpitations due to tachycardia followed by a sensation of light-headedness due to bradycardia. This tachycardia–bradycardia syndrome may be due to intermittent atrial fibrillation with rapid conduction followed by spontaneous conversion to a sinus rhythm with marked sinus bradycardia.

In a patient with cardiac arrhythmia, the diagnostic evaluation is the same whether the arrhythmias are due to hemochromatosis or some other condition. Evaluation includes an electrocardiogram followed by either Holter monitoring or even more prolonged cardiac event electrical monitoring. If these test results are not conclusive, cardiac catheterization and electrophysiologic testing may be needed.

Congestive heart failure

By the time cardiac hemochromatosis results in congestive failure, the diagnosis is often established because of other presenting symptoms. When congestive heart failure is the presenting symptom or sign of hemochromatosis[24], breathlessness (the cardinal manifestation of left ventricular failure) may progressively worsen as (i) exertional dyspnea; (ii) orthopnea; (iii) paroxysmal nocturnal dyspnea; (iv) dyspnea at rest; and then (v) acute pulmonary edema.

Patients whose iron accumulation is greater in the right ventricle than the in left ventricle may have right heart failure. Dyspnea is not prominent in isolated right heart failure because pulmonary congestion is usually absent. In these patients, presenting symptoms are more likely to be associated with systemic fluid retention, including peripheral edema, weakness and fatigue. Congestive hepatomegaly may produce discomfort, generally described as a dull ache or heaviness in the right upper quadrant or epigastrium. This pain is caused by stretching of the hepatic capsule and may be severe when the liver enlarges rapidly in acute right heart failure.

Physical signs of congestive heart failure in cardiac hemochromatosis include elevated jugular venous pressure, pulmonary rales, positive hepato-jugular reflux, congestive hepatomegaly, peripheral edema, pleural effusions, and ascites. Cardiac findings may include cardiomegaly with an enlarged and laterally displaced point of maximum impulse, an S_3 or S_4 gallop, pulsus alternans, accentuation of the P2 heart sound, or the presense of systolic murmurs. Systolic murmurs are common in

heart failure due to the mitral or tricuspid regurgitation that may accompany left ventricular dilatation. Often these murmurs diminish or disappear when cardiac compensation is restored.

Diagnostic procedures

The three main non-invasive studies to establish the diagnosis of congestive heart failure are the chest x-ray, echocardiogram, and magnetic resonance imaging. The principal invasive procedures available are right- and left-heart catheterization and endomyocardial biopsy.

Chest X-ray

The two features of the chest X-ray that are useful in the diagnosis of congestive heart failure due to cardiac hemochromatosis are the size and shape of the cardiac silhouette and evidence of increased pulmonary hydrostatic pressure or pulmonary edema. An enlarged cardiac silhouette is a relatively specific indicator of increased left ventricular end-diastolic volume. With elevation of left atrial pressure and pulmonary venous and capillary pressures, interstitial and perivascular edema develops. This is initially more prominent in the lung bases.

When pulmonary capillary pressure exceeds 20 mm Hg (normal <10), interstitial pulmonary edema occurs. When pulmonary capillary pressure exceeds 25 mm Hg, alveolar edema with a cloud-like appearance, and concentration of the fluid around the hilai in a butterfly pattern, and large pleural effusions, may occur. With elevation of systemic venous pressure, the azygos vein and superior vena cava may also enlarge. There is no specific finding on chest X-ray that distinguishes the congestive failure of hemochromatosis from other causes of congestive failure.

Echocardiogram

The most useful diagnostic test to evaluate left ventricular function in congestive heart failure due to cardiac hemochromatosis is the two-dimensional echocardiogram. Left ventricular diameter and fractional shortening provide an assessment of the size and systolic function of the left ventricle. Left ventricular volumes and ejection fraction can be determined from two-chamber and four-chamber echocardiograms[25]. The presence or absence of left ventricular hypertrophy and signs of diastolic dysfunction can also be determined by echocardiography.

The echocardiographic features of 24 patients with cardiac involvement in hemochromatosis was evaluated by Olsen et al.[26]. Although the patients did not have signs or symptoms of congestive failure at the time of presentation, 37% had structural and functional echocardiographic abnormalities attributable to hemochromatosis. The abnormalities included chamber dilatation and global systolic dysfunction. Increased wall thickness was not a feature in these patients. Of these patients, more than 50% were dead within 2½ years because of congestive heart failure[26].

As in patients with other forms of infiltrative cardiomyopathy, such as amyloidosis, a myocardial echo reflectance pattern may be observed in patients with hemochromatosis. This has the appearance of an enhanced speckled pattern within the myocardium. This speckled pattern may be quantitatively evaluated through measurements of integrated back scatter[27]. The integrated back scatter in patients with hemochromatosis is significantly higher than in controls ($35+/-14\%$ vs. $21+/-6\%$, $p<0.001$).

In addition to the functional abnormalities described above, in patients whose iron deposition results in a restrictive cardiomyopathy, echocardiography plus Doppler cardiography can be used to establish the presence of restrictive cardiomyopathy[28]. Restrictive physiology with diastolic dysfunction is established by the presence of E/A reversal[25].

In summary, echocardiography allows assessment of subtle cardiovascular changes in hemochromatosis before they present clinically or are detectable by routine chest X-ray. These changes include increased myocardial reflectance, increased left ventricular dimensions in the absence of increased left ventricular wall thickness, and decreased left ventricular function resulting in reduced ejection fraction. This simple non-invasive test allows serial evaluation of cardiac function in iron-loaded individuals.

Magnetic resonance imaging

Stored intracellular iron enhances magnetic susceptibility. This property is the basis on which tissue iron can be detected by nuclear magnetic resonance. Magnetic resonance imaging (MRI) is sometimes employed to identify increased iron in the liver or spleen[29, 30]. Intracellular iron decreases MRI signal intensity within tissues. The degree of reduction in signal intensity allows estimation of the amount of iron in the myocardium. In addition to identifying iron loading, MRI can also be used to assess ventricular function in patients with hemochromatosis[31]. Although these functional findings can usually be obtained with echocardiography, in patients whose echocardiographic images are poor, evaluation by MRI may be helpful.

Cardiac catheterization and endomyocardial biopsy

In a few patients who have other confounding medical problems, definitive evaluation of cardiac status requires catheterization and endomyocardial biopsy.

Right heart catheterization

Right heart catheterization of hemochromatosis patients allows pressure measurements that clarify the patient's hemodynamic status. Elevated central venous or right atrial pressures (>5 mmHg) imply intravascular volume overload. An elevated right ventricular pressure (>30 mmHg) may indicate pulmonary hypertension, which may occur as a result of increased pulmonary vascular resistance or as a result of an increase in left ventricular end-diastolic pressure. An elevation of pulmonary artery pressures (mean >19 mmHg) also gives evidence of pulmonary hypertension.

Pulmonary capillary wedge pressure (PCWP) provides an indirect measurement of left atrial diastolic pressures. An elevated PCWP (>12 mmHg) is either associated with elevated left ventricular end diastolic pressure or the presence of mitral stenosis.

In addition to pressure measurements, right heart catheterization allows measurement of cardiac output. Using the combination of right heart pressure measurements and cardiac output, it is possible to determine if a patient has symptoms related to congestive heart failure, if the congestive heart failure is associated with a high or low output state, and if pulmonary or arteriolar vascular resistances are abnormal.

Left heart catheterization

Left heart catheterization of hemochromatosis patients allows evaluation of left ventricular size, regional wall motion, ejection fraction and of mitral and aortic valve abnormalities. After completion of left ventriculography, selective coronary arteriography may be performed to prove or disprove the presence of coronary ischemic heart disease, which is the most common cause of congestive heart failure.

Right ventricular endomyocardial biopsy

The definitive technique to identify cardiac hemochromatosis is right ventricular endomyocardial biopsy of the interventricular septum. Histologic grading of the amount of iron in these samples is quite predictive of progression to congestive heart failure and of the success of chelation therapy or phlebotomy therapy[32–35].

Management

The management of patients with cardiac hemochromatosis can be separated into the general management of the cardiac complications and the primary or secondary prevention of progressive cardiac complications through treatments designed to arrest or reduce levels of myocardial iron deposition.

General management of cardiac complications

Conduction defects

Patients with cardiac hemochromatosis often present with bradycardic symptoms, which are best treated by temporary transthoracic or transvenous pacing. All medications that decrease the heart rate should be discontinued, including digoxin, beta-blockers and calcium channel blockers. Permanent pacemaker implantation is required for patients who have persistent, symptomatic sinus bradycardia or second- or third-degree heart block.

Cardiac arrhythmias

The management of patients with iron-related atrial or ventricular arrhythmias is similar to other patients with the same arrhythmia, regardless of the underlying cause. Atrial fibrillation is treated by rate control with either digoxin, calcium channel blockers, or beta-blockers, and chronic anticoagulation using warfarin. Conversion to sinus rhythm using a variety of membrane-active anti-arrhythmic agents may also be considered, although the risk of pro-arrhythmic events may be increased in patients with diffuse myocardial iron deposition. Patients presenting with other supraventricular arrhythmias may also be treated with appropriate AV nodal blocking agents or membrane-active anti-arrhythmic agents.

In patients with iron-related ventricular arrhythmias, treatment is similar to patients with similar arrhythmias due to other causes. Patients presenting with frequent premature ventricular contractions or asymptomatic non-sustained ventricular tachycardia are managed with either watchful waiting or by administration of a beta-blocker. Patients with symptomatic and prolonged non-sustained ventricular tachycardia or sustained ventricular arrhythmias may require immediate hospitalization and treatment with intravenous lidocaine. These patients may need to undergo electrophysiologic studies to determine whether and which anti-arrhythmic agents may be appropriate and whether placement of an automatic implantable cardiac defibrillator is indicated.

Congestive heart failure

The management of patients with congestive heart failure is determined by the diagnostic separation of the patients into either congestive heart failure that is due to systolic dysfunction, or due to diastolic dysfunction. Fig. 28.3 is an algorithm that may be used to treat patients with congestive failure. For patients with congestive failure due to systolic dysfunction, the three mainstays for treatment are digoxin, cautious administration of diuretics, and aggressive treatment with after-load reducing agents, of which angiotensin converting enzyme inhibitors are the most effective, if tolerated.

Digoxin is beneficial in most patients with dilated cardiomyopathy, but cardiomyopathies caused by a myocardial infiltrative process such as amyloidosis are made worse by digoxin.

Monitoring of sodium and water intake is important. Recent studies have documented the importance of the careful addition of a beta-blocker to prevent the progression of cardiomyopathy. This should only be done after stabilization of the patient's symptoms. In patients with severe congestive failure (ejection fraction $<30\%$), anticoagulation with warfarin should be considered. Other medications which may have a negative effect on myocardial contractility (such as the calcium channel blockers verapamil, diltiazem, and nifedipine) should be avoided.

In patients with congestive failure due to diastolic dysfunction, a primary mode of therapy is diuresis. Positive inotropic agents such as digoxin are contraindicated in the congestive failure of diastolic dysfunction. Afterload reducing agents are not recommended in patients with diastolic dysfunction, although in some cases angiotensin converting enzyme inhibitors may result in some benefit. Agents which have a negative effect on myocardial contractility, such as beta-blockers or verapamil, may show benefit in some patients. Anticoagulation is usually not required in patients with congestive failure due to diastolic dysfunction alone.

Specific treatment of hemochromatosis

An important question is whether or not ventricular function improves after phlebotomy or chelation therapy. There are a number of patients in whom phlebotomy arrested the course of cardiomyopathy or improved ventricular function as assessed by clinical symptoms or the echocardiogram[8, 36].

Some patients whose initial left ventricular percent fractional shortening is severely reduced prior to phlebotomy experience a decrease in left ventricular dimensions and their fractional shortening becomes normal[8, 37].

In another report, echocardiographic results were compared among patients with hemochromatosis (before and after iron depletion) and control subjects[37]. Hemochromatosis patients who had impaired systolic function prior to phlebotomy developed improved systolic function and decreased left ventricular mass after iron depletion. In general, reduction in total body iron stores may result in improvement in cardiac manifestations of hemochromatosis, which makes early recognition and treatment of this entity essential[38–43]. In most patients the function of the iron-loaded heart can be improved by iron depletion, especially when iron depletion therapy is instituted early in the course of the disease. Unfortunately, improvement does not always occur[2].

CASE STUDY

History of present illness A 35-year-old Caucasian woman was in her usual state of health until she presented to an outpatient emergency care center with progressive shortness of breath and palpitations for two days. She experienced increased fatigue, weakness, and difficulty sleeping during the previous several weeks. During the two days prior to evaluation, she was short of breath at rest and felt like she had to work to breath. She also noticed swelling in her ankles and reported a 15 pound weight gain during the previous 4–6 weeks, despite a poor appetite.

Past medical history She had no prior history of cardiac problems. She had an episode of iritis a few years earlier. She had been followed at a reproductive endocrinology clinic for amenorrhea the past 6–8 years. She had intermittent arthralgias involving both knees and ankles. Her only medication was ibuprofen for arthritis. She also had a history of panhypopituitism with hypothyroidism, adrenal insufficiency, amenorrhea, and infertility.

Family history There was no known family history of heart disease or of hemochromatosis. The patient's mother had diabetes mellitus type II and arthritis.

Social history She did not use tobacco and she did not drink alcohol.

Physical examination The patient was sitting on a stretcher in mild respiratory distress. Heart rate was 238 on the monitor and blood pressure was 106/64. Oxygen saturation was 97% on a 100% non-rebreathing mask. Extremities were cool and cyanotic. Her color was ashen to pale. HEENT exam was unremarkable. Neck exam

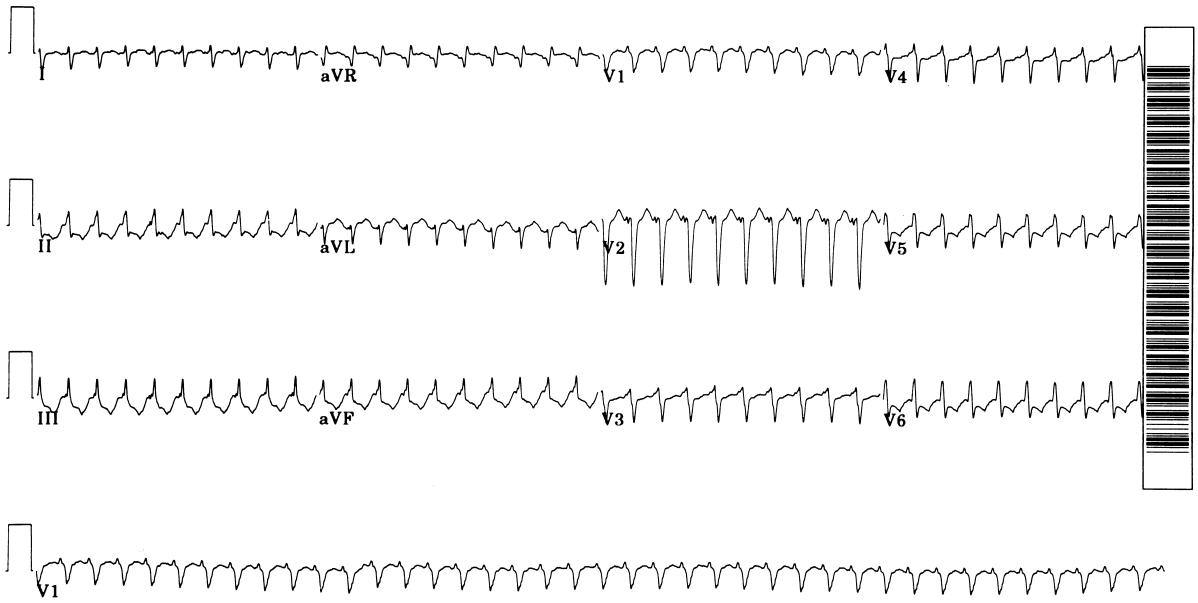


Fig. 28.4. The initial 12-lead electrocardiogram of the patient with cardiac hemochromatosis presented in case study. The arrhythmia is atrial flutter with 1:1 conduction at a rate of 233 beats per minute. A non-specific inter-ventricular conduction delay is present in all leads, and T-wave abnormalities are present in leads II, III, AVF, and $V_{5–6}$, suggestive of inferolateral ischemia.

revealed no thyromegaly or adenopathy. Jugular venous pressure was elevated at >15 cm of water. Thorax was non-tender to percussion. Lungs revealed bibasilar crackles. Cardiovascular exam revealed a laterally displaced apical impulse which was enlarged and sustained. There was a prominent S3 gallop and a Grade III/VI holosystolic murmur at the apex. Abdomen was soft and bowel sounds were present. Liver was palpable 4 cm below the right costal margin. No tenderness was detected. Extremities revealed 1–2+ pitting edema in the lower extremities. Neurologic exam was non-focal.

Laboratory data WBC was 10300/μl, Hb 15 g/dl, VPRC 46 ml/dl, platelets 186000/μl, Na 141 mmol/l, K 5.4 mmol/l (normal 3.5–5.0), BUN 17 mg/dl, creatinine 0.7 mg/dl, total cholesterol 201 mg/dl, AST 85 U/l (normal <67), ALT 91 U/l (normal <84), and glucose was 203 mg/dl (normal <110). The electrocardiogram (Fig. 28.4) revealed atrial flutter with 1:1 conduction and a heart rate of 239 bpm. There was significant right axis deviation. There were marked S–T abnormalities, possibly consistent with inferior subendocardial injury. There was poor R wave progression in the precordial leads. Portable chest X-ray (Fig. 28.5) revealed marked cardiomegaly, hazy vessels, pulmonary vascular redistribution in the upper lung fields, and a pleural effusion, all consistent with acute pulmonary edema due to congestive heart failure.

Hospital course The initial working diagnosis was viral myocarditis or idiopathic cardiomyopathy. Included in the differential diagnosis were autoimmune causes, amyloid cardiomyopathy, and possible hemochromatosis. She was transferred from the outpatient center to the hospital emergency room where her rapid atrial flutter was cardioverted using intravenous ibutilide. She was then transferred to the intensive care unit where her rhythm reverted to atrial flutter, so she was given intravenous diltiazem for rate control. The regular atrial flutter soon deteriorated to atrial fibrillation with a rapid ventricular response. In an attempt to convert her out of atrial fibrillation, she was given intravenous procainamide. Attempts at electrical cardioversion using 50 and 360 joules was not successful. The anti-arrhythmic was switched to intravenous amiodarone, but again cardioversion was not successful and the patient remained in atrial fibrillation.

An echocardiogram (Fig. 28.6) revealed a markedly dilated left ventricle with normal wall thickness. The left ventricle demonstrated severe reduction in global function with an ejection fraction of <30% (normal 55–78). The right ventricle was normal in size and thickness but demonstrated a moderate reduction in global function. There was mild to moderate left and right atrial enlargement. All of the cardiac valves were normal in anatomy, but mild mitral regurgitation and moderate tricuspid

regurgitation were present. The remainder of the echocardiographic exam, including the aortic root and pericardium, appeared normal and there was no evidence of pericardial effusion. The inferior vena cava was distended, consistent with intravascular volume overload.

In addition to treatment of her arrhythmia, she also was begun on treatment for acute congestive heart failure including continuous intravenous infusions of furosemide and captopril. Her clinical status continued to deteriorate. She required more than 50% oxygen by face mask to maintain an arterial oxygen percent saturation in the mid-1980s. Milrinone, an intravenous positive inotropic agent, was started, but without success.

Because of continued deterioration, right heart catheterization was performed and documented progressive deterioration of cardiac function, with a cardiac output of 2.4 l/min (normal 4–6) and a cardiac index of 1.6 l/min/m^2 (normal 2.6–3.5), despite an elevated pulmonary capillary wedge pressure of 25 mmHg (normal <10). Because of continued atrial fibrillation with a rapid ventricular response, she underwent AV nodal radiofrequency ablation to slow her rate, followed by a pacemaker implantation. Atrio-ventricular nodal ablation was successful, but she remained hypoxic and developed progressive hypotension. She underwent endotracheal intubation and an intra-aortic balloon pump was placed. During this procedure, she developed cardiac asystole. She underwent prolonged resuscitation, including multiple doses of epinephrine, chest compression, and total cardiopulmonary support with a peripheral bypass machine. She was transferred to the thoracic intensive care unit in the hopes of finding a cardiac transplant donor or for consideration of placement of a total artificial heart. In spite of aggressive measures, she could not maintain a blood pressure and she expired 48 hours after seeking medical attention.

Results of iron studies that returned after her death revealed a markedly elevated serum ferritin concentration of 5350 ng/ml (normal females <81). Results of an endomyocardial biopsy performed at the time of cardiac catheterization revealed iron laden myocardial cells, confirming the diagnosis of hemochromatosis. Her siblings were advised to seek medical evaluation for possible hemochromatosis.

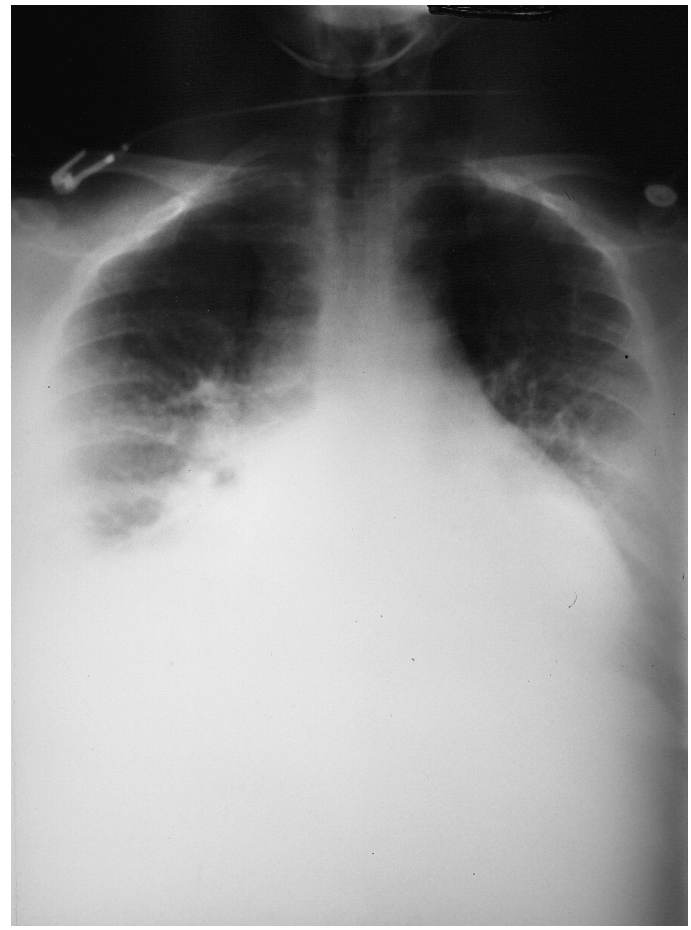
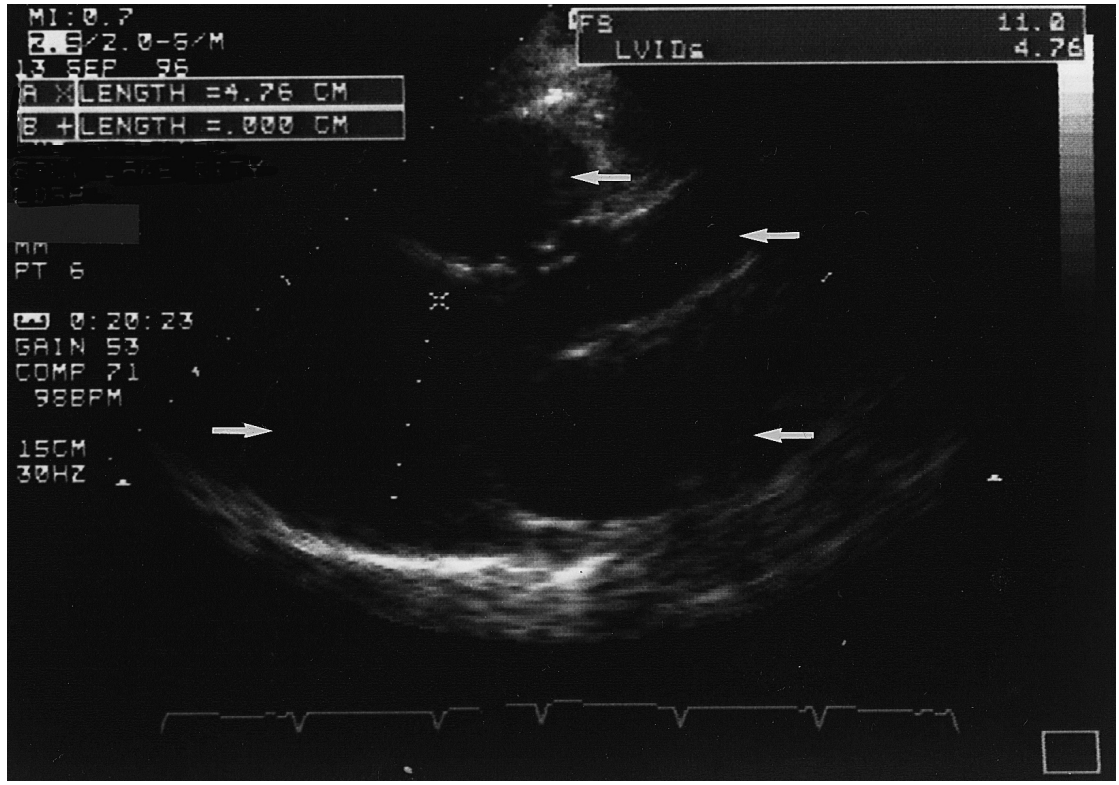


Fig. 28.5. Chest X-ray (upright position) of patient with cardiac hemochromatosis presented in case study. The heart is markedly enlarged and there are alveolar infiltrates, peri-hilar hazy infiltrates, right pleural effusion, widening of the superior mediastinal vessels, and increased blood flow in the vessels of the superior lung fields, all compatible with pulmonary edema.

Comments

This case study demonstrates, with startling clarity, the importance of making the diagnosis of hemochromatosis early on, when individuals have no symptoms of illness. This case also demonstrates that patients with hemochromatosis may have severe cardiac manifestations that lead to the diagnosis of hemochromatosis, including atrial arrhythmias, dilated cardiomyopathy, and severe congestive heart failure. Her arthralgias, hypopituitarism, and premature amenorrhea occurred long before her cardiac decompensation. If these had been evaluated thoroughly, the diagnosis of hemochromatosis could have been established and iron depletion therapy could have prevented the development of lethal cardiac complications.

(*a*)



(*b*)

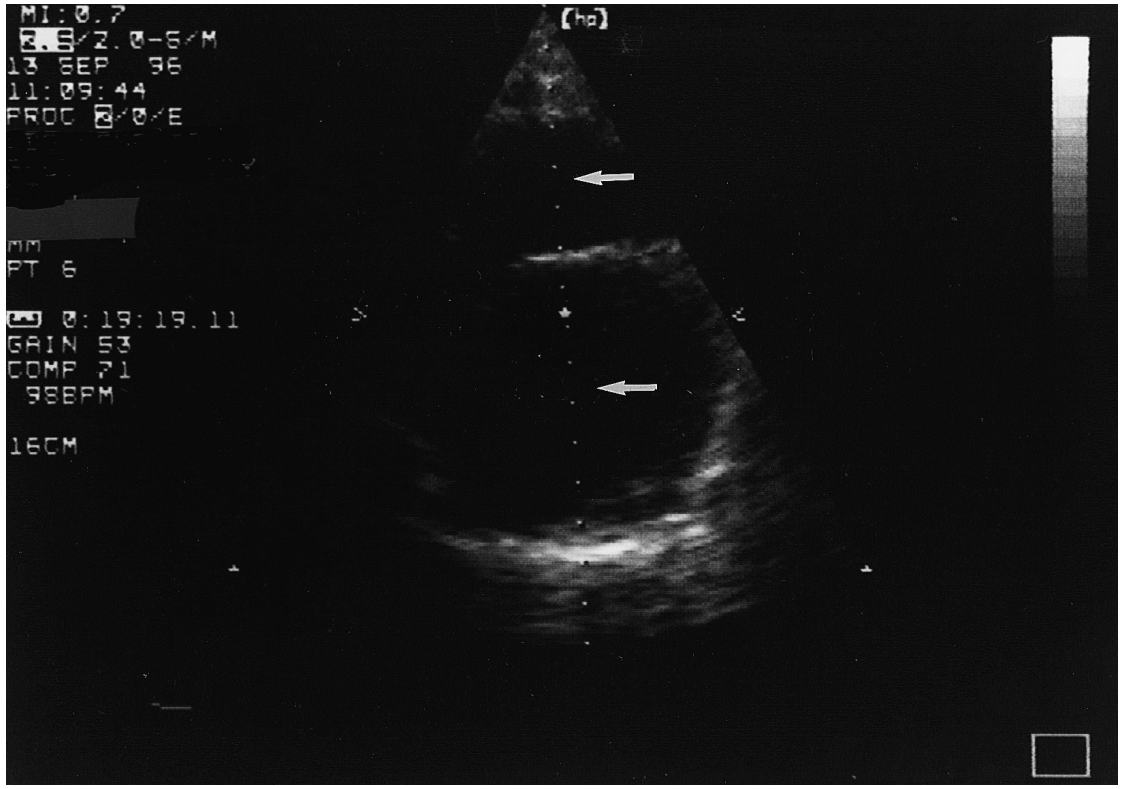
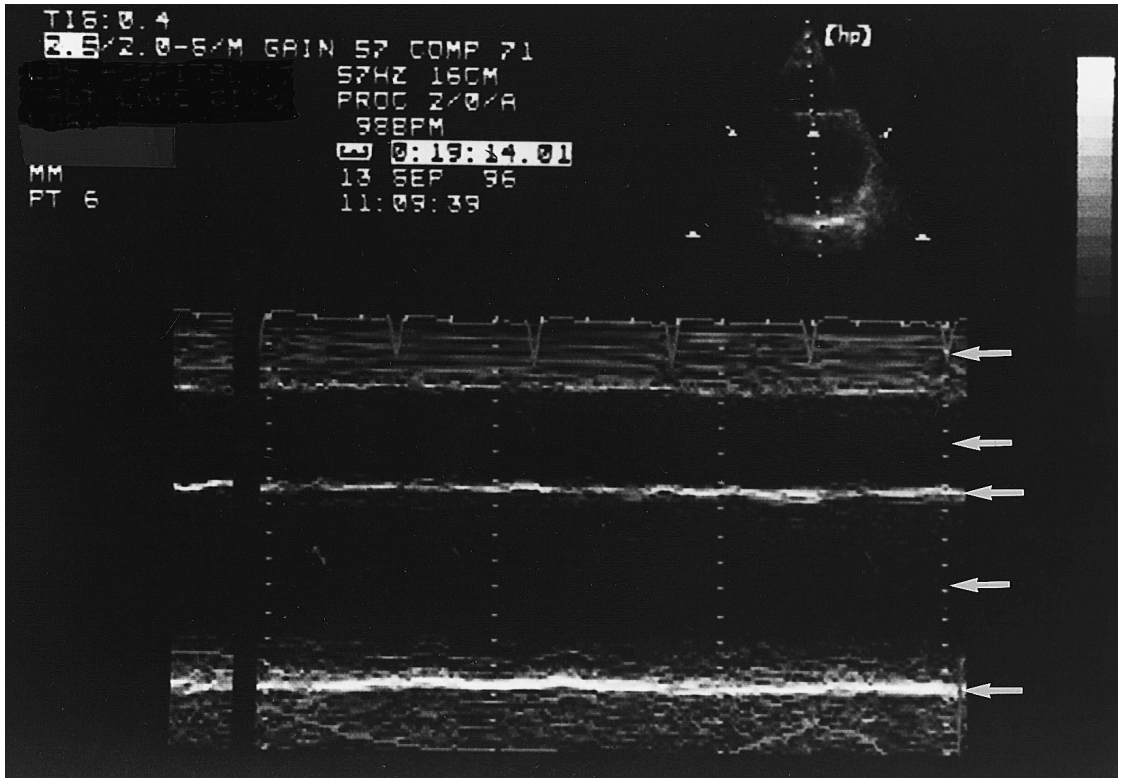
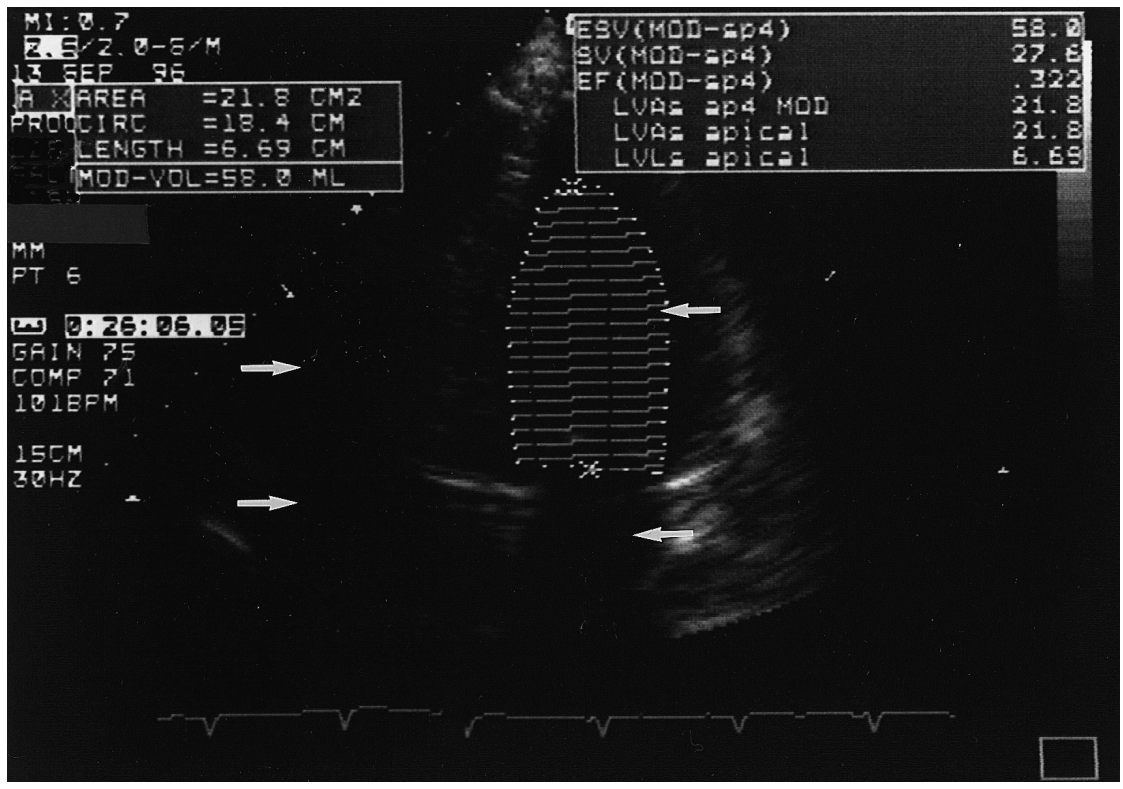


Fig. 28.6. Echocardiogram (2D and M-mode) of case study patient with cardiac hemochromatosis.
(*a*) Parasternal long axis view: top center arrow indicates right ventricle; bottom left indicates markedly enlarged left ventricle; top right arrow indicates aorta; bottom right arrow indicates left atrium.
(*b*) Short axis view: top arrow indicates right ventricle; bottom arrow indicates markedly enlarged left ventricle.

(*c*)



(*d*)



(*c*) M-mode tracing includes five arrows: top arrow points to anterior wall of right ventricle; second arrow points to moderately enlarged right ventricle; middle arrow refers to marked hypokinesis of the interventricular septum; fourth arrow points to markedly enlarged left ventricle; bottom arrow indicates marked hypokinesis of the posterior wall of the left ventricle.
(*d*) Top left arrow points to right ventricle; bottom left arrow points to right atrium; top right arrow points to left ventricle (cross-hatched area) with large volume of end-systolic blood; the calculated ejection fraction from this apical four-chamber view is very low at 32% (normal 55–78%); bottom arrow points to left atrium.

Conclusions

The cardiac manifestations of hemochromatosis may have a serious impact on a patient's health. Both structural and functional cardiac abnormalities occur in hemochromatosis, including dilated and restrictive cardiomyopathy, as well as resting and exercise-induced left ventricular systolic dysfunction. These abnormalities can often be ameliorated or reversed following iron removal by therapeutic phlebotomy. Accordingly, it appears that a reversible structural or metabolic defect is in part responsible for the cardiac abnormalities in the heart failure of hemochromatosis. Histologic study reveals that iron deposition within myocardial cells can occur with a minimum of fibrosis or inflammation, at least initially. Iron deposits are greatest in the ventricles, less in the atria, and least in the conduction tissue of the heart.

The ventricles usually increase in size and undergo a slight increase in mass, with reduction in systolic function–dilated cardiomyopathy. Less frequently, the ventricles are normal in size but there is impaired filling–restrictive cardiomyopathy. Both conditions can result in congestive heart failure. Supraventricular arrhythmias may be related to the amount of iron deposition in atrial myocardium but also may reflect ventricular dysfunction and heart failure. The functional left ventricular abnormalities can antedate clinical disease and may be unveiled by exercise radionuclide angiography, echocardiography or magnetic resonance imaging. Iron removal by repeated venesection can cause regression of cardiac enlargement, of hemodynamic abnormalities, and of exercise-induced ventricular dysfunction. The iron laden heart is not a strong heart, but a weak one. Iron depletion therapy can often make it strong again.

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29

Estimate of the frequency of morbid complications of hemochromatosis

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Introduction

There are many descriptions of the organ damage associated with late-stage hemochromatosis[1–12]. The frequency of some of the symptoms of illness, abnormal physical examination findings and laboratory abnormalities in hemochromatosis patients who were identified in different ways are summarized in Table 29.1. The purpose of this chapter is to compare the morbidity of hemochromatosis in homozygotes who were not identified due to illness and in probands who were identified due to illness.

Selection bias in identification of hemochromatosis homozygotes

There is a strong ascertainment bias in the identification of hemochromatosis probands. The ascertainment of probands who seek medical attention due to illness is biased for the presence of morbidity. Those who are found to have hemochromatosis only because they participated in a screening study have an ascertainment bias for good health[13–15]. A comparison of differences in the morbidity among groups of probands whose ascertainment was biased either for illness or for good health appears in Tables 29.2 and 29.3[10, 16]. The great differences in the morbidity among groups of sick probands and probands who were identified during screening clearly demonstrate the effect of ascertainment bias on the presentation of hemochromatosis.

The diagnosis of hemochromatosis is established much more frequently among men than in women[11, 17, 18]. This also represents an ascertainment bias explained, in part, by differences in the amount of iron that accumulates in male and in female homozygotes. Male homozygotes, as a group, have a greater body iron burden than female homozygotes. Normal women may lose 10–15 grams of iron during their lifetime due to menstruation and pregnancies[2]. These physiologic iron losses protect many, but not all, female homozygotes from developing the symptoms of illness, the abnormal physical exam findings, and the laboratory abnormalities that are common in males who have hemochromatosis. For this reason, fewer women are ascertained with hemochromatosis than men.

French/Canadian study of the effect of age and gender on morbidity

A recent retrospective study was designed to study the effects of gender on the clinical findings in hemochromatosis homozygotes[10]. The study provided a comparison of the incidence of the complications of hemochromatosis among male and female homozygotes in tertiary referral centers in France and Canada. A total of 352 hemochromatosis homozygotes were evaluated, including 176 men and 176 women who were matched for age. The women had a higher incidence of fatigue, and a modestly greater incidence of skin bronzing than the men. The most common presenting symptoms of illness among the men were related to hepatic disease. Normal values of both transferrin saturation and serum ferritin concentration were present in six percent of the women; none of the men had both a normal transferrin saturation and a normal serum ferritin concentration. The women had a mean serum ferritin concentration value that was approximately 50% as high as that in the men. The women had only two-thirds as much mobilizable storage iron, even though the hepatic iron concentration values were similar in the men and the women. Diabetes mellitus and hepatic cirrhosis were about one-half as common in the women as in the men. The authors concluded that there was less expression of hemochromatosis among women than men, but that some women developed progressive iron accumulation

Table 29.1. Clinical observations in subjects with hemochromatosis[a]

	Sheldon 1935[1]	Finch 1955[2]	Milder 1980[3]	Edwards 1982[4]	Adams 1991[6]	Milman 1991[7]	Fargion 1992[8]	Niederau 1996[9]	Moirand 1997[10]
Number of patients	311	787	34	41	118	179	212	251	352
Males	295	711	34	26	94	140	181	224	176
Females	16	76		15	24	39	31	27	176
Symptoms (percentage of patients)									
Weakness	13	70	73	22	9	79		82	54
Weight loss		44	53	7		69			
Arthralgias			47	56	13	44		44	40
Abdominal pain	26	29	50	20	6	34		56	
Loss of libido and/or impotence	6	14	56	24[b]	1	41		36	
Amenorrhea						10		15	
Cardiac complaints		33	35	39					
Asymptomatic			15	37	18				
Physical and laboratory findings (percentage of patients)									
Skin pigmentation	84	85	82	49		70	35	72	52
Hepatomegaly	92	93	76	54		84	75	81	
Abnormal hepatic function tests			54	61	13	92			
Cirrhosis	92		94	41		84	69	57	20
Hepatoma	6	14	18	2.4			12		
Splenomegaly	55	50	38	37		12		10	
Diabetes	79	82	53	12	10	47	30		12
Testicular atrophy		16	50	20					
Hypogonadism, documented			40[b]	20[b]			20[b]		
Hypogonadotrophic hypogonadism			100[b]	80[b]					
Arthropathy			44	68			13		
Cardiac arrhythmia		35	26	7			20	35	14
Congestive heart failure		33	35	2.4		15			

Notes:

[a] These symptoms of illness, abnormal physical examination findings, and laboratory abnormalities were observed in patients in nine studies from seven countries[1–4, 6–10]. Results expressed as percent.

[b] Findings in males only; not all males were studied.

and advanced disease despite the iron losses of menstruation and pregnancy[11]. The authors also concluded that women should be included in screening for hemochromatosis.

Avoidance of ascertainment bias in estimation of morbidity

The morbidity of hemochromatosis can be estimated without ascertainment bias only among a group of clinically unselected homozygotes. Clinically unselected hemochromatosis homozygotes are the relatives in whom the diagnosis is established through family screening after a proband is identified. These individuals are not selected either for illness or for good health. There are two known reports of the morbidity of hemochromatosis that included at least 70 homozygotes identified without ascertainment bias[19, 20].

Canadian study of morbidity in unselected homozygotes

The morbidity of hemochromatosis was studied recently in 74 Canadian homozygotes who were discovered during family studies or during screening, including 48 males and 28 females[19]. The age of the homozygotes was five to nine years greater in the male and female probands compared to the discovered homozygotes of the same sex. A comparison of the results of transferrin saturation, serum ferritin concentration, hepatic iron concentration, and hepatic iron index values in probands and in discovered homozygotes appears in Table 29.4.

Table 29.2. Clinical observations in hemochromatosis probands identified due to illness or during Utah screening studies[a]

Abnormality	59 sick probands (%)	38 screening probands (%)	Value of p^b
Symptoms			
Asymptomatic	0	90	<0.0001
Abdominal pain	48	5	<0.0001
Weakness, lethargy	54	5	<0.0001
Palpitations	37	5	<0.001
Impotence (males)	25	3	<0.008
Weight loss	17	5	<0.016
Clinical findings			
Skin pigmentation	71	19	<0.0001
Arthropathy	48	10	<0.0001
Hepatomegaly	56	3	<0.0001
Liver enzyme elevation	68	8	<0.0001
Liver iron stain grade 3–4[c]	98	21	<0.0001
Hepatic cirrhosis	42	0	<0.0001
Diabetes mellitus	27	0	<0.001
Cardiomegaly	14	0	<0.037
Documented hypogonadism	16	0	<0.026

Notes:
[a] Data summarized from [11].
[b] p values calculated by chi-square test.
[c] Scale of 0–4; normal grade 0–1.

Utah morbidity study in 86 clinically unselected homozygotes

Definition of clinically unselected homozygotes

All of the available first-degree relatives of 99 hemochromatosis probands were evaluated by HLA typing, serum iron concentration, percent saturation of transferrin and serum ferritin concentration. 86 siblings (45 males, 41 females) were identified who were HLA-identical to the proband in the family. These individuals were considered to be clinically unselected homozygotes because they were identified only during family studies. They were not selected either for illness or for good health. The clinically-unselected homozygotes underwent a history, physical examination, serum chemistry testing, measurement of serum or plasma hormone concentrations, other blood tests, radiographic studies, electrocardiography, and needle biopsy of the liver among those who consented. A preliminary report of the disease-related morbidity of hemochromatosis among these clinically unselected homozygotes has been published[20].

Table 29.3. Hepatic parenchymal cell stainable iron in symptomatic and asymptomatic hemochromatosis probands[a]

Probands	Age (range) years	n	Stainable hepatic iron[b] 0	1	2	3–4
Sick	49 (13–80)	59	0	0	1	58
Screening	29 (20–57)	38	6	15	9	8
Value of p^c				<0.0001		

Notes:
[a] Data summarized from [16].
[b] On a scale of 0–4, normal grade 0–1.
[c] p value calculated by chi-square test.

Definition of iron overload and of disease-related morbidity

Iron overload was defined as the presence of an hepatic parenchymal cell stainable iron grade of 2–4 (normal = 0–1), or a serum ferritin concentration greater than 325 ng/ml for men, and greater than 125 ng/ml for women. Morbidity was defined as the presence of organ damage, including arthropathy, hepatomegaly, abnormal hepatic function tests without a history of alcohol abuse or hepatitis, hypogonadism, hepatic fibrosis, or cirrhosis.

Disease-related morbidity among clinically unselected homozygotes and among probands

The clinically unselected homozygotes (Table 29.4) had an intermediate amount of disease-related morbidity that was greater than the probands who were ascertained through screening, and less than the probands who were ascertained because of illness (Table 29.1).

Effect of age and sex on morbidity among clinically unselected homozygotes

Males experienced disease-related morbidity much more frequently than clinically unselected females homozygotes. This result was expected, because iron is the cause of the morbidity of hemochromatosis, and males with the disorder usually are much more iron-loaded than females. The other major determinant of morbidity among unselected homozygotes was increasing age. Of the males under age 40, 80% were iron-loaded (based on elevation of serum ferritin concentration or hepatic iron stores), but only 12% had evidence of organ damage. After age 40, 95%

Table 29.4. Age, iron phenotype, and clinical features of 194 Canadian hemochromatosis homozygotes[a]

	Males		Females		
	Discovered[a]	Probands	Discovered[a]	Probands	Normal values
Number	46	95	28	25	
Age	48	53	54	63	
Transferrin saturation, %	70	83	68	72	<55
Serum ferritin, μg/l	876	2658	524	1555	M <300 F <200
Hepatic iron concentration, μmol/g dry weight	225	321	208	292	<35
Hepatic iron index	4.7	6.6	4.1	5	<1.9
Weakness, %	72	60	67	59	
Skin pigmentation, %	9	49	4	36	
Hepatomegaly, %	12	60	9	61	
Arthritis, %	26	42	25	52	
Testicular atrophy, %	7	24			
Cardiac failure, %	6	15	4	35	
Diabetes mellitus, %	14	36	8	22	
Hepatic disease, %	3	46	8	48	
Cardiac arrhythmia, %	3	20	0	29	
Hepatocellular carcinoma, %	1	5	0	5	

Note:

[a] These data include observations on 120 probands and 74 other homozygotes discovered during evaulation of relatives or during screening, not due to illness. These data were adapted from ref. [19]; frequency (%) was estimated from bar graphs. Numerical data not presented in original text.

of males were iron-loaded and 50% had evidence of disease-related morbidity. Of the women under age 40, 39% were iron-loaded, but none had evidence of organ damage. Seventy-four percent of the women over age 40 were iron-loaded, and 13% had evidence of morbidity.

Significance of estimates of morbidity in unselected homozygotes in screening strategy

After age 40, 95% of clinically unselected male homozygotes and 74% of female homozygotes were iron loaded. The implication of these findings is that men and women should be screened prior to age 40 in order to identify and treat iron-loaded homozygotes before the onset of the disease-related morbidity of hemochromatosis.

Hemochromatosis gene mutations

A mutation common in hemochromatosis was recently identified[21]. The mutation results in a cysteine to tyrosine conversion at amino acid 282 (C282Y) in the gene termed *HFE*, and is homozygous in 82–90% of hemochromatotics in most studies[15, 21–23]. The frequency of this mutation varies among different populations[15, 24]. A second mutation, resulting in a histidine to aspartic acid conversion at amino acid 63 (H63D), is less common among persons with hemochromatosis and is somewhat controversial with respect to its contribution to an abnormal iron phenotype. Early data suggested that this mutation occurred with similar frequency in both hemochromatosis and normal populations[21]. A more comprehensive analysis compiling data from several different populations suggested a slightly higher frequency of H63D among hemochromatosis patients[23]. Recent studies suggest that compound heterozygotes may have a higher risk of iron overload than single heterozygotes for the C282Y mutation[25, 26]. Whether the H63D mutation contributes to iron overload will be resolved when greater numbers of patients are examined and more complete clinical data are combined with the molecular genotyping for the two mutations.

To date, no other mutations in the *HFE* gene have been associated with hemochromatosis. Individuals having both the iron and clinical phenotype of hemochromatosis, but lacking C282Y or H63D mutations, will be of interest to identify non-*HFE* mutations leading to iron overload.

Table 29.5. Disease-related morbidity of hemochromatosis among 86 clinically unselected homozygous relatives of homozygous probands[a]

Findings	Of 45 males (%)	Of 41 females (%)
Arthropathy	13	2
Hepatomegaly	11	2
Hepatic enzyme elevation	11	2
Documented hypogonadism	2	2
Hepatic iron stain grade[b]	% of 39 biopsies	% of 21 biopsies
0	0	14
1	5	14
2	18	38
3–4	77	33
Hepatic fibrosis	8	0
Hepatic cirrhosis	18	0

Notes:
[a] From ref. [19]; used with permission of journal.
[b] Scale of 0–4; normal grade 0–1.

Although the C282Y *HFE* mutation cannot account for all cases of iron overload, it is clear from studies on unselected hemochromatosis homozygotes that C282Y homozygotes have a high risk of developing morbidity associated with iron overload[15].

Variability of expression among probands and unselected homozygotes

Because the iron accumulation of hemochromatosis occurs over several decades, it is expected that the vast majority of children, adolescents and premenopausal women who have two mutant hemochromatosis alleles will not be iron loaded and will not have morbidity at the time of testing. The best explanation for the variability of expression of hemochromatosis in the two studies that estimated the disease-related morbidity of hemochromatosis discussed in this chapter and in refs[19, 20] is the effect of sex and age. Clinically unselected female homozygotes develop less morbidity than males, and at an older age (Tables 29.4, 29.5).

Relevance of DNA testing to future estimates of morbidity

The ability to screen young populations for mutations in the *HFE* gene is important. The data from unselected homozygotes indicate that, with time, nearly all males and most females will develop iron-related morbidity. However, a significant proportion of clinically unselected homozygotes do not have any indication of disease-related morbidity. This finding favors the existence of other factors and perhaps other genes that influence iron metabolism.

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Juvenile hemochromatosis

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Definition

The term 'juvenile hemochromatosis' is used to describe a particularly severe form of genetic hemochromatosis, complicated by heart failure and endocrine disorders with manifestations in persons less than 30 years of age. Juvenile hemochromatosis should be distinguished from another iron overload syndrome of early onset known as neonatal hemochromatosis. The relationship of juvenile hemochromatosis to the common adult form of genetic hemochromatosis and to neonatal hemochromatosis is unclear at present. Elucidation of the etiology of these particular forms of severe and potentially fatal iron overload syndromes can be expected from the recently discovered hemochromatosis gene (*HFE*).

Incidence and prevalence

There is little information about the incidence and prevalence of clinical disease with symptomatic onset before the age of 30 years. The pattern of juvenile hemochromatosis was first recognized and described by French authors in the early 1930s and termed 'le syndrome endocrine-hepato-cardiaque'[1, 2]. The early French cases have been summarized by Royer de Vericourt in his thesis, published in 1935[3] when Sheldon's famous monograph[4] also appeared, in which he expressed his belief that hemochromatosis is the result of an inborn error of metabolism.

In the review of Finch and Finch[5] published in 1955, only 3.5% of their 787 patients were younger than 30 years. A review of the literature pertinent to juvenile hemochromatosis published in 1979 by Lamon et al.[6] reviewed 52 cases. Since 1979, at least 13 additional cases have been described[7–12]. Unlike adult genetic hemochromatosis, juvenile hemochromatosis appears to affect males and females equally[6] (Table 30.1) Because most of the published data on juvenile hemochromatosis are based on single case reports, its prevalence and distribution in different ethnic populations is difficult to estimate. The great majority of, if not all, cases published so far are confined to the populations of Northern European origin, as is the case for the more common adult form of hemochromatosis.

Clinical features

Juvenile hemochromatosis presents a pattern of parenchymal organ damage that is indistinguishable from adult hemochromatosis except for a greater incidence of cardiac damage[13] and hypogonadotropic hypogonadism[7], and a lower frequency of diabetes mellitus and hepatic damage[6, 14, 15]. Testicular atrophy or amenorrhea are the presenting symptoms. Heart failure due to cardiomyopathy is the main cause of death ([6, 15]; Table 30.1) whereas primary hepatocellular carcinoma is the most frequent cause of death in treated patients with the common form of hemochromatosis[16].

Hypogonadism

In a survey collected from the literature, Goosens[15] analyzed 55 patients whose first signs of hemochromatosis occurred before the age of 30 years, and compared these to 1098 adult-onset cases. Testicular atrophy and amenorrhea were the presenting features in 94% of the juvenile hemochromatosis subjects, whereas hypogonadism was present in only 17% of the adult hemochromatosis control population with disease onset at >40 years of age.

In 53 patients with juvenile hemochromatosis accumulated from the literature up to 1979[6], 64% (34 of 53 subjects)

had hypogonadism, compared to 30% (236 of 787 subjects) of the published series of adult hemochromatosis cases of all ages reported by Finch and Finch in 1955[5].

In non-alcoholic males with hemochromatosis, and in many males with juvenile hemochromatosis, testicular atrophy and amenorrhea probably occur due to insufficient gonadotropin secretion due to iron accumulation in gonadotropic cells of the pituitary gland[6, 7, 9–11, 15, 27, 28].

For example, the inadequate gonadotropin response to the luteinizing/hormone-releasing/factor (LHRH) of a 20-year-old German male with juvenile hemochromatosis is depicted in Fig. 30.1. Hypogonadotropic hypogonadism due to pituitary abnormality is also found in most patients with thalassemia major who are severely iron overloaded owing to regular red blood cell transfusions[29].Thus hypogonadism in juvenile hemochromatosis of both sexes appears to be the result of a primary, isolated inadequate gonadotropin release, rather than due to direct testicular or ovarian damage by excessive iron[6, 7, 27].

It is, however, not clear whether isolated gonadotropin insufficiency is the only reason for hypogonadism in juvenile hemochromatosis. Because loss of libido and testicular atrophy are also prominent features in alcoholic cirrhosis[27], the severity of hepatic disease might have additional effect on hypogonadism, via hyperestrogenism, at least in males. This has been shown to be unlikely, however, considering that gynecomastia due to impaired androgen–estrogen metabolism in males occurs in >60% of alcoholics, but is extremely rare in hemochromatotics[27]. The equal frequency of hypogonadism in males and females with juvenile hemochromatosis also suggests that primary hypothalamic–pituitary dysfunction occurs in this disorder. Unfortunately, at the time when clinical manifestations of hypogonadism are apparent in juvenile hemochromatosis damage is irreversible in most cases[6, 7].

Table 30.1. Causes of death in juvenile hemochromatosis (case reports from the literature)

References	Sex	Age of onset (years)	Age of death (years)	Cause of death
1	M	18	20	Heart failure
17	M	19	21	Heart failure
19	F	19	21	Infection
20	M	15	17	Heart failure
21	M	14	14	Occlusion of the portal vein
22	F	18	—	Alive
23	M	4?	21	Hepatic coma
18	M	19	19	Heart failure
24	M	17	24	Heart failure
25	M	13	—	Alive
26	F	23	—	Alive
26	F	21	—	Alive
13	F	?	20	Heart failure
13	F	?	28	Heart failure
13	M	?	24	Heart failure
13	M	?	19	Heart failure
15	F	25	—	Alive
6	F	16	—	Alive
11	M	22	22	Heart failure
7	M	20	21	Heart failure
7	M	18	19	Heart failure
7	F	18	—	Alive
7	M	17	—	Alive
10	F	20	21	Heart failure
8	M	22	—	Alive
9	F	21	—	Alive

Note:
numbers: order of references.

Cardiomyopathy

Cardiac failure due to cardiomyopathy is the second most common presenting feature of juvenile hemochromatosis. Symptoms of cardiac failure in younger subjects suffering from hemochromatosis frequently develop suddenly and progress rapidly. In Lamon's series[6] of 53 cases, cardiomyopathy was present in 58.5 %, significantly greater than the frequency of 35% in an adult hemochromatosis control group. Cardiac failure has also been the main cause of death in the majority of patients (80%) reported in the literature up to 1975[15]. Like the adult form of hemochromatosis, congestive heart failure and cardiac arrhythmias are the most common complications of cardiomyopathy in juvenile hemochromatosis. However, symptoms of congestive heart failure may be misdiagnosed if other manifestations of hemochromatosis are absent[13, 25].

Histologic studies reveal that iron deposition occurs mainly within the myocardial cells, and is associated with minimal fibrosis or inflammation[7, 30, 31]. The myocardial iron concentration is much higher in the subendomyocardial region. This pattern of iron distribution may be of importance regarding possible sampling errors during monitoring of hemochromatotic cardiomyopathy by sequential endomyocardial biopsies[8]. Conducting tissue is generally less affected by iron deposition than the contractile myocardium[8, 30, 31]. The ventricles generally increase in size and mass, and have reduced systolic function (dilated cardiomyopathy). Less frequently, the ventricles may be normal in size, but there is impaired filling (restrictive cardiomyopathy)[31, 32]. Both conditions result in

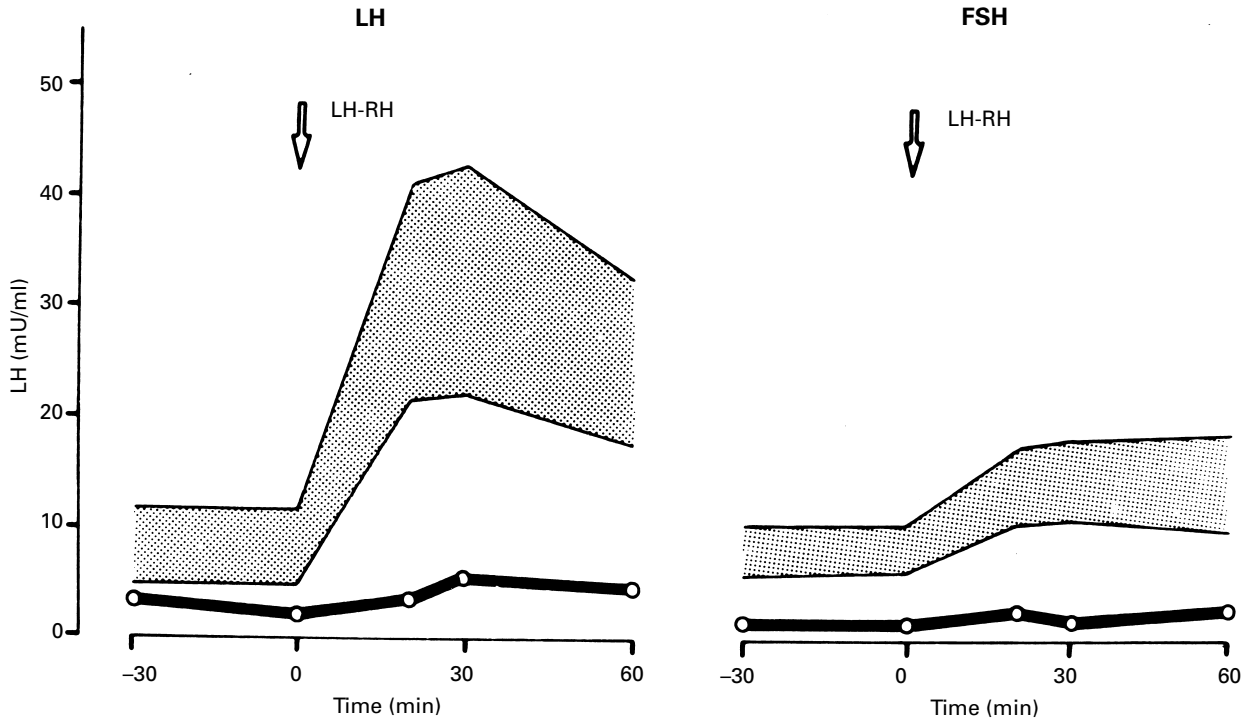

Fig. 30.1. Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) after i.v. injection of 100 μg reagent (LH-RH, Relefact®) in a 21-year-old male patient with juvenile hemochromatosis, showing inadaequate gonadotropin response. Shaded areas represent normal response range[28].

congestive heart failure, and there is a positive relationship between the severity of myocardial iron load and cardiac dysfunction[30].

Disturbances of cardiac rhythm also occur in juvenile hemochromatosis, and, when present, are a grave prognostic sign. Most common are ventricular extrasystoles; supraventricular and ventricular tachycardias, atrial fibrillation, and heart block may also occur. There may be other electrocardiographic changes such as low voltage or left axis deviation.

Monitoring of cardiac function has been facilitated by the advent of echocardiography to assess ventricular pathophysiology. Radionuclide angiography is another non-invasive technique that can be used to evaluate left ventricular systolic function at rest and to assess the functional reserve of the ventricles during exercise[31]. Determination of the myocardial iron content by magnetic resonance imaging (MRI) may soon become useful for the evaluation and follow-up of patients with myocardial iron overload due to hemochromatosis, and may be helpful in avoiding sampling errors of endomyocardial biopsies due to the inhomogenous distribution of iron in the myocardium[8].

According to Lamon's review of the literature[6], liver involvement (expressed by hepatomegaly) is slightly less frequent (83%) in juvenile hemochromatosis than in symptomatic patients with hemochromatosis diagnosed after the age of 30 years (94%). In contrast to Lamon's analysis, Goosen[15] reported that hepatomegaly was present in 100% of subjects with juvenile hemochromatosis, whereas in adult-onset cases the frequency was 93%. In both reviews, diabetes mellitus was observed less frequently in juvenile hemochromatosis[6, 15]. A comparison of long-term complications of diabetes in the two groups is not available. Skin pigmentation was present with similar frequency in juvenile and adult-onset forms of hemochromatosis. Hemochromatotic arthropathy appears to be largely a complication of older patients. Only 9% of patients (5 of 53) in Lamon's review[6] had symptoms of arthropathy. The significance of this low frequency of joint involvement in young patients is difficult to assess, because hemochromatotic arthropathy was first described in 1960[33] and may have been missed in earlier reports.

The natural history of juvenile hemochromatosis is very similar to that of patients with thalassemia major treated with chronic red blood cell transfusion (Fig. 30.2). The

obvious parallelism in incidence and expression of clinical features and the similarity in age relationship strongly indicates that iron overload per se is the major factor responsible for the clinical expression of disease in both conditions.

Genetics of juvenile hemochromatosis

Well-documented cases of juvenile hemochromatosis have been reported in the last 60 years in France, Italy, Germany, Great Britain, Switzerland, the Netherlands, Scandinavia, Spain, Canada, USA, Argentina, South Africa and Australia[15]. The distribution of case reports is consistent with the theory of North European origin of the adult-onset form of hemochromatosis and of juvenile hemochromatosis. It also indicates that there may be a strong relationship, if not identity, between both forms of hemochromatosis.

In the classical adult form of hemochromatosis, males are predominantly affected, but juvenile hemochromatosis seems to affect males and females equally[6].Whether juvenile hemochromatosis differs in its pattern of inheritance is not known. Autosomal recessive inheritance seems likely[6, 7, 15, 26, 39]. This pattern of inheritance is supported by the findings of equal sex distribution, clinical expression of the disease in siblings with absence of symptoms in parents of affected individuals, and the presence of consanguinity in some families[6, 15]. Autosomal dominant inheritance cannot, however, be excluded[6, 18]. Studies of possible HLA linkage so far are limited and inconclusive. Whether there is a different genetic defect in the young patients or whether juvenile hemochromatosis is a more severe form of the adult disease remains unclear. However, all HLA haplotypes of proven cases of juvenile hemochromatosis published so far differ completely from the ancestral haplotype HLA-A3,-B7(n-B14) (Table 30.2). Moreover, a report indicates that five patients with juvenile hemochromatosis were negative for the recently discovered hemochromatosis candidate gene *HFE* on chromosome 6[12, 34].The discovery of the C282Y and H63D mutations of the *HFE* gene will permit more detailed insights into the fundamental defect or defects responsible for the abnormal iron accumulation in hemochromatosis. If the assumption that cases of juvenile hemochromatosis do not share the mutated *HFE* candidate gene typical of the adult form of hemochromatosis is correct, more than one genetic mechanism for the syndrome of hemochromatosis must be hypothesized.

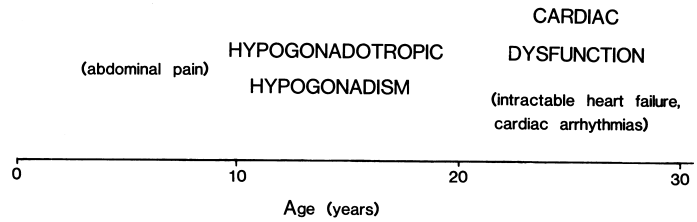

Fig. 30.2. Scheme of the natural history of juvenile hemochromatosis. The temporal relationship is schematic and may be substantially different in different patients[7].

Dietary and other extrinsic factors

The two extrinsic factors that merit the most attention for the clinical expression of hemochromatosis are the amount of iron intake and alcohol consumption. Major sources of iron include diets rich in meat content, iron cooking utensils and containers, water with a high iron content, iron medications or 'tonics' and alcohol[13]. Excessive alcohol consumption is probably the single most important extrinsic factor, because it appears to affect both the body iron burden and the degree of tissue damage.

In most of the case reports on juvenile hemochromatosis, there are no suggestions of excessive iron ingestion from the diets, cooking utensils, or drinking water. Unlike adult hemochromatotics unusually high alcohol consumption has been reported in only one case (a 24-year-old Danish male patient with hemochromatosis)[8]. His alcohol abuse occurred between the age of 17 and 22 years, and had ceased two years before clinical manifestation of hemochromatosis. There were no cases of hemochromatosis, or endocrinologic or cardiologic disorders in this patient's family.

In Lamon's review of juvenile hemochromatosis[6], 29 of the 53 patients reported were the only family members with clinically manifest hemochromatosis. In two Italian and two German cases[7], the parents had no signs of clinically manifest iron overload. The fact that no single case of fully developed disease in different generations has been observed in various families[7, 15, 18, 25], makes it extremely unlikely that environmental factors play a relevant role in the early manifestation of iron overload in juvenile hemochromatosis.

An effect of iron fortification of dietary materials (such as flour) on the time and extent of clinical expression of juvenile hemochromatosis also seems to be very unlikely. Juvenile hemochromatosis was observed many years before fortification programs were introduced, and the disease has been described most frequently in France, where iron fortification of the diet has never been used in

Table 30.2. HLA class I haplotype in five cases of juvenile hemochromatosis

	Case				
	1	2	3[a]	4[a]	5
Sex	f	m	m	m	m
Age of onset (y)	18	17	19	19	24
% Satur. of TIBC	90	93	88	78	98
Serumferritin	4400	3280	—	2100	4170
Liver iron (scale + to ++++)	++++	++++	++++	++++	++++
Hypogonadism	++	++	++	++	++
Heart	Arrhythmia	Arrythmia	Congestive heart failure	Congestive heart failure	Restrictive cardiomyopathy
HLA-haplotype	A9, w30, 31 B12, 13	A9, w30, 31 B12, 13	A19, — B35, 17	A19, — B35, 19	A 1, 2 B5, 7
Outcome	Alive	Alive	Death	Death	Alive (HTX)
References	7	7	7	7	8

Note:
[a] identical twins.
HTX = heart transplantation.

a national program. Even very efficient iron fortification programs such as that carried out in Sweden have a minor influence on the expression of hemochromatosis[35]. The only dietary factor of possible importance for individual iron balance in hemochromatosis (besides alcohol abuse) is the proportion of meat in the diet. There is greater accumulation of storage iron in young homozygotes in Australia than in Swedish male homozygotes (Fig. 30.3). This suggests that there is an influence of the meat content of the diet on disease expression in iron-loading gene(s) carriers, taking into account that the average meat consumption is much higher in Australia than in Sweden[35].

Iron absorption studies in patients with juvenile hemochromatosis are few. In one of identical twins[7], intestinal iron absorption was studied using ^{59}Fe – test dose absorption measurements by whole-body counting. At the time of diagnosis, with no previous phlebotomy, and with histologically proven tissue iron overload, a patient absorbed 100% of an oral test-dose of 0.5 mg of radioactive iron (normal range for adult men $28 \pm 9\%$). In another male patient with juvenile hemochromatosis aged 21 years, Alper et al.[36] performed radioiron studies. This patient, who had myocardial failure, sexual hypoplasia, and hepatic enlargement, retained 60% (20.4 mg) of an orally administered dose of 35 mg of iron. According to these limited data, the absorption pattern in cases of juvenile hemochromatosis seems to differ considerably from that of patients with the adult form of hemochromatosis, in whom absorption rates in advanced stages are in the normal range in the majority of cases[37]. However, further absorption studies are needed to confirm this striking difference in iron absorption.

Treatment

Regular phlebotomy is the treatment of choice in juvenile hemochromatosis. The manifestations of organ damage (sexual retardation, cardiac and hepatic failure, and diabetes mellitus) should be managed using conventional therapy. Because hypogonadism and heart failure are prominent presenting features in juvenile hemochromatosis (Table 30.2, Fig. 30.2), children and young adults with this type of organ damage should always be investigated for clinical signs of iron overload. The measurement of serum iron concentration, transferrin saturation, and serum ferritin concentration may be sufficient to evaluate whether iron overload is present. Because hypogonadotropic hypogonadism due to iron overload may result in untreatable infertility, and because cardiomyopathy may rapidly lead to death, knowledge about juvenile hemochromatosis is of more than academic interest and should be known in more detail by clinicians. There is a limited chance to detect this rare form of severe hemochromatosis early enough through family studies, because a great proportion of patients reported have not had other affected

family members[6]. Late (or even postmortem) diagnosis of juvenile hemochromatosis has been described in many case reports[6–11, 13, 15, 18, 28]. Careful hematologic assessment is also required in young subjects to exclude iron-loading secondary to congenital dyserythropoietic disorders such as thalassemia intermedia and sideroblastic anemias.

The management of organ damage in juvenile hemochromatosis and in the adult form of hemochromatosis depends very much on the stage of organ dysfunction present at time of diagnosis. Early institution of phlebotomy treatment to remove iron overload prevents heart failure, whereas fertility can be restored by therapy with gonadotropins[6, 7, 9, 25, 38]. Mortality is unusually great in juvenile hemochromatosis, and cardiac failure is the most prominent cause of death (Table 30.1)[3, 15, 28]. In cases with advanced cardiomyopathy, aggressive bleeding may become a serious, life-threatening problem[28]. These patients cannot tolerate phlebotomy easily and severe hypotension and arrhythmias may develop if the blood volume is not maintained while therapeutic phlebotomy is being performed. Replacement of the plasma that has been removed is therefore recommended in such patients. Ascorbic acid has an additional adverse effect on cardiac function in iron-loaded subjects and should be avoided in iron mobilization treatment regimens[40].

Although the beneficial effect of iron removal by phlebotomy on the cardiac function has been documented in several cases[25, 38], patients with manifest cardiac involvement may also be successfully treated with continuous infusions of desferrioxamine (DFO; ~4 g/24 h). Even heart transplantation may be considered in juvenile hemochromatosis patients with severe cardiac disease who fail to improve with conventional treatment[8]. In the first patient who underwent successful heart transplantation, this became necessary despite intensive iron mobilization treatment by phlebotomy and DFO administration. MRI determination of the myocardial iron content is possible, and may be a useful supplement in the follow-up of these patients[8].

A 26-year-old Italian woman with a five-year history of secondary amenorrhea due to juvenile hemochromatosis was treated successfully by phlebotomy and gonadotropin therapy, resulting in a twin pregnancy with uncomplicated term delivery and postpartum clinical course[9]. Even when pituitary function has been irreversibly impaired in males, testicular function can still be initiated by treatment with gonadotropins at least for a few years[7].

Other therapeutic recommendations do not differ from those given for patients with typical adult hemochromatosis. There is no place for a low-iron diet during or after phlebotomy therapy. Regular drinking of black as well as green tea together with daily meals, however, may be recommended, because it reduces the intestinal iron absorption of non-heme iron in the diet of patients with hemochromatosis significantly[41, 42].

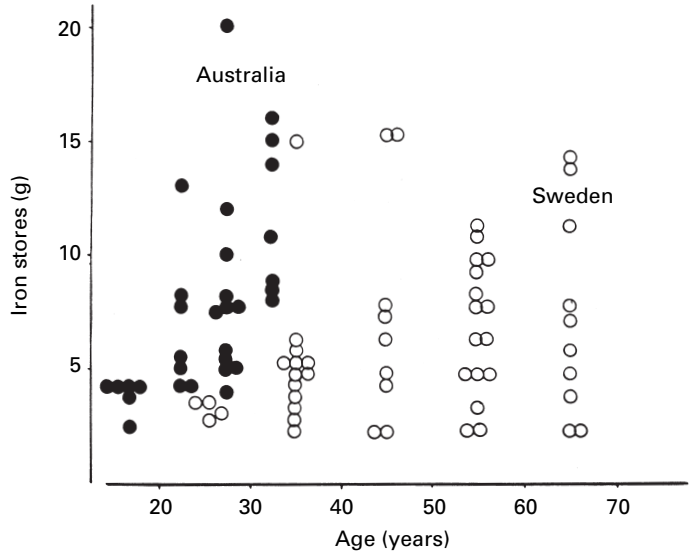

Fig. 30.3. Mobilizable iron stores as a function of age in Swedish male homozygotes (○) and young Australian homozygotes (●)[35].

Course and prognosis

The natural history of genetic hemochromatosis has changed considerably over the last sixty years. In Sheldon's review in 1935[4], diabetes mellitus was the cause of death in approximately half of the patients, and carcinoma of the liver in only 7%. Fifty years later, primary hepatic carcinoma has become the most important cause of death in persons with the adult form of hemochromatosis[16]. In cases of juvenile hemochromatosis, cardiac failure has always been the main cause of death (Table 30.1). In his review of 55 cases of juvenile hemochromatosis published between 1932 and 1975, Goosens[15] demonstrated that 80% of deaths were due to cardiac failure. Of the 26 cases shown in Table 30.2, 16 (62%) died in their early twenties. Among these 16 cases, 13 (81%) died from cardiac failure, usually one to two years after the onset of clinical symptoms (Table 30.1). These data demonstrate that patients with juvenile hemochromatosis have a particularly bad prognosis. However, damage to tissues such as cardiac muscle can be arrested by removal of iron with significant improvement of cardiac function[25]. Even gonadal function, relatively unchanged by phlebotomy treatment in most adult-onset patients[40], has been improved by phlebotomy treatment in

juvenile hemochromatosis, provided therapy can be started early enough[9, 15]. For those juveniles with very advanced cardiac involvement, heart transplantation may also be considered as a life-saving treatment modality[8]. However, advanced and life-threatening organ damage is best prevented by earlier diagnosis of juvenile hemochromatosis.

Conclusions

Juvenile hemochromatosis is an iron overload syndrome very similar to the adult form of hemochromatosis, but distinguishable by the early onset and by the particularly high incidence of hypogonadotropic hypogonadism and cardiac damage as presenting clinical features. There is anecdotal evídence that intestinal iron absorption is unusually high in juvenile hemochromatosis, and that some genetic pattern such as the ancestral haplotype HLA-A3,-B7 (n-B14) and the *HFE* mutation are absent in this iron-overload syndrome. However, it is uncertain whether there is a different defect in the younger patients, or whether the juvenile disease is a more severe form of the adult disease.

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Part VI

Therapy of hemochromatosis and iron overload

31

Management of hemochromatosis[a]

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Introduction

The complications of iron overload in hemochromatosis can be avoided by early diagnosis and appropriate management. Therapeutic phlebotomy is used to remove excess iron and maintain low normal body iron stores, and it should be initiated in men with serum ferritin levels of 300 g/l or more and in women with serum ferritin levels of 200 μg/l or more, regardless of the presence or absence of symptoms. Typically, therapeutic phlebotomy consists of (i) removal of 1 unit (450 to 500 ml) of blood weekly until the serum ferritin level is 10 to 20 μg/l and (ii) maintenance of the serum ferritin level at 50 μg/l or less thereafter by periodic removal of blood. Hyperferritinemia attributable to iron overload is resolved by therapeutic phlebotomy. When applied before iron overload becomes severe, this treatment also prevents complications of iron overload, including hepatic cirrhosis, primary liver cancer, diabetes mellitus, hypogonadotrophic hypogonadism, joint disease, and cardiomyopathy. In patients with established iron overload disease, weakness, fatigue, increased hepatic enzyme concentrations, right upper quadrant pain, and hyperpigmentation are often substantially alleviated by therapeutic phlebotomy. Patients with liver disease, joint disease, diabetes mellitus and other endocrinopathic abnormalities, and cardiac abnormalities often require additional, specific management. Dietary management of hemochromatosis includes avoidance of medicinal iron, mineral supplements, excess vitamin C, and uncooked sea foods. This can reduce the rate of iron reaccumulation; reduce retention of non-ferrous metals; and help reduce complications of liver disease, diabetes mellitus, and *Vibrio* infection. This comprehensive approach to the management of hemochromatosis can decrease the frequency and severity of iron overload, improve quality of life, and increase longevity.

Diagnosis and initial evaluation

Diagnosis of hemochromatosis

Persons with hemochromatosis have an inherited propensity to absorb excess iron; most persons are of European origin and are homozygotes or compound heterozygotes for a mutant gene or genes on chromosome 6p[1, 2]. Hyperferremia and increased iron saturation of transferrin are essential attributes of hemochromatosis. A transferrin saturation of 60% or more for men and 50% or more for women on at least two occasions in the absence of other known causes of elevated transferrin saturation suggests the diagnosis of hemochromatosis[1, 2] and permits affected persons to be identified before iron overload develops. Normal or subnormal serum transferrin saturation values occur in unusual circumstances[3]. Many persons who have hemochromatosis without iron overload are children, young adults, and premenopausal women. Although iron overload often develops in patients with hemochromatosis, the demonstration of hepatic or systemic iron overload and associated complications is not needed to confirm the diagnosis (Table 31.1)[1, 2, 5].

Evaluation of iron overload

Iron overload develops primarily because mechanisms to eliminate excess iron are limited. Many persons, particularly men, eventually develop severe iron overload. Women are at lower risk, partly because of iron losses during menstruation, childbirth, and lactation[1, 2]. The severity of iron

[a] This work originally appeared in the *Annals of Internal Medicine* 1998;129:932–9, and is reproduced here by permission.

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Table 31.1. Evaluation of patients with hemochromatosis and iron overload

Abnormality	Indicated evaluations
Weakness, fatigue, malaise	Evaluation for liver, endocrinologic, and cardiac disorders, and for anemia
Elevated hepatic enzyme concentrations	Evaluation for hepatitis B, hepatitis C, and other liver disease (such as drug-related hepatopathy, α_1-antitrypsin deficiency, or metastatic cancer); liver biopsy
Hepatomegaly or hepatic pain	Evaluation for hepatitis B, hepatitis C, and other liver diseases; liver biopsy; measurement of α-fetoprotein concentration; hepatic imaging procedures
Arthralgias or arthropathy	Radiography of affected joints; evaluation for non-hemochromatosis-associated arthropathy (such as gout and rheumatoid arthritis)
Hyperglycemia or diabetes mellitus	Fasting serum glucose and glycosylated hemoglobin concentrations; evaluation for diabetes complications, (such as renal dysfunction, neuropathy, and retinopathy)
Thyroid dysfunction	Measurement of thyroid-stimulating hormone, triiodothyronine, thyroxine, and antithyroid antibody concentrations; thyroid scanning
Impotence, premature amenorrhea	Measurement of luteinizing hormone, follicle-stimulating hormone, and sex hormone concentrations; computerized tomography of anterior pituitary to evaluate for possible pituitary tumor
Cardiac symptoms	Electrocardiogram; echocardiography; assesssment of ventricular ejection fraction and exercise tolerance; coronary arteriography

overload is most often determined by measuring the serum ferritin level, although inflammation or cancer can elevate this level in the absence of iron overload. Approximately 90% of excess iron is retained in the liver. Therefore, many patients benefit from analysis of liver biopsy specimens to identify liver disease and to determine the presence or absence of cirrhosis, which directly affects prognosis. Biopsy specimens should be evaluated for iron by histochemical methods (Perls staining) and quantitative techniques (atomic absorption spectrometry)[1–7]. The quantity of iron removed by therapeutic phlebotomy is a valuable retrospective indicator of the severity of iron overload[8]. Radiologic imaging techniques are too insensitive for the evaluation of most young, asymptomatic persons with little or no excess hepatic iron[1, 2]. The hepatic iron index is useful in distinguishing persons who are homozygous for hemochromatosis from heterozygotes and persons with other hepatic disorders[5, 9]. Some patients have coincidental conditions that augment iron absorption and thus increase iron overload (for example, excessive dietary iron supplementation, excess ethanol ingestion, porphyria cutanea tarda, or hemolytic anemia)[1, 2, 10, 11]. Because serum iron variables in patients with viral hepatitis can mimic those in patients with hemochromatosis and because some patients have both disorders, persons with hemochromatosis must often be evaluated for hepatitis[12–14].

Medical evaluation before treatment

From each patient, physicians should collect information that includes a review of current and past symptoms and health problems, especially those related to liver, joint, and heart disease; diabetes mellitus and other endocrinopathic conditions; sexual function; and skin pigmentation[1, 2]. A dietary history should focus on general dietary habits and food choices, use of dietary supplements, and ingestion of ethanol. Any history of blood donation, receipt of blood transfusion, and illness associated with blood loss should be documented. The details of menstruation, childbirth, lactation, menopause, and hysterectomy are important (women taking oral contraceptives may have decreased menstrual blood loss or may absorb less dietary iron). The history should include inquiries about family members, especially first-degree relatives. The physical examination must include assessment of the liver, joints, heart, endocrine status, and skin coloration. Certain sequelae of iron overload may require additional specific evaluations to assess management needs (Table 31.1).

Therapeutic phlebotomy

Described in 1952, therapeutic phlebotomy was the first successful treatment for iron overload due to hemochromatosis[15] and is still the preferred treatment for this condition today[1, 2]. The removal of 1 unit of blood (450 to 500 ml)

Table 31.2. Criteria for initiating therapeutic phlebotomy in homozygotes or heterozygotes for hemochromatosis gene or genes and other persons with a hemochromatosis phenotype, regardless of genotype[a]

Patient	Serum ferritin level (μg/l)
Persons <18 years of age	≥200
Women	
Reproductive years, non-pregnant	≥200
Reproductive years, pregnant[b]	≥500
Post-reproductive	≥300
Men, all ages	≥300

Notes:

[a] All persons with porphyria cutanea tarda should undergo iron depletion therapy to reduce the serum ferritin concentration to 10–20 μg/l.

[b] Unless substantial cardiac or hepatic dysfunction attributable to iron overload is present, induction of therapeutic phlebotomy should be deferred until the end of pregnancy (a normal term pregnancy removes approximately 1 g of iron from the mother).

results in the loss of 200 to 250 mg of iron. Although iron chelation and erythrocytapheresis have also been used[16, 17], therapeutic phlebotomy is safer, more efficient, and more economical[1, 2].

Selection of patients for treatment

Most persons with hemochromatosis benefit from therapeutic phlebotomy (Table 31.2). Rarely, children and adolescents have severe iron overload (often associated with cardiac and anterior pituitary failure) and need aggressive therapeutic phlebotomy for removal of 1.5 to 2.0 units weekly, if possible[18–20]. Withholding therapeutic phlebotomy from older patients on the basis of age alone is not justifiable. In asymptomatic persons with iron overload (Table 31.2), therapy must not be delayed until symptoms develop. However, some patients are not candidates for treatment because they are intolerant toward phlebotomy or have limited life expectancy. Patients with severe, refractory anemia require iron chelation therapy[21]. Approximately 8% of white persons of western European descent inherit one detectable hemochromatosis gene and thus are heterozygotes[22]. Of the 1% to 3% of heterozygotes who develop iron overload[23], many have a coincidental disorder that increases iron absorption or alters iron metabolism[1, 2, 14]; others may have an additional hemochromatosis mutation or mutations undetectable by current testing methods[24]. Many persons with porphyria cutanea tarda have skin lesions that are alleviated with therapeutic phlebotomy, and many are heterozygous for *HFE* mutations[2, 25–27]. No study has shown the benefits of therapeutic phlebotomy in other persons with iron overload who are heterozygotes or compound heterozygotes for the hemochromatosis gene or genes. However, we recommend that all persons with iron overload who have a clinical phenotype consistent with hemochromatosis, regardless of genotype, receive therapeutic phlebotomy and management similar to that recommended for homozygotes for 'classic' hemochromatosis (Table 31.2).

Performance of therapeutic phlebotomy

Therapeutic phlebotomy should be done by experienced persons and should be supervised by a physician. It is usually performed in a physician's office but can be done in a medical laboratory, a blood bank, or a patient's home. However, comprehensive management of hemochromatosis is usually accomplished best in a physician's office. For many patients, compliance with treatment is proportional to the skill of the phlebotomist and the confidence of the patient in the treatment staff and environment. Adequate hydration and avoidance of vigorous physical activity for 24 hours after treatment minimize the effects of hypovolemia caused by therapeutic phlebotomy. Persons with a hemoglobin concentration less than 110 g/l or a hematocrit less than 0.33 before treatment are more likely to have symptoms of hypovolemia and anemia, and phlebotomy is less efficient in removing iron in these patients. However, many patients with chronic hemolytic anemia and iron overload tolerate phlebotomy well. The hemoglobin concentration or hematocrit and volume (or weight) of blood removed with each phlebotomy session should be documented.

Frequency and duration of therapeutic phlebotomy

Depletion of iron stores typically involves the removal of 1 unit of blood weekly until mild hypoferritinemia occurs[1, 2]. Some men and persons with large body mass can sustain removal of 1.5 to 2.0 units of blood weekly. Some women, persons with small body mass, elderly persons, and patients with anemia, cardiac problems, or pulmonary problems can sustain removal of only 0.5 units of blood weekly. After a few weeks of therapeutic phlebotomy, erythroid hyperplasia permits more blood to be removed more often in many patients. Although recombinant human erythropoietin therapy also enhances erythrocyte production, this therapy should be reserved for patients who also have renal dysfunction or anemia of chronic disease[28]. Life expectancy may be substantially decreased

in patients in whom iron depletion by phlebotomy cannot be completed within 1 year[29]. Serum ferritin and hepatic iron levels permit a relative estimation of the amount of therapeutic phlebotomy required for iron depletion[2]. On average, men require twice as many units of therapeutic phlebotomy as women do[24, 30, 31]. Older persons typically have more severe iron overload, as do persons who are homozygous for *HFE* mutation C282Y[2, 24, 32]. Hormonal factors, diet, abnormalities that alter iron absorption, and blood loss also influence the severity of iron overload[33]. Persons who have been regular blood donors often have less severe iron overload than do nondonors[1, 34]. The serum ferritin level is the most reliable, readily available, and inexpensive way to monitor therapeutic phlebotomy; the serum iron level and the transferrin saturation are less suitable[1, 2]. In general, patients who have higher serum ferritin levels have more severe iron overload and need more phlebotomy. Among patients who have serum ferritin levels greater than 1000 μg/l before treatment, it is sufficient to quantify the serum ferritin level every 4 to 8 weeks during the initial months of treatment. The serum ferritin level should be measured more often in patients who have received many phlebotomy treatments and in those who have mild or moderate iron overload at diagnosis. In all patients, serum ferritin levels should be quantified after each additional one or two treatments once the value is 100 μg/l or less. Progress of treatment is also monitored by assessing hemoglobin concentration and hematocrit, their recovery rates, and mean corpuscular volume. Iron depletion is complete when the serum ferritin level is 10 to 20 μg/l or when the hemoglobin concentration is less than 110 g/l or the hematocrit is less than 0.33 for more than 3 weeks (in patients without chronic anemia). These values indicate that mild iron deficiency has been induced and that potentially pathogenic iron deposits have been removed. Sustaining overt iron deficiency by phlebotomy is not justifiable. After iron depletion, the hemoglobin concentration and hematocrit are allowed to return to and remain within the normal range. Phlebotomy should be done throughout the patient's life to keep the serum ferritin level at 50 μg/l or less. This requires the annual removal of 3 or 4 units of blood in men and 1 or 2 units of blood in women, on average[8]. Some persons, especially elderly persons, may require no maintenance phlebotomy, but their serum ferritin levels should be monitored annually.

Complications often improved by therapeutic phlebotomy

Malaise, weakness, and ease of fatigue affect approximately 80% of patients with iron overload but are often not attributable to specific organ dysfunction. Iron depletion usually alleviates these symptoms, often early in the course of treatment (Table 31.3)[1, 2]. Liver-associated abnormalities (other than cirrhosis) usually resolve (Table 31.3)[1, 2], and benefit may also be seen with respect to manifestations of viral hepatitis[35, 36]. Diabetes mellitus some times improves, if temporarily, although it rarely disappears[37]. Cardiomyopathy (dilated or restrictive) and refractory arrhythmia caused by myocardial siderosis (with or without congestive heart failure) are usually alleviated or cured by the aggressive removal of stored iron[38–40]. The hyperpigmentation associated with iron overload gradually fades in most patients. Hyperferremia due to iron overload invariably resolves after iron depletion. Normal life expectancy has been noted among patients with hemochromatosis who do not have hepatic cirrhosis, diabetes mellitus, or cardiomyopathy and who undergo and maintain iron depletion by phlebotomy[29, 41–43]. Among patients who have these serious complications, therapeutic phlebotomy often improves quality of life and may increase longevity[29, 41–43].

Complications usually not improved by phlebotomy

Hepatic cirrhosis due to iron overload rarely resolves with therapeutic phlebotomy (Table 31.3)[44–46]. A change from a micronodular to a macronodular pattern of hepatic scarring is the likely explanation for the 'disappearance' of cirrhosis seen in a few patients[46]. Cirrhosis is associated with an increased risk for primary liver cancer, and the risk is not diminished by therapeutic phlebotomy (Table 31.3)[1, 2, 29, 47]. Significant improvement in joint function and alleviation of deformity after iron depletion are uncommon, and joint disease may progress despite therapy (Table 31.3)[1, 2, 48]. Some patients have exacerbation of joint pain during phlebotomy that resolves with depletion of iron stores. Hypogonadotrophic hypogonadism is usually not alleviated, but therapeutic phlebotomy has been associated with the restoration of normal pituitary and gonadal function if these complications are of recent onset[37, 49–52]. Thyroid disorders rarely respond to phlebotomy treatment[54]. When therapeutic phlebotomy is monitored properly by using the serum ferritin level, serum iron levels and transferrin saturation values usually remain elevated (Table 31.3)[3, 54].

Table 31.3. Results of therapeutic phlebotomy in patients with hemochromatosis

Complications of iron overload	Expected treatment outcome
None	Prevention of complications of iron overload; normal life expectancy
Weakness, fatigue, and lethargy	Resolution or marked improvement
Elevated serum concentrations of hepatic enzymes	Resolution or marked improvement
Hepatomegaly	Resolution often occurs
Hepatic cirrhosis	No change
Increased risk for primary liver cancer	No change[a]
Right upper quadrant pain	Resolution or marked improvement[b]
Arthropathy	Improvement in arthralgias sometimes occurs; change in joint deformity is rare; progression is sometimes seen
Hypogonadotrophic hypogonadism	Resolution is rare
Diabetes mellitus	Occasional improvement, often temporary
Hyperthyroidism, hypothyroidism	Resolution is rare
Cardiomyopathy	Resolution sometimes occurs
Hyperpigmentation	Resolution usually occurs
Hyperferritinemia	Resolution
Hyperferremia	Little or no change[c]
Excess absorption and storage of non-ferrous metals[d]	Little or no change
Infections with *Vibrio vulnificus* or other bacteria	Little or no change

Notes:

[a] Increased risk occurs only in persons with hepatic cirrhosis.

[b] Right upper quadrant pain in persons with hemochromatosis is often related to hepatic iron overload. In these cases, therapeutic phlebotomy usually results in marked improvement or resolution. However, right upper quadrant pain may also be caused by primary liver cancer, portal vein thrombosis, gallbladder disease, lesions in the hepatic flexure, or nephrolithiasis. Iron depletion alone will not alleviate right upper quadrant pain due to these causes.

[c] Serum iron levels may be normal or subnormal in persons with hemochromatosis due to severe iron deficiency, chronic inflammatory or infectious disease, vitamin C deficiency, or prolonged fasting[3].

[d] Cobalt, manganese, zinc, and lead.

Management of complications of iron overload without phlebotomy

Hepatic disorders

The estimated longevity of persons with hepatic cirrhosis due to hemochromatosis is reduced. Because hepatic biopsy is the most reliable means by which to diagnose hepatic cirrhosis[2, 41], this biopsy is important, especially in patients with hemochromatosis who have elevated serum concentrations of hepatic enzymes or a serum ferritin level greater than 750 μg/l. An additional biopsy specimen should be obtained if the first is unsatisfactory for interpretation, particularly if there is uncertainty about the presence of cirrhosis[1, 2]. However, routine hepatic biopsy after iron depletion is unnecessary. Some persons with clinical evidence of advanced hepatic cirrhosis and portal hypertension cannot undergo biopsy safely[14]. When a biopsy specimen is not available, prognosis and estimation of risks for primary liver cancer must be based on available data. Coincidental hepatic disorders occur in approximately 5% of patients with hemochromatosis, and persons with hepatic abnormalities atypical of hemochromatosis need further evaluation (Table 31.1)[14]. Patients with cirrhosis and other important hepatic disease (such as ethanol-associated hepatic injury or active viral hepatitis) may have persistently elevated serum concentrations of hepatic enzymes or more rapid deterioration of hepatic function than would be expected from iron overload alone. Hepatic failure and hemorrhage from esophagogastric varices cause substantial illness and require routine medical and surgical management[1, 2].

Primary liver cancer

Primary liver cancer causes 10% to 30% of hemochromatosis-related deaths, and it is the leading cause of death in patients with hepatic cirrhosis[29, 55, 56]. With few exceptions,

only patients with cirrhosis develop primary liver cancer. The risk is increased approximately 200-fold in patients with cirrhosis, particularly those older than 55 years of age, those seropositive for hepatitis B surface antigen, and those who drink excess ethanol[56–58]. Serum concentrations of α-fetoprotein are elevated in one third of patients[1, 2], and occult cancers are sometimes visualized with hepatic ultrasonography. We recommend that patients with hepatic cirrhosis have measurement of α-fetoprotein levels and hepatic ultrasonography every 6 months, although the efficacy and cost-effectiveness of this testing are not clearly established. Any patient with cirrhosis who develops hepatic pain, increasing liver size, unexplained fever, or weight loss should be evaluated for primary liver cancer. Treatment methods for primary liver cancer in hemochromatosis, including liver transplantation, are reviewed elsewhere[59–64].

Joint disease

Hemochromatosis-related joint disease often progresses despite therapeutic phlebotomy[48, 65]. Many patients need joint rest, treatment with salicylates or nonsteroidal anti-inflammatory drugs, or physical therapy. Surgical replacement, usually of the hip or the knee, is necessary in some patients. Other hereditary or acquired joint disorders can also cause progressive discomfort and disability in patients with hemochromatosis after therapeutic phlebotomy is complete, and these disorders should be evaluated and managed independently (Table 31.1).

Endocrinopathic disease

Diabetes mellitus in patients with hemochromatosis should be treated as it is in other patients. In men with hypogonadotrophic hypogonadism, sexual potency and renewed vigor often return with monthly testosterone replacement. Severe iron overload in women of reproductive age is frequently associated with hypogonadotrophic hypogonadism. After iron depletion, menstruation and successful pregnancy can occur with appropriate gonadotrophin and other hormone therapy[52]. In older women, estrogen and progesterone replacement therapy should be considered. Thyroid disorders are more frequent among men with hemochromatosis than among men in the general population[53]. The management of thyroid dysfunction is the same for patients with and patients without hemochromatosis (Table 131.)[53].

Table 31.4. Diet and general management recommendations for persons with hemochromatosis

Avoid supplemental iron
Consume red meats in moderation
Consume ethanol in moderation[a]
Limit supplemental vitamin C to 500 mg daily
Use mineral supplements for specific deficiencies only
Cook shellfish from warm-water areas

Note:
[a] This recommendation applies to persons without liver abnormalities. Persons with evidence of liver injury due to hepatic iron loading, such as elevated serum concentrations of hepatic enzymes, hepatomegaly, or hepatic cirrhosis, should consume little or no ethanol.

Cardiac disorders

Severe cardiomyopathy and arrhythmias are typical of the massive iron overload that sometimes occurs in teenagers or young adults. Medical therapy to control congestive heart failure and minimize serious arrhythmias must be applied until vigorous therapeutic phlebotomy (possibly combined with iron chelation therapy) relieves the myocardial siderosis. In middle-aged or elderly persons, cardiac dysfunction is occasionally due to iron overload, but atherosclerosis of coronary arteries or other forms of heart disease are more common (Table 31.1)[14, 66].

Dietary recommendations

Iron intake and absorption

Patients should consume in moderation foods that contain large concentrations of bioavailable iron, such as red meats and organ meats[67, 68], and they should not use iron supplements, including multivitamins with iron (Table 31.4). Dietary changes intended to minimize or eliminate iron ingestion are usually unnecessary and are often not feasible. Furthermore, the 0.5 to 1.0 mg of iron absorbed daily in excess of normal in most persons with hemochromatosis is small in comparison with the 200 to 250 mg of iron per unit of blood removed weekly by therapeutic phlebotomy. Substances in foods and drinks, including tannates, phytates, oxalates, calcium, and phosphates, can bind iron and inhibit its absorption[67, 68]. The consumption of large quantities of tea, which contains tannates, can decrease iron absorption[69]. However, altering a patient's diet in an attempt to inhibit iron absorption is unnecessary

unless the patient cannot tolerate therapeutic phlebotomy (persons with severe thalassemia, for example, cannot tolerate this therapy).

Ethanol

Ethanol sometimes increases iron absorption[70, 71], and certain alcoholic drinks, especially red wines, also contain relatively high concentrations of iron[70, 71]. Ingestion of 30 g or more of ethanol daily can potentiate hepatic injury due to iron overload[57, 72] and increases the relative risk for primary liver cancer in persons with cirrhosis[57]. Patients with evidence of hepatic injury should consume little or no ethanol. Others should consume ethanol in moderation (Table 31.4).

Vitamin and mineral supplements

Vitamin C (ascorbic acid) increases the intestinal absorption of inorganic iron[73]. However, vitamin C deficiency sometimes occurs in untreated patients with hemochromatosis but resolves after iron depletion[74]. Rarely, ingestion of large quantities of vitamin C has been associated with fatal cardiac arrhythmias in persons with iron overload, presumably as a result of oxidative injury caused by mobilization of stored iron[75]. There is no rationale for discouraging patients with hemochromatosis from consuming fresh fruits and vegetables containing vitamin C, but it seems prudent to advise them to limit ingestion of vitamin C in supplements to 500 mg/d (Table 31.4). In patients with hemochromatosis, absorption of inorganic forms of some nonferrous metals, including cobalt, manganese, zinc, and lead, is increased[76–82]. Excess inorganic cobalt is rapidly excreted[79]. Manganese and zinc, however, are deposited in the liver and other tissues[80, 82]. Lead, which is toxic in small amounts, is retained for prolonged periods[77]. The role of these metals in the pathogenesis of symptoms and tissue injury associated with hemochromatosis has not been elucidated, but we suggest that persons with hemochromatosis use dietary supplements containing these metals only if a specific nutritional deficiency has been shown (Table 31.4)[83]. Because blood concentrations of zinc, manganese, and lead are low, therapeutic phlebotomy is not effective in reducing retention of these metals[76, 77].

Shellfish

Vibrio vulnificus occurs naturally in many warm coastal waters, including those along the US shore in the Gulf of Mexico, and it sometimes contaminates shellfish harvested from these areas. This spiral organism can cause infection when ingested in raw or improperly cooked contaminated shellfish or when introduced into the open wounds of persons who handle contaminated seafood or bathe in contaminated waters[84–86]. Bacteremia due to *V. vulnificus* in patients with hemochromatosis may be related to the availability of iron for microbial metabolism or to the presence of hepatic cirrhosis[85], and it is often fatal [84–86]. Persons with hemochromatosis should consume seafood from potentially contaminated waters only if it is thoroughly cooked, and they should take other indicated measures to prevent infection (Table 31.4). Therapeutic phlebotomy probably does not reduce susceptibility to infections with *Vibrio* species.

Comprehensive dietary management

Many patients appreciate being given a list of dietary recommendations that includes the amounts of iron in servings of common foods and beverages. Some patients rigidly (and unnecessarily) avoid certain dietary items, usually on the basis of iron content. Many patients have iron overload-associated or coincidental medical conditions (such as diabetes mellitus, hepatic dysfunction, anemia, hyperlipidemia, hypertension, or obesity) that also require changes in diet. In patients with complex dietary requirements, a dietitian who understands hemochromatosis can help formulate an effective diet that is acceptable to the patient. Diets do not enhance iron excretion, and patients must understand that there is no substitute for iron depletion therapy.

Conclusions

Physicians can expect to diagnose and treat increasing numbers of persons with hemochromatosis because awareness of the frequency and diagnostic criteria of hemochromatosis and the multisystem disease caused by iron overload is increasing. Early diagnosis and therapeutic phlebotomy to maintain low normal body iron stores can prevent all known complications of hemochromatosis. Most patients with hemochromatosis who have developed iron overload before diagnosis also benefit from therapeutic phlebotomy. Many will need additional evaluation and treatment of complications of iron overload, including hepatic disorders, joint disease, diabetes mellitus and other endocrinopathic conditions, and cardiac dysfunction. Proper dietary management can decrease the rate of iron reaccumulation and can reduce or avoid complications of hepatic disease, diabetes mellitus, and *Vibrio* infection in patients with hemochromatosis. This

comprehensive approach to the management of hemochromatosis can decrease the frequency and severity of iron overload, improve quality of life, and increase longevity.

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Chelation therapy in iron overload

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Introduction

The efficacy of phlebotomy in the treatment of hemochromatosis is well established and described in detail elsewhere in this volume. Each pint of blood removed represents approximately 200 mg of iron, and there is no other method that could be compared with the efficacy and availability of this mode of intervention. However, phlebotomy requires an intact hemopoietic system and is not suitable for patients with hereditary and other chronic anemias who require blood transfusions for their survival. For these patients, iron chelating therapy is the only available method of protection against the life-threatening consequences of severe transfusional iron overload. Because aggressive iron-chelating treatment can reverse existing iron-induced cardiomyopathy, iron chelation (in addition to phlebotomy) may also be considered in newly diagnosed hemochromatosis patients with cardiomyopathy in whom an immediate threat of fatal complications may not allow sufficient time for the effective removal of iron by phlebotomy alone. The iron-chelating drug deferoxamine (DF) had a major impact on the life expectancy of children with thalassemia. In this chapter the pharmacology of DF will be reviewed, the compartments of body iron available for chelation defined, the mechanism of iron-induced myocardial injury discussed, the impact of long-term DF therapy on life expectancy in transfusion iron overload discussed, and the development of orally effective iron chelators of potential clinical usefulness reviewed.

Deferoxamine pharmacology

The discovery of deferoxamine (DF), the useful iron chelator for the management of transfusion iron overload, was accidental. As described by Keberle[1], ferrioxamine B was first identified as an antagonist to ferrimycin, a fermentation product with antibiotic properties. Due to its solubility in water and high iron content, ferrioxamine B was considered for use as an iron donor, but its intravenous injection to human volunteers was followed by the quantitative recovery of a dark-brown soluble iron complex in the urine. This observation was rightly attributed to the very high affinity of the compound to iron, and led to the idea of exploiting this affinity for promoting urinary iron excretion by the iron-free substance – deferoxamine. To increase its water solubility, deferoxamine B hydrochloride was later replaced by the deferoxamine B methane sulfonate salt, the form in which DF is marketed for clinical use.

DF is a colorless, crystalline substance produced by *Streptomyces pilosus*. It consists of three hydroxamic acids terminating in a free amino group (Fig. 32.1) enabling it to form water-soluble salts[2]. It forms a complex with ferric (Fe^{3+}) iron at a 1:1 molar ratio with a stability constant of 10^{31}. The affinity of DF to ferrous (Fe^{2+}) iron and to other metal ions is much lower, and the stability constants range from 10^{2} to 10^{14}. The complexation of DF to iron and the resultant change in configuration renders DF extremely stable and resistant to enzymatic degradation[3]. In contrast to DF, its iron complex ferrioxamine is unable to penetrate cells and is confined to the extracellular compartment, as illustrated by a plateau in its serum concentration after nephrectomy[2]. In healthy dogs, 98% of labelled ferrioxamine is recovered in the urine. By contrast, the iron-free DF is able to penetrate various tissues, particularly the liver, and is catabolized rapidly.

Tritium-labeled DF was employed in early studies to determine the fate of the drug in vivo. In dogs, approximately 70% of DF is excreted in the urine within three days, and over half of this excretion is in the form of metabolites[2]. DF is excreted in the urine by glomerular filtration and tubular secretion, whereas ferrioxamine is partly

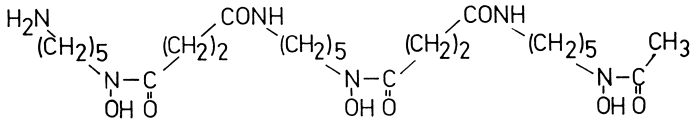

Fig. 32.1. Deferoxamine B.

reabsorbed after its glomerular filtration[4]. The disappearance curve of DF in nephrectomized dogs is complex, and is the result of a combination of penetration into various tissues and metabolic breakdown. Tissue concentrations of labelled DF in dogs after intravenous injection are highest in the bile and brain, intermediate in spleen and kidney, and lowest in the heart, lungs and fatty tissues. Despite the great efficiency of the liver in clearing DF from the circulation, DF is not retained in the liver, but is rapidly concentrated in the bile. Similar to urinary excretion, most of the biliary excretion is in the form of DF metabolites. In the urine, the major DF metabolite is compound C in which the original amino group of DF has been replaced by a carboxyl group. Although various parenchymatous organs are active in DF catabolism, the most active tissue in DF breakdown is the plasma[3].

In man, plasma DF levels following rapid intravenous injection drop to half the initial concentration within 5–10 min[5]. By contrast, slow subcutaneous infusion of DF, in a manner identical with the current methods of DF administration to patients, results in a gradual buildup of plasma DF concentrations that reach a plateau in approximately 12 h. This is accompanied by a parallel increase in plasma ferrioxamine concentrations. When DF is infused intravenously continuously for 48 h at a dose of 50 mg/kg/d to thalassemia patients[6], steady-state plasma DF values range from 2 to 96 μmol/l (mean 18.2 ± 2.1 μmol/l). Reported plasma half-lives of DF differ widely. Apart from using different methods of DF measurement, these variations may also be attributed to using different methods of administration (subcutaneous, intramuscular, or intravenous) and bolus vs. continuous infusions. There is a direct correlation between total 48 h urinary iron excretion and peak plasma concentrations of ferrioxamine during continuous DF infusion. Similarly, following bolus DF injection, a direct linear correlation was found between 2 h plasma ferrioxamine levels and the logarithm of 24 h urinary iron excretion[7]. Due to its poor intestinal absorption, the oral effectiveness of DF for promoting storage iron excretion is limited[2]. Although a slight consistent increase in urinary iron excretion may be achieved at oral doses ranging from 3 to 9 g/d[8], the cost-effectivenes of such treatment is very low. However, oral DF treatment is effective in blocking the intestinal absorption of inorganic iron[9] and oral DF treatment is highly recommended for the emergency treatment of acute oral iron poisoning[10].

Chelatable iron

Most of the body iron in normal subjects is unavailable for chelation. Hemoglobin iron, representing more than two-thirds of all iron, is resistant to DF chelation[11]. Likewise, transferrin-bound iron is a very poor source for in vivo chelation by DF even though the stability constant of DF with Fe^{3+} exceeds that of transferrin. By exclusion then, the most likely source of chelatable iron is that stored in tissues in the form of ferritin or hemosiderin, or a labile iron compartment in dynamic equilibrium with the former.

The intracellular labile iron pool

Current models of iron acquisition, sequestration, and storage by mammalian cells are based on a regulated adjustment of membrane transferrin receptor and cytosolic ferritin levels. Iron in transit between these two iron-binding proteins is believed to exist in a weakly bound low-molecular-weight complex[12–14] that is also available for interaction with iron-chelating drugs[15–18]. This chelatable labile iron pool (LIP) is assumed to be sensed by cytosolic iron-responsive proteins (IRP) that coordinately repress ferritin mRNA translation and increase transferrin receptor mRNA stability[19].

Measurement of the intracellular chelatable iron pool has been associated with major technical difficulties[14]. Analytical techiques relying on cell-disruptive steps are of limited use due to the dynamic nature of the cytosolic iron pool. Likewise, uncertainty exists regarding the relative levels of di- and trivalent iron in the cytosolic chelatable pool. Physicochemical measurements of tissue iron implicated iron(II) as the dominant low-molecular form in the cytosol[20], but chemical determinations of iron in tissue extracts yielded variable results reflecting the propensity of iron(II) for oxidation[21].

Study of the LIP has been facilitated by the recent development of a method allowing the continuous monitoring of intracellular fluorescence associated with the metal-sensitive probe calcein[22]. The fluorescence of calcein is quenched by its binding to iron(II) and recovers when it yields iron to more potent iron chelators capable of penetrating the cell. Using this method, it was shown that in K562 cells the cytoplasmic concentration of iron(II) ranges from 0.3 to 0.5 μM, and its mean transit time through the chelatable pool is 1–2 h. The ostensible absence of chelatable iron(III) implies the existence of vigorous intracellular

reductive mechanisms[14]. Further studies in K562 cells have shown the existence of efficient regulatory mechanisms preventing fluctuations in the size of the labile iron pool under conditions of moderate iron deprivation and iron loading. However, massive iron loading (100–200 μM ferric ammonium citrate) at concentrations similar to those used in previous studies of iron-loaded heart cells, causes an apparently uncontrollable expansion in the chelatable pool that is not matched by the sequestrating capacity of cellular ferritin[23]. This expanded LIP is an obvious target of iron chelating drugs.

Role of iron stores in RE and parenchymal cells

In iron overload, excess iron may be deposited in almost all tissues, but most iron is found in association with two cell types: reticuloendothelial (RE) cells located in the spleen, liver, and bone marrow, and parenchymal tissues represented mainly by hepatocytes. In contrast to iron accumulation in RE cells which may be relatively harmless, parenchymal siderosis may result in significant organ damage. It is important therefore to determine whether DF (or any other chelating drug) interacts preferentially with one of these two cellular storage compartments.

The source of iron, and the proportion of iron retained in ferritin stores or recycled into the circulation from the two cell types, is quite different. RE cells are unable to assimilate transferrin iron and they derive their iron from the catabolism of hemoglobin in non-viable erythrocytes[24]. Most of this catabolic iron is recycled within a few hours. In contrast, hepatic parenchymal cells maintain a dynamic equilibrium with plasma transferrin, with iron uptake predominating when transferrin saturation is high, and iron release predominating when serum iron concentration and transferrin saturation are low[25]. In contrast to RE cells, the turnover of parenchymal iron stores is extremely low. In general, iron overload associated with increased intestinal absorption such as hemochromatosis results in predominant parenchymal siderosis, whereas, in conditions wherein iron overload is caused by multiple blood transfusions, the primary site of siderosis is the RE cells. Considerable redistribution of iron may take place subsequently.

Several studies employing selective storage iron labels in rats have shown that DF interacts preferentially with hepatocellular iron stores and that the contribution of RE cells to DF-induced iron excretion is limited[11, 26, 27]. Because hepatocytes are unable to incorporate circulating ferrioxamine (^{59}Fe-DF), the biliary excretion of ^{59}Fe-DF is evidence of intrahepatic chelation of biliary radioiron. In contrast, urinary radioiron excretion is derived entirely

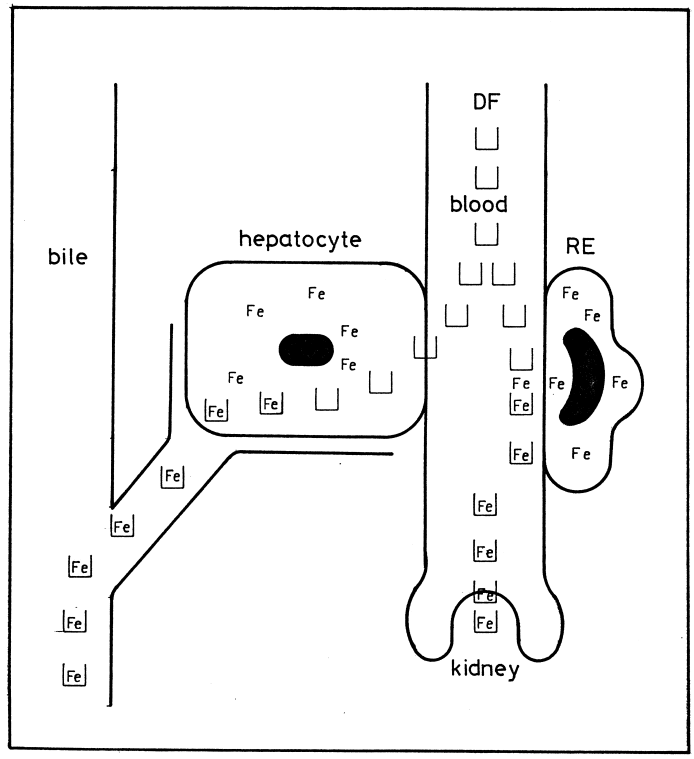

Fig. 32.2. Alternative pathways of in vivo iron chelation by deferoxamine (DF). Iron excretion is derived from (*a*) in situ interaction with hepatocellular iron (Fe) with subsequent biliary excretion, or (*b*) chelation of iron derived from RBC catabolism in reticuloendothelial (RE) cells with subsequent urinary excretion.

from circulating ferrioxamine that, in turn, may originate from the chelation of iron in RE stores, or iron-in-transit to or from circulating transferrin[26].

A number of experimental and clinical observations support the assumption that the urinary excretion of chelated iron is derived mainly from RE cells. Studies in hypertransfused rats using continuous DF infusion to capture all chelatable iron have shown that most of the radioiron excretion derived from the RE label is recovered in the urine, in contrast to hepatocellular radioiron excretion which is confined entirely to the bile[28–30]. Moreover, when DTPA or IRC011, water-soluble synthetic chelators that do not enter cells, are employed in the same experimental model, there is no enhancement of hepatocellular iron excretion. However, the enhancement of urinary RE radioiron excretion is similar, or higher than that observed previously with DF[31]. Figure 32.2 represents a schematic description of the 'alternative pathways' hypothesis derived from these observations: DF obtains iron for chelation by one of two alternative mechanisms: (a) in situ interaction with hepatocellular iron and subsequent

biliary excretion; and (b) chelation of iron derived from erythrocyte catabolism in the RE system with subsequent urinary excretion. The observations described do not permit a firm conclusion regarding the question of whether RE-derived iron is chelated by DF within the RE cell or following its release into the plasma in the form of non-transferrin iron[30].

Role of non-transferrin plasma iron (NTPI)

The original description of the existence of a chelatable, low-molecular-weight plasma iron fraction in patients with severe iron overload[32] has met considerable skepticism. However, subsequent studies have confirmed the existence of NTPI using a variety of methods[33–39]. NTPI is found only after complete saturation of circulating transferrin. NTPI promotes the formation of free hydroxyl radicals and accelerates the peroxidation of membrane lipids in vitro[39]. Improved methods for the direct quantitation of NTPI in patients undergoing chelation therapy with DF, using nitriloacetic acid ultrafiltration and ultraviolet detection with high-performance liquid chromatography[33], have been developed. Studies by Al-Rafaie et al in thalassemia patients have shown that long-term treatment with DF or deferiprone (L1) results in a marked decrease in their NTPI levels[40]. More recent studies by Porter et al.[6]. have clearly demonstrated that plasma NTPI is removed by intravenous DF therapy in a biphasic manner and that upon cessation of DF infusion it reappears rapidly, lending support to the continuous, rather than intermittent, use of DF in high-risk patients. The rate of low-molecular-weight iron uptake by cultured rat heart cells is over 300-fold greater than that of transferrin iron[41]. Such uptake resulted in increased myocardial lipid peroxidation and abnormal contractility, and these effects were reversed by in vitro treatment with DF. Recognition of NTPI as a potentially toxic component of plasma iron in hemochromatosis is useful in designing better strategies for the effective administration of DF and other iron chelating drugs.

Mechanism of myocardial iron toxicity

Iron-induced myocardial disease is a major complication of hemochromatosis, and the most critical life-limiting factor in thalassemia major and other iron-loading anemias[42]. Failure to produce a satisfactory animal model of hemochromatosis has hindered research on the pathogenesis of iron toxicity. Consequently, an experimental model of rat myocardial cells in culture for studying the harmful effects of iron and the protective effects of iron chelation was developed[41, 43–48]. A unique feature of these cultured cells is their ability to differentiate into spontaneously contracting cells, providing an opportunity to study simultaneously the biochemical and functional effects of iron toxicity in heart cells.

The role of iron in promoting the conversion of superoxide and hydrogen peroxide into the highly toxic free hydroxyl radicals through the Haber–Weiss reaction has been extensively studied[49, 50]. Increased lipid peroxidation is the most easily measurable effect and is usually regarded as the most significant event in the pathogenesis of cellular damage. Increased in vivo lipid peroxidation has been asociated with increased formation and tissue concentrations of malonyldialdehyde (MDA), of conjugated dienes in tissues, or increased respiratory excretion of low-molecular-weight alkanes[51, 52], all of which represent products of lipid peroxidation. The peroxidation of polyunsaturated fatty acids results in the formation of highly reactive aldehydes such as malonyldialdehyde and 4-hydroxynonenal, leading to the formation of covalent links to proteins, or protein adducts[53]. These links involve mainly the amino group of lysine residues and sulfhydryl groups[54].

A number of cellular lipid membrane structures have been considered as possible targets for iron-induced peroxidative damage. Decreased latent activity of lysosomal enzymes implying increased fragility of the lysosomal membrane has been demonstrated in vitro[55] and in biopsy specimens obtained from the livers of patients with hemochromatosis and secondary iron overload[56]. It has been suggested that iron-induced free radical formation is responsible for the disruption of lysosomal membranes resulting in the release of hydrolytic enzymes into the cell and ultimately in cell death [57]. Our own studies, employing lysosomal β-hexosaminidase as an indicator of iron-induced lysosomal damage, have shown that in vitro iron-loading of cultured heart cells is associated with a marked increase in total β-hexosaminidase activity, and that this may be attributed to increased lysosomal fragility suggested by the loss of lysosomal latency and increased free enzyme activity[58].

Another organelle implicated in iron toxicity is the cytoplasmic or, in the case of heart cells, sarcolemmal membrane. Disruption of the sarcolemmal membrane in cultured mouse myocardial cells occurs after exposure to increased concentrations of environmental oxygen and iron[59]. Likewise, reversible sarcolemmal damage was implicated in the iron-induced reduction in beating rate of cultured rat myocardial cells[43]. Our studies of sarcolemmal thiolic enzymes in iron-loaded heart cells have shown a loss of 5′-nucleotidase and Na^+,K^+ATPase activity attributed to the direct, or indirect (via lipid peroxidation

products) effects of increased oxidative stress on sarcolemmal thiolic enzymes[60]. In the absence of major alterations in the composition of sarcolemmal lipids, the decrease in sarcolemmal 5′-nucleotidase and ATPase activity implies that iron-induced peroxidative damage to membrane proteins may represent an important mechanism in the pathogenesis of altered myocardial function the iron-loaded heart. Since Na^+,K^+ATPase plays a significant role in cellular calcium homeostasis through the Na/Ca exchange mechanism, inactivation of Na^+,K^+ATPase by iron toxicity may provide a direct link between the structural and functional abnormalities observed in iron-loaded cells.

Finally, mitochondrial swelling and amorphous densities developing in the matrix space in association with intracristal deposition of iron in rat hepatocytes occur after oral iron administration[61]. Studies by Bacon et al. in rats with chronic iron overload have shown a progressive reduction in ADP-stimulated respiration and respiratory control rates at hepatic iron concentrations >1000 μg/g and increased conjugated diene formation, suggesting a cause-and-effect relation between iron-induced lipid peroxidation and impaired mitochondrial function[62]. These authors have also shown a 70% reduction in cytochrome C oxidase activity and a loss of hepatic ATP and ADP levels in this model[63]. An increased concentration of hepatic mitochondrial malondialdehyde was caused by a combination of increased production and decreased catabolism due to inhibition of mitochondrial aldehyde dehydrogenase activity[64]. In cultured, iron-loaded rat heart cells, there was a marked inhibition in the function of segments II to III of the mitochondrial inner membrane respiratory chain, monitored by measuring the activity of succinate-cytochrome C oxidoreductase[65]. This was completely reversed by in vitro DF treatment of the cultured heart cells.

The toxicity of iron to heart cells is not a simple function of iron concentrations. It may be aggravated or inhibited by a number of coexistent variables: reduction of ferric to ferrous iron promotes hydroxyl radical formation via the iron-driven Haber–Weiss reaction. Ascorbic acid, a natural reducing agent accelerates iron-induced lipid peroxidation in biological systems[66, 67]. Moreover, clinical observations indicate that ascorbate supplementation may aggravate or accelerate the development of cardiac disease in patients with iron overload (see below)[68]. Conversely, α-tocopherol, a natural lipid-soluble antioxidant, can interrupt the chain reaction of membrane lipid peroxidation initiated by free radicals and interfere with iron-induced lipid peroxidation in liposomes[45, 46, 67]. DF removes iron directly from iron-loaded heart cells, inhibits lipid peroxidation[41, 44, 45], and reverses the abnormalities in cellular contractility and rhythmicity induced by iron in vitro. Finally, iron plays a key role in anthracycline cardiotoxicity. Redox cycling of doxorubicin produces superoxide and peroxide that are converted into highly toxic hydroxyl radicals by the iron-driven Haber–Weiss reaction[69]. This can be prevented by iron-chelating treatment. Iron aggravates and iron chelators limit the damage caused by doxorubicin to cultured heart cells[70, 71]. Although the therapeutic implications of these observations are evident, a key issue for future studies is to show that DF treatment decreases cardiotoxicity without interfering with the anti-cancer effect of anthracycline therapy.

The dilemma of ascorbate supplementation

Ascorbate deficiency results in a block in reticuloendothelial iron release associated with a shift of iron stores from ferritin to hemosiderin, and a decrease in serum iron concentrations[72–74]. Conversely, ascorbate treatment in scorbutic patients has a pronounced effect on internal iron redistribution: serum iron concentration increases 2–3-fold within 6 h after ascorbic acid dosing[72], followed by an increase in serum ferritin concentrations[75, 76]. In a series of pioneering studies conducted in iron-overloaded Bantu patients, Bothwell and associates showed that ascorbate deficiency in this form of iron overload is very common[72]; that it is caused by increased catabolism of ascorbate and its oxidative conversion into oxalate[77]; that DF-induced urinary iron excretion is impaired in ascorbate deficiency, but improves after ascorbate treatment in direct proportion to the severity of iron overload[78]; and that ascorbate treatment has no effect on DF-induced iron excretion in normal subjects[78]. Thalassemia patients with transfusion iron overload often have low leukocyte ascorbic acid concentrations[75, 79] and in such patients, ascorbate supplementation results in a marked improvement in DF-induced iron excretion[80]. In consequence, ascorbate supplementation has been adopted as a useful adjunct to DF therapy.

The mechanism of the ascorbate-associated enhancement of DF effect is an expansion of the chelatable intracellular chelatable iron pool, as shown by in vitro studies in K562 cells[81]. These findings are in accordance with the observed increase in serum iron concentration, transferrin saturation, and serum ferritin concentration in ascorbate-repleted scorbutic patients[72, 75, 76]. Expansion of the chelatable iron pool, however, is also associated with an increased availability of toxic, low-molecular-weight iron which, in combination with ascorbate, could enhance free radical formation and lead to catastrophic consequences.

Clinical observations indicate that this may indeed be the case. Thalassemia patients treated with DF in combination with oral ascorbate (500 mg/d) supplementation had striking (and not always transient) deterioration in left ventricular function[68, 82]. Others[83] have reported a paradoxical, rapid aggravation of cardiomyopathy in patients receiving combined DF and high-dose ascorbate therapy. Likewise, several case reports have described rapid progression of congestive cardiomyopathy in hemochromatosis patients after self-administration of ascorbic acid at 500 to 1000 mg per day[84, 85]. These observations imply that secondary ascorbate deficiency in chronic iron overload may represent a fortuitous self-protective mechanism, and that the potential benefits of increased DF-induced urinary iron excretion may be outweighed by the risk of aggravating iron toxicity by ascorbate supplementation.

In addition to promoting mobilization of storage iron, ascorbate may have a more direct role in iron-induced tissue damage. In a microsomal system, the rate of iron-induced lipid peroxidation is strongly stimulated by ascorbic acid[86]. Likewise, studies in isolated rat liver mitochondria showed impaired mitochondrial inner membrane respiratory complex activity by the co-administration of iron and ascorbate[87]. In iron-loaded heart cells, ascorbate prevents the beneficial effects of DF on lipid peroxidation[48]. As a reducing agent, ascorbate has two opposing effects on iron toxicity. It can serve as a donor antioxidant and prevent free radical-mediated oxidative damage[88]. However, it can also reduce iron and enhance its pro-oxidant catalytic effect in the Haber–Weiss reaction. At high concentrations, ascorbate is an anti-oxidant, whereas at low concentrations it is a pro-oxidant. The position of the crossover effect is a function of catalytic metal concentrations. Thus, at high tissue iron concentrations, the prooxidant effect of ascorbate predominates. Hence, ascorbate is particularly dangerous in the presence of severe iron overload[89].

In view of such overwhelming evidence implicating ascorbic acid in the aggravation of iron toxicity, it has been recommended that ascorbate supplementation not be started prior to the implementation of effective DF therapy, and that the daily dose of ascorbate should not exceed 200 mg[68, 90]. It is doubtful, however, whether reducing the dose of ascorbate completely abolishes toxicity, because studies of lipid peroxidation in iron-overloaded spleens showed maximal malonyldialdehyde formation at the lower range of ascorbate concentrations; very high concentrations had an anti-oxidant effect[66]. The need for prospective controlled studies for establishing the role, if any, of ascorbate supplementation in iron-chelating programs has been emphasized[68].

Results of long-term chelating therapy

The pioneering studies of Barry et al demonstrating the ability of long-term intramuscular DF treatment to arrest hepatic siderosis and fibrosis in thalassemia[91] and the introduction of portable pumps for continuous subcutaneous infusion that increases the efficacy of DF, resulted in the adoption of chelating programs by a large number of centers responsible for the care of persons with thalassemia. Ideally, justification for such long-term chelating programs costing of $6000–12 000 US$ per patient year should rest on the demonstration of improved survival of treated patients by prospective, controlled clinical trials. Such studies, however, are not available and are not forthcoming.

Witholding DF therapy from thalassaemia patients for the sake of research is no longer ethically acceptable. Experience with long-term DF therapy in thalassemia patients has been the subject of a number of excellent recent reviews[92–94].

Method of treatment

The effect of treatment, documented by urinary iron excretion, is directly proportional to the severity of iron overload. Hence, treatment in subjects without iron overload will result in limited iron excretion. However, treatment should not be introduced too late if the objective is the prevention of iron toxicity. It is recommended that treatment be started when serum ferritin concentrations reach approximately 1000 mg/l, which usually occurs after the first 10–20 transfusions[92]. DF is infused via a thin subcutaneous needle inserted to the arm or abdomen nightly, connected to a portable pump over 8–12 h, 5–7 times per week, at a daily dose of 20–60 mg/kg. Urinary iron excretion of 0.5 mg/kg/d is usually sufficient to ensure negative iron balance. Response to treatment may be assessed by serum ferritin concentration measurements, hepatic biopsies[95], computed tomography[96], magnetic resonance imaging[97] or magnetic susceptibility (SQUID)[98, 99]. Serum ferritin concentrations are disproportionately low in patients with co-existent ascorbate deficiency[100] and high in patients who have active hepatic disease or inflammation[99]. Nevertheless, serum ferritin concentration is the most available and inexpensive tool for the long-term monitoring of chelating efficiency, and excellent protection from cardiac complications is achieved when serum ferritin concentrations are kept <2500 mg/l[101].

Desferrioxamine toxicity

DF is remarkably non-toxic, but a number of complicatons may arise in relation to excessive DF dosage, especially in subjects with limited iron overload. These complications are dose dependent although their mechanism is poorly understood. A decreased growth rate was first described by Piga et al.[102], especially in young children with low serum ferritin concentrations. Abnormal skeletal growth has also been described, manifested in metaphyseal bone deformities most commonly seen in the distal ulnar, radial and tibial bones[103]. Improved growth rates are resumed upon decreasing DF dosage. Similarly, high-frequency sensorineural hearing loss has been observed in thoroughly chelated young patients with low serum ferritin concentrations[104–106]. Reducing DF dosage was followed by recovery in patients with mild hearing defects, but not with severe auditory impairment. At very high DF dosage, ocular, pulmonary, and renal toxicities have occurred[107–110]. None of these complications precludes continued chelation therapy. They are preventable if proper monitoring is practiced to detect early signs of toxicity. Because they are dose dependent and inversely related to the degree of iron overload, their risk can be minimized by proper dose adjustment. One such method for dose adjustment is to limit the daily dose of DF/kg body weight to a DF to ferritin ratio of 0.025[111]. A rare but serious complication of DF is *Yersinia* sepsis, a non-virulent organism that utilizes chelated iron as a virulence factor. It occurs in 0.7 % of chelated patients. Prompt diagnosis of *Yersinia* sepsis may limit the severity of this life-threatening complication[112].

Impact on survival

To appreciate the impact of DF therapy on the survival of thalassemia patients, one must first examine the life expectancy and causes of death in patients with thalassemia who have been untreated or incompletely treated with DF. In a study of 1100 thalassemia patients treated at seven Italian teaching hospitals, 30% of patients born before 1970 (and hence prior to the modern era of regular transfusion and chelation) died before reaching the age of 20 years[93]. The most important causes of mortality before age 10 years were infection and anemia. Both of these are non-iron-related and partly preventible by state-of-the art supportive therapy. Conversely, the most important cause of mortality in patients older than 10 years of age (and therefore with potentially life-threatening iron overload) was heart disease. Cardiac complications, including heart failure, arrhythmias, and pericarditis, were responsible for 79% of deaths. Hence, one would anticipate reduction in cardiac mortality as the most important beneficial outcome of effective long-term iron chelating therapy.

The survival of thalassemia patients in recent years is definitely better than in the pre-DF era. For example, in thalassemia patients receiving a hypertransfusion program combined with regular subcutaneous DF chelation, median survival has increased to 31 years from 18 years in patients treated formerly by a low-transfusion regimen and no chelation[113]. In Italy, where DF treatment has been introduced for systematic use in 1978, extensive information on survival of more than 5000 patients with thalassaemia major is available and is monitored by a coordinated study group. In a report by Borgna-Pignatti et al.[114] on 942 Italian thalassaemia patients, progressive improvement in survival has been shown for cohorts born in the intervals 1960–1964, 1965–1969 and 1970–1974. The total mortality was 15.7% and heart disease was the cause of death in two-thirds of cases. Overall survival for patients born in 1970–1974 (and therefore subjected to chelation therapy from an early age) was 97% at 10 years and 94% at 15 years. Cardiac mortality at age 15 years in cohorts of thalassaemia patients born in 1960–1964, 1965–1969 and 1970–1974 was 12.3%, 10.0%, and 2.4%, respectively, indicating a marked reduction in cardiac disease after the introduction of adequate iron chelating programs in Italy.

In a more recent study of 1127 Italian thalassemia patients followed for a median period of 216 months, survival of patients born between 1970 and 1974 was 88% at age 20 years[115]. In contrast, of the patients born between 1965 and 1969 who were alive at age 10 years, 70% have reached age 20 and only 54% have reached age 25. This cohort-of-birth related improvement in survival is reflected in an inverse decrease in cardiac mortality, confirming the assumption that prevention of cardiac mortality is the most important beneficial effect of DF therapy (Fig. 32.3).

The strongest direct evidence supporting the beneficial effect of DF on hemosiderotic heart disease is the reversal of established cardiomyopathy in some advanced cases. Earlier experience in hereditary hemochromatosis has shown that the cardiomyopathy of iron overload is potentially curable by effective iron mobilization through phlebotomy. However, in transfusion iron overload, the course of established myocardial disease was uniformly fatal and, until recently, believed to be non-responsive to iron-chelating therapy. Several reports indicate that such patients may still be responsive to aggressive chelating treatment. Marcus et al.[116] first described the reversal of established symptomatic myocardial disease in three of five patients by continuous high-dose (85–200 mg/kg/d) intravenous DF therapy at the cost of severe reversible

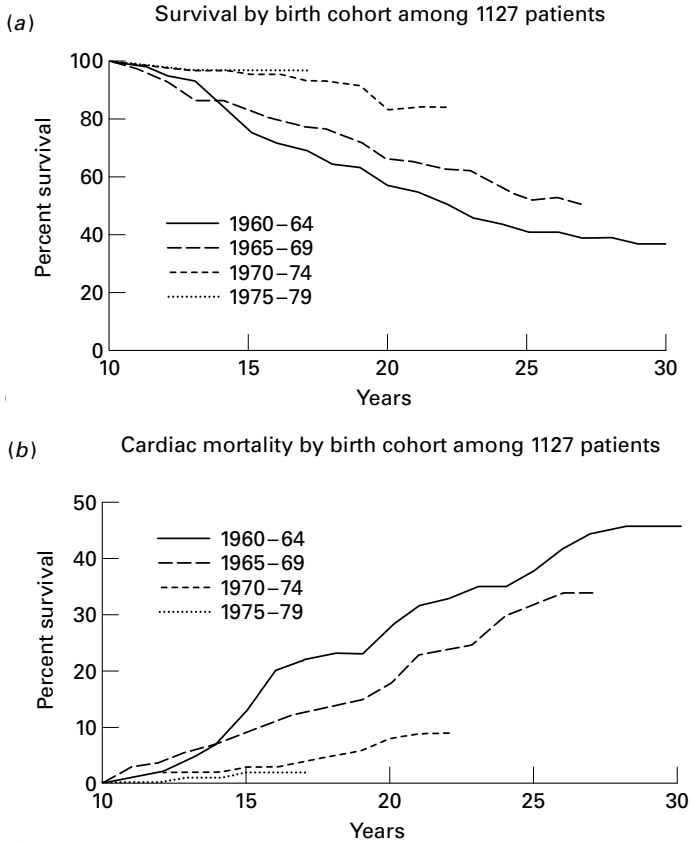

Fig. 32.3. Survival of thalassemic patients stratified by year of birth. Figure 32.3(*a*) shows a steady *improvement in survival* in direct correlation with year of birth. Figure 32.3(*b*) shows a mirror-image of the improvement in survival reflected in a steady *decrease in cardiac mortality.* (Modified from refs. [114] and [115].)

retinal toxicity. No similar toxicity occurred in three patients on high-dose subcutaneous and intravenous DF treatment who survived 2, 7 and 8 years after the onset of congestive heart failure[117]. Similarly, two patients with severe myocardiopathy who received intensive intravenous DF therapy have recovered, with a follow-up of over two years[118]. Continuous 24-h ambulatory intravenous infusion of DF through central venous ports using a portable infusion pump is a most suitable technique for the rapid reversal of established hemosiderotic heart disease. In addition, it is an excellent tool for improving patient compliance because it allows uninterrupted delivery of 6–12 g DF/d and the depletion of large iron stores[119, 120].

The search for orally effective chelators

The long-term use of DF has changed the quality of life and improved life expectancy of many thalassemia patients.

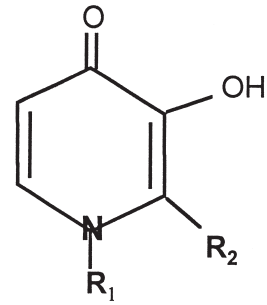

Fig. 32.4. General formula of the 3-hydroxypyrid-4-one bidentate chelators. In 1, 2-dimethyl-3-hydroxypyrid-4-one (deferiprone, L1, CP20) both R1 and R2 are represented by a methylic group.

However, effective DF treatment requires continuous subcutaneous infusion by portable pumps, and many patients are unable or unwilling to cope with the rigorous requirements of such treatment. In addition, the high cost of DF treatment makes it unavailable for the vast majority of patients with hereditary anemia requiring long-term transfusional therapy.

Due to these considerations, there is a great need for the development of alternative, orally effective iron chelating drugs. Several hundred candidate compounds have been screened over the last decade, using in vitro and in vivo animal models or a combination of both. These studies have led to the identification of several interesting compounds, but only a few are of possible clinical usefulness. The most outstanding of these orally effective compounds are deferiprone (L1), the polyanionic amines, and the substituted polyaza compounds.

Deferiprone (L1)

Only deferiprone has been introduced to clinical use as an orally effective substitute for DF for the long-term management of transfusion iron overload. 3-hydroxypyrid-4-one bidentate chelators[121, 122] bind to iron in a 3:1 ratio with a stability constant of 10^{37}, approximately six orders of magnitude higher than DF (Fig. 32.4). Increasing the size of the alkyl function substitution on the ring N atom enhances lipophilicity without altering affinity to iron. High lipophilicity is associated with increased chelating efficiency, but also carries the risk of increased toxicity.

The most important compound of this family is 1,2-dimethyl-3-hydroxypyrid-4-one, (deferiprone, CP20, or L1). Although clinical reports on the use of L1 in thalassemia and other patients were published by 1987, detailed animal studies on toxicity and pharmacokinetics have only become available subsequently. Peak plasma L1

concentrations are achieved within ≤ 1 h after oral administration[123, 124]. One of the most important factors controlling the in vivo pharmacokinetics and chelating efficiency of L1 is glucuronidation. This is much more active in man than in the rat, a difference that may explain both the higher toxicity and greater chelating efficiency of the hydroxypyridones in rats than in man[121, 125]. Different rates of glucuronidation may also explain the higher rate of fecal iron excretion in the rat than in man[126]. At a daily dose of 200 to 300 mg/kg given for several months, L1 caused bone marrow aplasia in mice, rats, dogs and monkeys, and involution of lymphatic tissues in rats, L1 also caused adrenal steatosis. These alterations were associated with a high rate of mortality[125, 127–130].

The results of long-term iron chelating therapy with L1 in thalassemia patients[101, 131–135] have been summarized in several recent reviews[136–138] and the combined experience of the four major European and Canadian groups pioneering the clinical use of L1 has been described in a report of the International Study Group for Oral Iron Chelators[139]. The latter report is based on questionnaires completed on patients treated at the four major centers involved in long-term L1 treatment, including clinical and laboratory data at initial evaluation and six-monthly updates. The source of L1 was highly variable, including two university research laboratories and three pharmaceutical firms. The study involved 84 patients, 74 of whom had thalassemia major or intermedia, representing a total of 167 patient–years of L1 treatment. Compliance was rated as excellent in 48%, intermediate in 36%, and poor in 16% of patients. With an L1 dose of 73 to 81 mg/kg/d, urinary iron excretion was stable, at ~0.5 mg/kg/d with no indication of a diminishing response with time. Serum ferritin concentrations decreased steadily over time from an initial mean ±1S.D. of 4207 ±3118 ng/dl to 1779 ±1154 ng/dl after 48 months ($p<0.001$). 17 patients abandoned L1 therapy. Major complications of L1 requiring permanent discontinuation of L1 therapy included agranulocytosis ($n=3$), severe nausea ($n=4$), arthritis ($n=2$) and persistent hepatic dysfunction ($n=1$). The remaining patients abandoned treatment due to low compliance ($n=3$) and conditions unrelated to L1 toxicity. Lesser complications permitting continued L1 treatment were transient mild neutropenia ($n=4$), zinc deficiency ($n=12$), transient increase in serum concentration of hepatic enzymes ($n=37$), moderate nausea ($n=3$), and arthropathy ($n=16$). There was no treatment-associated mortality. Two patients died, both while off-treatment. One died of hemosiderotic heart disease, and the other of *Pneumocystis carinii* pneumonia with AIDS. This study demonstrates the efficacy of L1 in long-term use for the treatment of transfusion iron overload, but also underlines the complications associated with treatment.

Recently, the clinical results of ISGOIC study have been updated[140–143]. Although the methods used for evaluating hepatic iron concentrations were different, all groups have reported failure to reduce hepatic iron concentrations in many patients below a threshold of 15 mg/g wet weight, identified in a previous study as associated with an increased risk of heart disease and early death[101]. Likewise, more than half of the patients in one study[141] still had serum ferritin concentrations >2500 μg/l, the threshold identified for increased risk of developing cardiac disease. Failure to achieve a steady decrease in storage iron on long-term L1 therapy in some of the patients is explained by the difference in efficacy between the two drugs on a weight per weight basis. In a metabolic balance study that compared combined urinary and fecal iron excretion in thalassemia patients receiving either 60 mg/kg DF or 75 mg/kg oral L1, mean iron excretion on L1 therapy was 65% of that on DF, although in some patients L1 was as effective or better than DF[144].

These data do not disqualify L1 as a suitable drug for the management of transfusion iron overload in thalassemia. However, they reveal that oral L1 treatment alone does not ensure sufficient protection in all patients and that careful monitoring is required to identify patients in whom additional parenteral chelating therapy is indicated. Concerns related to the accelerated development of hepatic fibrosis have been expressed based on observations made on a small numer of patients on long-term L1 therapy. This important issue needs urgent clarification by reviewing the hepatic status of all patients receiving long-term L1 treatment.

The polyanionic amines (HBED)

The search for improved, orally effective iron-chelating compounds has led to the re-discovery of a polyanionic amine synthesized over 30 years ago[145]: *N,N´*-bis (2-hydroxybenzoyl) ethylenediamine -*N,N´*-diacetic acid (HBED) (Fig. 32.5). It forms a hexadentate ligand with ferric iron by its secondary, or tertiary nitrogens and hydroxyl and carboxyl groups. The affinity constant for iron(III) of HBED is 39.6, and its affinity for other metals is relatively low. Conversion of the carboxylic groups of HBED to methyl esters results in a marked improvement in its intestinal absorption and a further increase in iron excretion.

HBED and dimethyl-HBED are remarkably non-toxic and their LD_{50} is >800 mg/kg[146, 147]. In hypertransfused rats, the dose range of 25–50 mg/kg HBED and dimethyl HBED were 12–15 times more effective than DF. Chemical measurements have shown that a single intramuscular

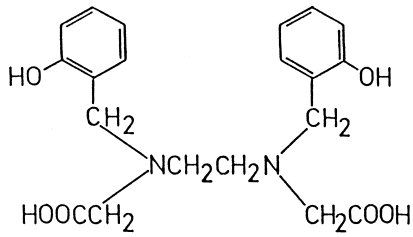

Fig. 32.5. HBED: *N, N′*-bis(2-hydroxybenzoyl) ethylenediamine-*N, N′*-diacetic acid.

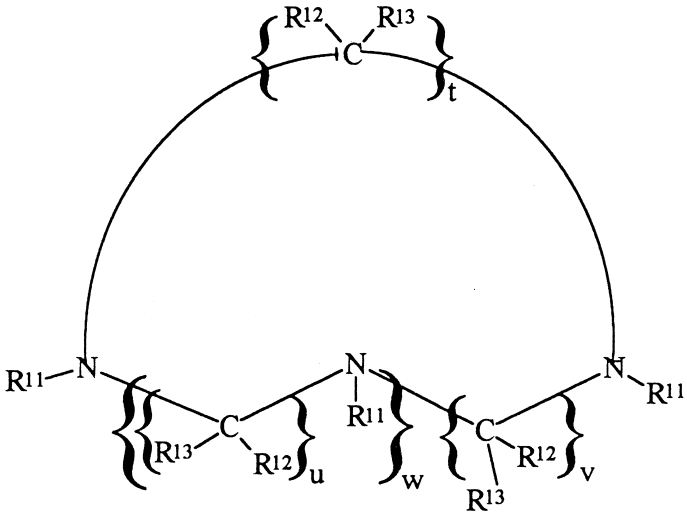

Fig. 32.6. The substituted polyaza compounds. The subject compound IRC011 (Ferofix A) is a member of the family of substituted cyclic polyaza compounds having the formula shown above where: R12 and R13 are H, t = u = v = 2 and w = 1 and R11 are methylene phosphonic acid groups (-CH_2-PO_3H_2)[7]. By varying the nature of R11, R12, R13, t, u, and w, analogues can be obtained possessing a wide range of properties.

dose of dimethyl-HBED 200 mg/kg decreased hepatic non-heme iron stores by 72%, and ferritin iron by 74%. Iron balance studies performed in a small number of thalassemia patients treated with HBED[148] have shown increased iron excretion after oral treatment in the urine and stool. Daily total iron excretion with 40 mg/kg/d HBED was 6–11 mg, but no further increase in excretion was achieved at a dose of 80 mg/kg/d. These limited amounts of iron excretion represented 28–48% of the excretion needed for achieving negative iron balance. Because the pro-drug dimethyl-HBED showed improved absorption and bioavailability in animal studies, this compound is now regarded as a promising candidate for clinical evaluation. Likewise, a new pro-drug formula of HBED has been developed and is presently undergoing intensive evaluation of its suitability for clinical use[149].

The substituted polyaza compounds (IRCO11)

The family of substituted cyclic polyaza compounds with the formula displayed in Fig. 32.6 was synthesized by Israel Resources Corporation Ltd., Haifa, Israel. IRC011 (Ferofix A) is a derivative in which R12 and R13 are H, t = u = v = 2 and w = 1, and R11 are methylene phosphonic acid groups (-CH2-PO3H2)[150]. The formula weight of the acid form is 411 Daltons, and of the monohydrate of the acid form, 429 Daltons. By varying the nature of R11, R12, R13, t, u, and w, analogues can be obtained possessing a wide range of properties.

The substituted polyaza compound IRCO11 has a number of promising features[150]: (a) it is a hexadentate chelator binding Fe(III) at a 1:1 ratio and therefore is without risk of possible toxic intermediate complexes, in contrast with L1 which is a bidentate chelator requiring 3 molecules of the drug for each Fe(III) ion[151]; (b) IRC011 is water-soluble and its stability constant with Fe(III) is over 1000 times that of DF; (c) its acute LD50 in mice exceeds 4.0 mM/kg compared to 0.44 mM/kg for DF; and (d) unlike DF, polyaza compounds with the structure of IRC011 do not contain any readily hydrolyzable covalent bonds, and are anticipated to resist in vivo biotransformation.

Using selective radioiron probes in hypertransfused rats, a major difference has been found in the in vivo activities of IRC011 and DF. In contrast to DF, which promotes the excretion of both hepatocellular and RE iron, the major source of iron mobilized by IRC011 is iron derived from the catabolism of erythrocyte hemoglobin in the RE system, and the efficiency of IRC011 in mobilizing RE iron is about four-fold higher than that of DF. Because the contribution of erythrocyte catabolism to the influx of iron to the plasma in thalassemia is incomparably higher than that of all other sources combined, it is reasonable to assume that erythrocyte catabolism is the predominant source of the toxic and readily chelatable non-transferrin bound plasma iron (NTBI) compartment[39, 40, 152–154]. Thus IRC011, with its superior ability to interact with catabolic erythrocyte iron, may not only improve the rate of urinary iron excretion in thalassemia, and may offer better protection of vital tissues from peroxidative damage by preventing the formation or eliminating NTBI.

Dose–response studies have shown a relative advantage of IRC011 compared with DF in RE iron mobilization, which was highest at the lowest dose employed. These findings imply that, due to its higher affinity to iron and anticipated resistance to catabolism, IRC011 may be effective at doses lower than presently required for DF or L1. Whether the greater efficacy of IRC011 in RE iron

mobilization may also represent a therapeutic advantage in humans is unproven.

Preliminary studies in mice indicate limited and variable intestinal absorption of IRCO11. Previous experience with the hydroxypyrid-4-one family of synthetic chelators has shown that lipophilicity is an important factor in determining the oral effectiveness of an iron chelator, and its ability to penetrate cell membranes[151, 155]. Because IRCO11 is a water-soluble compound, its membrane permeability is limited, but it may be a useful parent compound to a wide spectrum of polyaza analogues. Substitution of its synthetic arms by more lipophilic ligands may result in improved interaction with hepatocellular iron stores, and possibly better oral activity.

Current issues in chelator development

DF remains the drug of choice for the management of transfusion iron overload in thalassemia patients. However, its high cost and the inconvenience of its parenteral administration by portable pumps are major limitations underlying the need for developing alternative orally effective new iron chelating drugs. In the search for new and improved chelators, it is useful to remember some of the basic principles determining the safety and efficacy of iron chelators.

The stability of the chelator–iron complex

This is determined not only by the affinity of the drug to iron(III), but also by the nature of their interaction. DF is a hexadentate chelator interacting with iron at a 1:1 ratio and the formation of a neutral, stable complex preventing iron from participation in harmful chemical reactions such as hydroxyl radical formation through the Haber–Weiss reaction. By contrast, L1 is a bidentate chelator. It requires three molecules of L1 to one ferric iron to form a stable, neutral complex[156]. Hence, at suboptimal concentrations of L1, when either tissue iron concentrations are very high or drug concentrations too low, incomplete 1:1 or 2:1 complexes may be formed resulting in enhanced mobilization of potentially toxic iron. Thus, hexadentate chelators would, on theoretical grounds, always be preferable to bidentate or tridantate chelators.

The partition coefficient of a chelator

The partition coefficient of a chelator, or its relative solubility in water and lipids, determines its ability to cross lipid membranes. Lipophilic compounds penetrate cells with relative ease and improve the ability of a chelator to interact with intracellular iron stores. Unfortunately, as shown by the experience with the family of hydroxypyrid-4-ones (of which L1 is the dimethyl derivative), increased lipophilicity may also increase drug toxicity[157].

Molecular weight

This is a critical feature in designing new chelators with improved oral efficacy and is also important in determining the handling of iron after its chelation. Macromolecular complexes of iron chelators may be effective in binding iron, but may also interfere with its subsequent urinary excretion of iron. Likewise, new chelators with excellent in vitro interaction with chelatable iron in cell cultures may be ineffective orally if their size interferes with intestinal absorption.

Pro-drugs

These are an excellent solution for a number of requirements in the development of oral chelators. An outstanding example is the improvement of the hexadentate chelator *N,N´*-bis (2-hydroxybenzoyl) ethylenediamine-*N,N´*-diacetic acid (HBED) by converting its carboxylic groups into methyl esters[147]. By comparison with HBED, the pro-drug dimethylHBED is less polar and its intestinal absorption is improved significantly. After absorption, it is converted into HBED. Due to its gradual absorption, its overall effect in enhancing iron excretion is several-fold higher than that of HBED. Other hexadentate pro-drugs of HBED derivatives are presently under evaluation[149]. Protection against the harmful effects of circulating NTBI is optimal when the chelator is always present in the plasma. Similar to continuous subcutaneous or intravenous drug infusion, this effect may be achieved conveniently by employing orally effective pro-drugs in slow-release tablets.

Conclusions

The introduction of deferoxamine for regular use in the management of transfusion iron overload has changed the quality of life and life expectancy of many patients with thalassemia. However, effective DF treatment requires continuous subcutaneous infusion by portable pumps and many patients are unable or unwilling to cope with the rigorous requirements of such treatment. In addition, the high cost of DF treatment makes it unavailable for the vast majority of patients with hereditary anemia who need

long-term transfusion therapy. Consequently, there is great need for the development of alternative, orally effective iron-chelating drugs.

At present, only deferiprone (L1) has been used in a sufficient number of thalassemia patients to allow preliminary clinical evaluation. Although L1 induces negative iron balance, its use is associated with significant toxicity, especially agranulocytosis. Recent observations also raise concerns regarding the ability of L1 to induce sufficient depletion of tissue iron to offer protection from critical organ damage in a substantial fraction of patients. Consequently, there is a need for the continued development of new and improved orally effective iron chelators. Better understanding of the pathophysiology of iron toxicity and the mechanism of iron chelation is vital for the development of improved strategies of iron chelating therapy.

Acknowledgment

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33

Liver transplantation and hemochromatosis

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Introduction

Liver transplantation has become the treatment of choice of end-stage hepatic disease with long-term survival results better than any other therapeutic modality previously available. Because hemochromatosis is one of the most common genetic disorders and cirrhosis is often present at the time of diagnosis, it might be expected that hemochromatosis would be a common indication for liver transplantation. In fact, reports of liver transplantation for hemochromatosis continue to be a collection of case reports, and large centers report that hemochromatosis is an uncommon indication for transplantation[1–6]. Liver transplantation is not only a life-saving procedure for the patient, but may provide new insights into the pathogenesis of the hemochromatosis. The development of a DNA-based genetic test for the gene for hemochromatosis (*HFE*) has the potential to improve the diagnostic precision of making a diagnosis of hemochromatosis in the setting of end-stage hepatic disease[7].

Pre-transplant diagnosis of hemochromatosis

It has been increasingly recognized that end-stage hepatic disease of diverse etiologies can be associated with iron overload at the time of liver transplantation. In a series of 447 explant livers, 8.5% had iron overload in the range associated with hemochromatosis[8]. Surgical and intrahepatic portosystemic shunts may also contribute to iron overload. A series reported by Kowdley et al. suggested that 30% of patients with hemochromatosis coming to liver transplant had the diagnosis made at the time of transplantation[6]. All of these cases raise the question of what were the diagnostic criteria used to establish the diagnosis of hemochromatosis? If the diagnoses were based on hepatic iron concentration and hepatic iron index, it is likely that many cases of 'hemochromatosis' may have, in truth, been secondary iron overload. The best available criteria other than these measurements would include a pedigree study identifying an HLA-identical iron-loaded sibling, or DNA-based genetic testing for the C282Y hemochromatosis-associated mutation. Preliminary studies using genetic testing on explanted tissue from patients previously classified as having hemochromatosis at the University of Pittsburgh have demonstrated that only a minority of the cases were homozygous for the C282Y mutation. In a report of 30 cases suspected of having hemochromatosis prior to transplant, only five cases (14%) were homozygous for the C282Y mutation. In 30 patients with a hepatic iron index >1.9, only two patients (6%) were homozgous for the C282Y mutation[9]. Therefore it is with some caution that pathophysiological inferences are made from available case reports on liver transplantation in iron-overloaded patients that may, or may not, have hemochromatosis. The pre-transplant characteristics of putative hemochromatosis patients are summarized in Table 33.1. It has also been uncommon for unsuspected homozygotes to be discovered when genotyping has been done on all explanted livers. In a study of 101 explants done for non-hemochromatosis indications, there was one case of autoimmune hepatitis that was homozygous for the C282Y mutation, and only eight heterozygotes for C282Y[10].

In patients who have been diagnosed with hemochromatosis and have been iron-depleted by therapeutic phlebotomy, there appears to be some stabilization of liver function[11]. However, hepatocellular carcinoma can develop many years after iron depletion. Although tumors can be detected at an early stage by serial ultrasound and alpha-fetoprotein determinations, elderly hemochromatosis patients often cannot withstand a major hepatic

Table 33.1. Pre-operative profile of patients who underwent liver transplants for presumed hemochromatosis

Reference	*n*	Hepatic iron staining 4^+	Hepatic iron index >1.9	Pre-operative treatment	Family history	Genotyping (C282Y)	Hepatitis C	Alcoholism
[2]	6	5	—	6	—	—	—	—
[3]	1	1	—	—	—	—	—	—
[4]	9	6	9	1	—	—	2	6
[6]	37		20	—	—	—	3	6
[5]	4			2	—	—	1	3
[9]	35	—	—	—	—	2	2	8
[23]	1	1	1	—	—	1	—	—

resection, nor wait for an available liver transplant with current donor shortages. If therapies such as percutaneous ethanol ablation or chemo-embolization are shown to extend survival, the cost-effectiveness of screening for hepatocellular carcinoma would improve[12].

Pre-transplant treatment of hemochromatosis

The ideal transplant candidate with hemochromatosis has undergone complete iron depletion prior to surgery. The goal of therapy would be to optimize cardiac function with iron depletion, because cardiac complications have been reported commonly after liver transplantation in iron-loaded patients[5]. Immunological defects in iron-loaded patients may be compounded by post-transplant immunosuppression[13]. The link between the mutant gene product of the gene *HFE* (a class I MHC protein) and immune function has not yet been clearly established.

Blood can be removed at the rate of 500 ml once or twice weekly until the serum ferritin concentration is approximately 50 μg/l. Patients that present with advanced disease at the time of transplant may neither have the time or stamina to endure a long course of phlebotomy treatments. The oral agent, deferiprone (L1), has been reported to have potential hepatic toxicity in thalassemia patients and should probably be avoided until the results of further studies are available[14, 15]. The role of deferoxamine infusions in this setting has not been well studied, and drug toxicity is a concern in elderly patients with hepatic failure. There have been cases treated perioperatively with parenteral chelation therapy[5]. The goal of therapy is to deplete extrahepatic iron stores to improve cardiac pump function and arrhythmias.

Patients with a small solitary hepatocellular carcinoma complicating hemochromatosis may still be considered for liver transplantation. Most centers recommend immediate treatment with chemo-embolization or percutaneous ethanol ablation to slow tumor growth while awaiting a suitable liver donor.

Results of transplantation

Whether all patients in these studies are considered to have hemochromatosis or not, patients that are transplanted with iron overload have a worse prognosis than most other patients transplanted for end-stage liver disease. If iron overload is an indirect measurement of disease severity, as has been proposed in hepatitis C, sicker patients ultimately have a higher mortality rate than more stable transplant candidates. However, the high frequency of deaths from cardiac complications and sepsis suggest a common etiology for post-transplantation mortality that may (or may not) be related to iron overload.

The actuarial survival of patients who undergo liver transplant for presumed hemochromatosis collected from a variety of case reports are compared to 343 non-hemochromatosis patients (Fig. 33.1). Long-term follow-up of 22 patients transplanted for hemochromatosis at four centers had a 5-year actuarial survival of only 38%. Congestive heart failure was the most common cause of death, followed by septicemia. There was no conclusive evidence of iron re-accumulation in patients in this group[16].

Pathophysiology of hemochromatosis and lessons from liver transplantation

If we assume that hemochromatosis is a systemic genetic disorder associated with increased iron absorption, one would predict that there would be a re-accumulation of iron after liver transplantation. However, short-term

follow-up studies have not produced convincing evidence of iron re-accumulation in the transplanted liver[17, 18]. In the four cases in which hepatic iron concentration was reported after transplantation, all values were within the normal range[18]. Iron staining of hepatic tissue has been patchy and often appears in macrophages rather than hepatocytes. Whether the interval after transplantation is too short or immunosuppression affects iron absorption has not been clearly established. Another possibility is that iron re-accumulation occurs in extrahepatic sources. Postmortem studies in four patients demonstrated marked extrahepatic iron deposition in the heart, pancreas, and endocrine glands[4]. If it is assumed that the *HFE* gene product plays a role in intracellular iron homeostasis, the newly transplanted liver presumably has normal *HFE* protein and this may be able to regulate hepatocyte iron normally. Excess iron would then pass through the liver to the heart and other organs. The heart would be directly exposed to increased iron levels, and this could explain the cardiac toxicity frequently reported after liver transplantation[16, 19, 20].

A 47-year-old man underwent emergency liver transplantation at our hospital after a four-week illness associated with fatigue, jaundice, and abnormal serum concentrations of hepatic enzymes. A transvenous hepatic biopsy performed immediately before transplant demonstrated marked parenchymal iron overload with a hepatic iron concentration of 336 μmol/g and a hepatic iron index of 5.9. The patient died three weeks after transplantation. Surprisingly, postmortem examination did not demonstrate excess iron in the heart, pancreas, or endocrine glands. Family studies revealed iron overload in his sister and son, and a slightly elevated serum ferritin concentration in his brother (Fig. 33.2). Genotyping demonstrated that the proband, his brother, sister, and son were each homozygous for the C282Y mutation. The brother had a hepatic iron concentration at the upper limit of normal (hepatic iron index = 0.8) and was considered to be a non-expressing homozygote. This case would not support the extrahepatic re-accumulation of iron. The effect of immunosuppression on iron absorption in normal persons or in patients with hemochromatosis has not been evaluated. It is possible that production or function of the *HFE* protein that is related to immune proteins is suppressed by transplant immunosuppression.

There have been several cases of inadvertent transplantation of an iron-loaded liver into a normal donor[5, 18, 21, 22]. The hepatic iron concentration after liver transplantation in such a case is illustrated with an extended follow-up period of three years (Fig. 33.3). Intestinal radioiron absorption was normal in this case at 45 days post-transplant[23]. These cases strongly suggest that an intrahepatic defect of hemochromatosis was not transplanted into the new recipient.

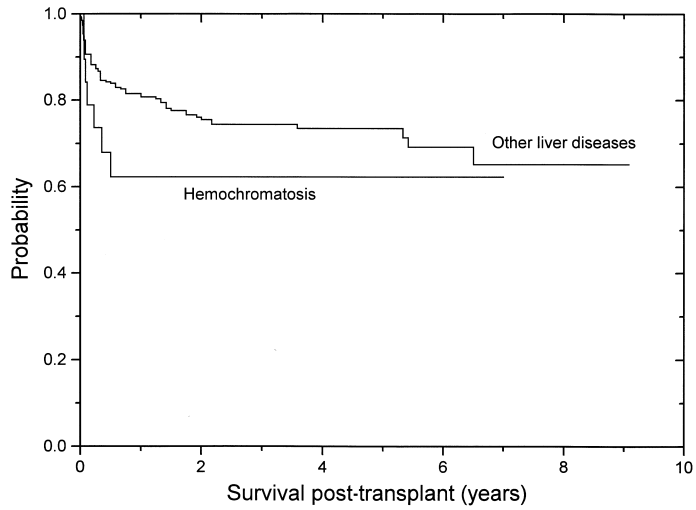

Fig. 33.1. Actuarial survival of patients transplanted for presumed hemochromatosis from a variety of case reports ($n = 19$) compared to 343 non-hemochromatosis patients.

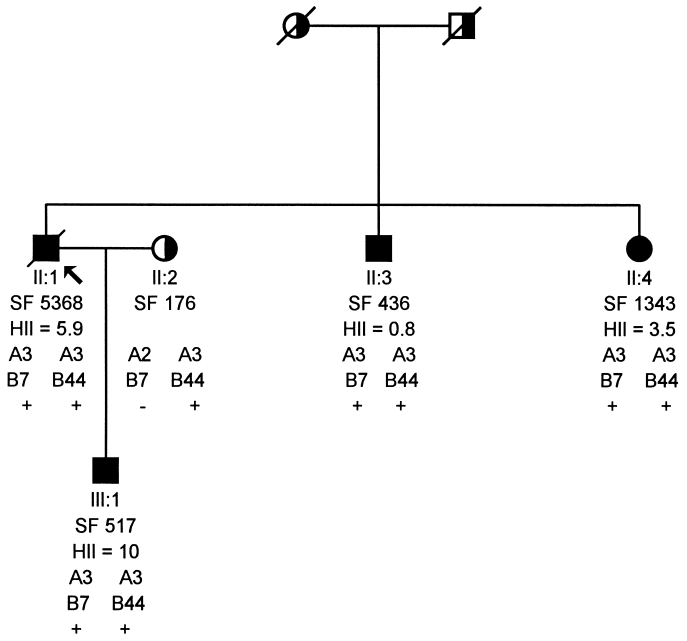

Fig. 33.2. Family pedigree of a patient (II:1) who is homozygous for the C282Y mutation and underwent liver transplantation. His brother (II:3), sister (II:4) and son (III:1) are also homozygous for the C282Y mutation. (Legend in descending order: SF = serum ferritin concentration (normal range 15–300 μg/L); HII = hepatic iron index; HLA-A and -B typing and C282Y mutation, + + = homozyote, + − heterozygote, − − normal).

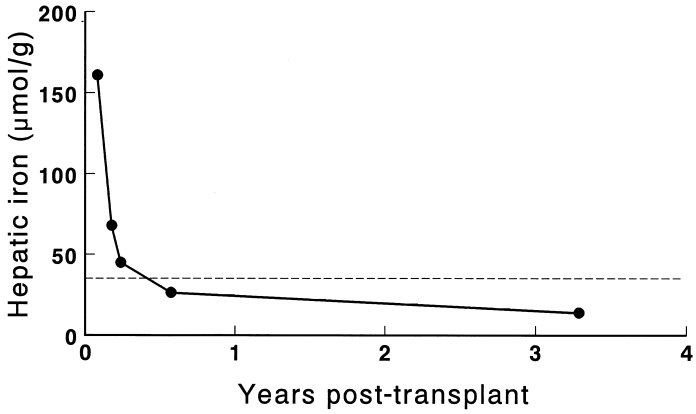

Fig. 33.3. Hepatic iron concentration post-liver transplant in a patient that inadvertently received an organ from a hemochromatosis donor. The dotted line represents the upper limit of normal of hepatic iron concentration (36 μmol/g).

Conclusions

Hemochromatosis is an uncommon indication for liver transplantation. As population screening becomes more common, early diagnosis and treatment should prevent many patients from developing hepatic cirrhosis. As genetic testing becomes more available, many cases previously considered to be hemochromatosis will be more appropriately classified as secondary iron overload. Clinical investigation of cases with a well-established genetic or pedigree diagnosis should be encouraged to increase our understanding of the pathogenesis of hemochromatosis.

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Survival in hemochromatosis

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Introduction

Hemochromatosis is one of the most frequent inborn errors of metabolism. Survival analyses in long-term studies show that, in the absence of hepatic cirrhosis and diabetes mellitus, iron removal by phlebotomy therapy prevents further tissue damage and is associated with a normal life expectancy. Patients with massive, long-lasting iron overload have a worse prognosis than those with less severe iron excess. It is probable that the duration and severity of iron overload determine the degree of tissue damage and thus the patient's prognosis. Iron removal, in general, ameliorates hepatic disease, weakness, and cardiac abnormalities, and also prevents the progression of endocrine alterations. Therapy, however, does not reverse arthropathy or insulin-dependent diabetes mellitus. Most deaths in patients with hemochromatosis are caused by hepatic cancer that often occurs years after completion of iron removal. In patients with hemochromatosis, hepatic cirrhosis, cardiomyopathy, and diabetes mellitus are also more frequent causes of death than in the general population. Further strategies have to evaluate the design of screening programs in order to diagnose more patients in the pre-cirrhotic and asymptomatic stage.

Therapy

The most important advancement in the management of hemochromatosis is phlebotomy therapy. In recent years, increasing attention has been paid to earlier diagnosis and treatment and, thus, to the prevention of irreversible tissue damage due to iron overload. Several long-term studies have evaluated the outcome in patients who were diagnosed in the pre-cirrhotic stage. It is unclear whether treatment of such patients prevents the development of hepatic cancer, a major cause of death in cirrhotic patients (even when their excessive iron has been removed).

Therapy usually starts with a rigorous schedule of phlebotomies until the complete removal of iron can be documented. Initially, 500 ml blood should be removed at least weekly. In our series of German patients, the number of phlebotomies necessary to remove excess iron during the initial treatment period varies from 50 to 150 during a period of 9 to 24 months[1, 2]. Iron depletion can be documented by repeat hepatic biopsy, or by biomagnetometry or specialized magnetic resonance tomography. Phlebotomy therapy must be continued for the rest of the patient's life. Four to ten phlebotomies per year are required to prevent re-accumulation of excess iron. This is the only effective method to remove grossly increased iron stores. Phlebotomy therapy is far more effective than using the iron chelator desferioxamine (that may also have serious side-effects). Desferioxamine therapy may be combined with phlebotomy in rare cases of severe cardiomyopathy, in which rigorous phlebotomy treatment alone may not be possible. Otherwise, desferioxamine therapy should be restricted for use in patients with secondary iron overload and anemia in whom phlebotomies are not possible. In genetic hemochromatosis, there is little rationale for an iron-free or iron-reduced diet.

Survival analyses

Impact of phlebotomy treatment

The prognosis of untreated hemochromatosis is poor[3–7]. Although the beneficial effect of iron removal has never been proven by controlled trials, several studies strongly suggest that early diagnosis and iron removal markedly improve survival and may offer the patients without

Table 34.1. Survival data for patients with hemochromatosis treated by phlebotomy

References	Publication year	Number of patients	% with cirrhosis	Percentage survival at			
				5 years	10 years	15 years	20 years
[6]	1976	85	79	66	32	8	n.d
[11]	1980	34	n.d.[a]	70	70	30	30
[1]	1985	163	69	92	76	59	49
[9]	1991	85	31	87	81	71	71
[10]	1992	212	68	83[b]	65[b]	n.d.	n.d.
[2]	1996	251	57	93	77	62	55

Notes:
[a] n.d. not determined. Of the 34 patients in the Milder Study, only 33 had hepatic biopsy. However, 31 of these 33 patients had cirrhosis.
[b] data are approximated from survival in cirrhotic and non-cirrhotic patients, because survival for total group of patients not given in the paper.

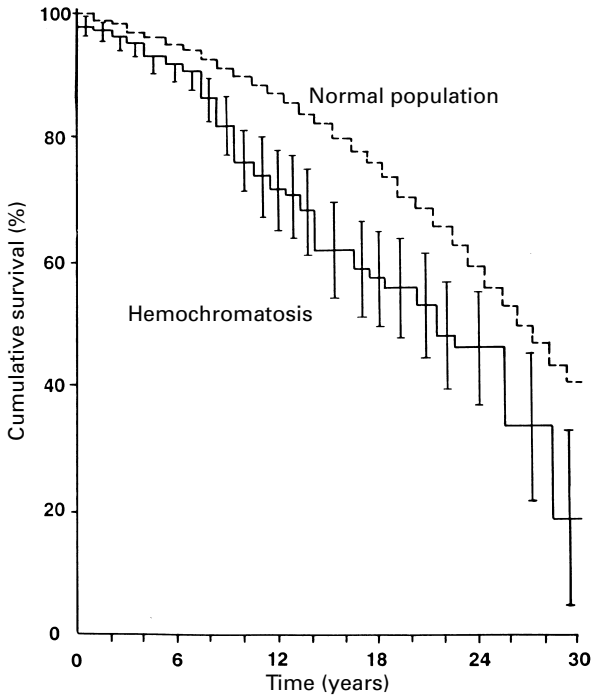

Fig. 34.1. Cumulative survival in 251 patients with hereditary hemochromatosis. A 95% confidence interval was calculated by adding twice the standard deviation from the means (vertical bars). Survival was significantly reduced in patients with hemochromatosis when compared with the expected survival rates for an age- and sex-matched normal population; the expected survival rates were outside and above the confidence interval calculated for survival in patients. (Adapted from ref.[2].)

hepatic cirrhosis a normal life expectancy[1, 2, 4–10]. There are no data on the natural history of untreated hemochromatosis in non-cirrhotic patients. Such patients may differ from those in whom cirrhosis develops, and their 'normal life expectancy' may not be a direct result of phlebotomy therapy. Although there is no experimental or epidemiological support for this hypothesis, the issue cannot be resolved due to ethical considerations: it is accepted treatment to deplete patients with hemochromatosis of their excess iron.

Impact of liver cirrhosis and diabetes mellitus

Several long-term survival analyses have been carried out in the last decade[1, 2] that include large numbers of patients followed for as long as 33 years. In general, the survival rates have increased in the last three decades (Table 34.1). The differences in survival rates among the studies are likely explained by differences in the percentage of patients diagnosed with and without hepatic cirrhosis. In particular, there was a high percentage of non-cirrhotic patients in the Canadian cohort, and thus the survival rates were high[9] (Table 34.1). In the long-term studies, the observed survival in patients with hemochromatosis was reduced when compared to the survival expected for a sex- and age-matched general population until 1996. Thus, the total group of hemochromatotic patients still had a significantly reduced life expectancy compared with the normal population (Fig. 34.1). However, in contrast to the total group of patients, survival in non-cirrhotic patients was similar with the survival expected in a sex- and age-matched normal population[1, 2, 10, 11] (Fig. 34.2). This was also true for the subgroup of patients that did not have diabetes mellitus at the time of diagnosis (Fig. 34.3). Thus,

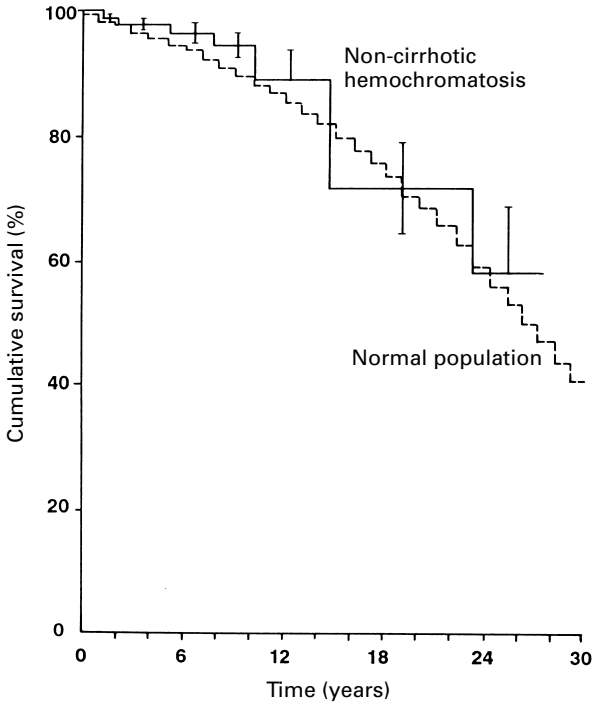

Fig. 34.2. Cumulative survival in 109 non-cirrhotic patients with hemochromatosis. A 95 per cent confidence interval was calculated by adding twice the standard deviation from the means (see vertical bars). Survival in patients with non-cirrhotic hemochromatosis was decreased when compared with the rate expected; the confidence interval for their survival overlapped the rates expected for an age- and sex-matched normal population. (Adapted from ref.[2].)

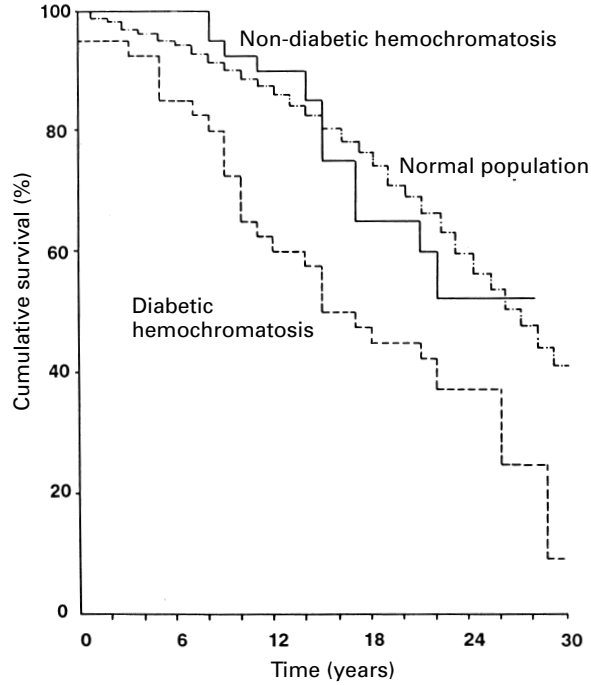

Fig. 34.3. Cumulative survival in 120 diabetic and 131 non-diabetic patients with hemochromatosis. Survival was significantly less in diabetic patients than in non-diabetic patients ($p \leq 0.01$, log-rank test). The mean age and distribution of age were similar in both groups (46.0 ± 12.1 (S.D.) years [range 24–75 years] in diabetic patients and 45.4 ± 12.4 years [range 18–77 years] in non-diabetic patients). The survival of non-diabetic patients was similar to that of a sex- and age-matched normal population (broken-dotted line), whereas diabetic patients had reduced survival (expected survival rates lying outside the confidence intervals are not shown in this figure). (Adapted from ref.[2].)

patients who are diagnosed in the non-cirrhotic or non-diabetic stage and treated by phlebotomy had a normal life expectancy[2]. In all recent long-term studies, cirrhotic patients have a significantly worse prognosis than non-cirrhotic patients when survival between the two subgroups was compared by a log-rank test (Fig. 34.4)[1, 2, 9, 10]. Similarly, non-diabetic patients have a better prognosis compared to patients who had diabetes mellitus at diagnosis (Fig. 34.3)[1, 2].

The prognosis of patients with hemochromatosis is surprisingly good, even if they already have cirrhosis. Their life expectancy appears to be at least 5 years longer than that reported in older studies[6, 11, 12], and 10–20 years longer than that reported for patients who have other forms of cirrhosis, especially that due to alcoholism[13–15]. The improved survival in recently reported cirrhotic patients as compared with cirrhotic patients in older reports is probably due to the fact that some recent patients were identified by 'screening methods' and thus at an earlier stage. The survival data of cirrhotic patients from Germany and Italy are virtually identical[1, 2, 10]. Both cirrhotic and diabetic patients showed a marked increase in mobilizable iron that exceeded that in non-cirrhotic and non-diabetic patients by more than 40%, respectively (Table 34.3).

Impact of laboratory iron parameters

Plasma iron concentration, transferrin saturation, serum ferritin concentration, and grade of hepatic iron staining are greater in cirrhotic than in non-cirrhotic cases[1,2]; however, these parameters cannot be used to make predictions concerning the course of the disease in individual patients because the values for one group overlapped those for the other.

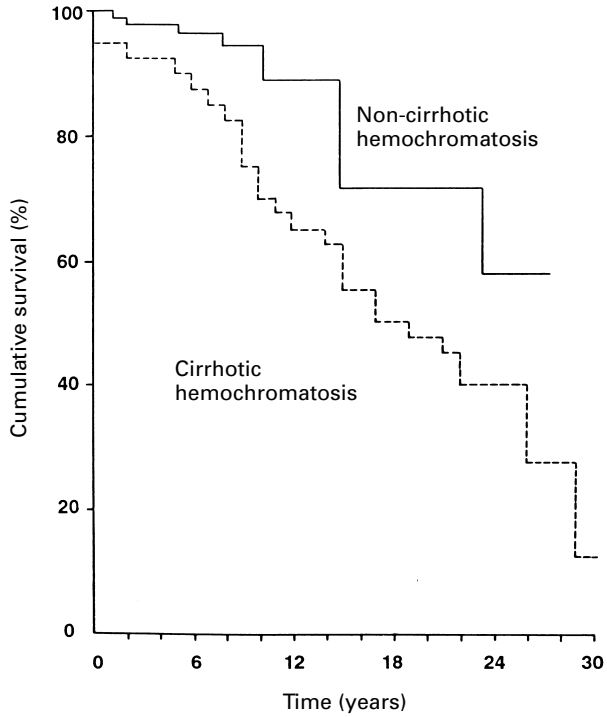

Fig. 34.4. Cumulative survival in 142 cirrhotic and 109 non-cirrhotic patients with hemochromatosis. Survival was significantly less in the cirrhotic patients than in the non-cirrhotic patients ($p \leq 0.01$, log-rank test). The mean age and distribution of age were similar in both groups (46.2 ± 11.9 (S.D.) years [range 24–75 years] in cirrhotic patients and 45.1 ± 11.8 years (range 18–77 years) in non-cirrhotic patients). (Adapted from ref.[2].)

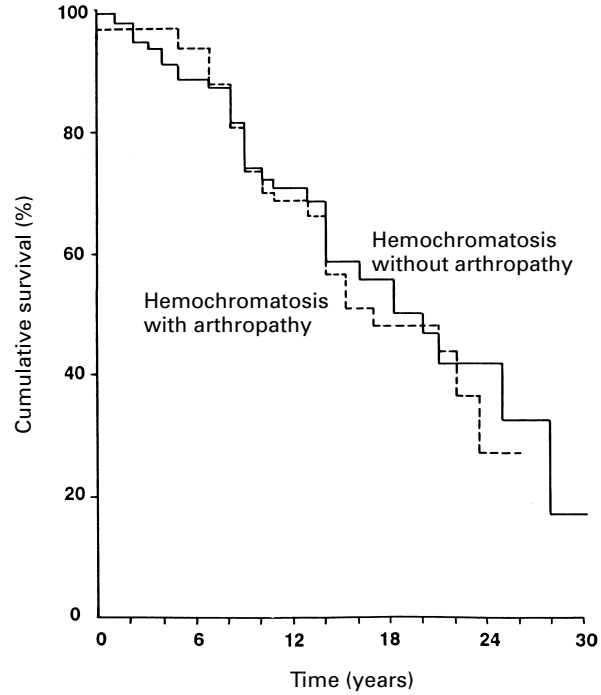

Fig. 34.5. Cumulative survival in 111 hemochromatotic patients with arthropathy and 140 patients without arthropathy. Survival was similar for patients with and without arthropathy ($p > 0.2$, log-rank test). (Adapted from ref.[2].)

Impact of sex

Prognosis of hemochromatotic patients was not statistically influenced by sex in any of the recent large series [1,2,9,10] (Fig. 34.5).

Impact of arthropathy

In the German series, arthralgia was equally common in cirrhotic and non-cirrhotic patients, whereas symptoms due to hepatic disease, diabetes mellitus, and cardiac or endocrine complications were more common in patients with cirrhosis[2]. This suggests that arthralgia is often an early symptom. This hypothesis is also supported by data that demonstrate that the amount of mobilizable iron was similar for patients with and without arthropathy (Table 34.2), and by data that show that the percentage of patients with arthralgia did not change during the last decades although the percentage of non-cirrhotic patients markedly increased (Table 34.3)[2].

In a cohort of Canadian patients, arthropathy was associated with an improved survival (risk factor 0.24 in a multivariate analysis), while advanced age and hepatic cirrhosis were associated with reduced survival[10]. In the most recent German series, only diabetes mellitus and hepatic cirrhosis were associated with reduced survival in the multivariate analysis; arthropathy had no significant impact on survival[2] (Fig. 34.6). In both the German and the Canadian series, arthritis was an early symptom and was associated with pre-cirrhotic hemochromatosis. The higher percentage of pre- or non-cirrhotic patients in the Canadian than in the German series (69% vs. 43%, respectively) may explain why arthropathy was associated with a significant increase in survival only in the Canadian patients[2,9].

Impact of alcohol consumption

Alcohol abuse has been reported to reduce survival in hemochromatosis patients[10,11]. However, our own data from a large cohort of German patients failed to show an effect of alcohol consumption on survival[2]. This may be due to the fact that only a small percentage of our patients in both series consumed >80 g of alcohol per day[1,2].

Table 34.2. Iron removed by phlebotomies in 185 patients with liver biopsy-documented iron depletion[a]

All patients ($n=185$)	21.2 ± 1.1		p value
Subgroups	present	absent	
Liver cirrhosis	25.7 ± 1.7	14.8 ± 1.5	≤ 0.001
Diabetes mellitus	26.3 ± 1.7	16.5 ± 1.5	≤ 0.001
Arthropathy	21.4 ± 1.7	20.9 ± 1.8	> 0.2
Outcome	survived ($n=150$)	died ($n=35$)	
	19.4 ± 1.7	29.1 ± 2.6	≤ 0.01
Causes of death	liver cancer ($n=15$)	other causes ($n=20$)	
	32.9 ± 2.9	26.1 ± 2.7	≤ 0.05

Notes:
[a] The amount of iron removed was calculated based on the assumption that 500 ml blood contains 250 mg of elemental iron. Data are shown as grams of iron (mean ± S.E.). Adapted from ref.[2].

Table 34.3. Clinical features and mobilizable iron in hemochromatosis patients during three different time periods

	1947–69 ($n=84$)	1970–81 ($n=84$)	1982–91 ($n=83$)	Total ($n=251$)	χ^2	p
Liver cirrhosis	79.8% (67/84)	48.8% (41/84)	41.0% (34/83)	56.6% (142/251)	28.7	0.59×10^{-6}
Diabetes mellitus	73.8% (62/84)	39.2% (33/84)	30.1% (25/83)	47.7% (120/251)	35.6	0.18×10^{-7}
Electrocardiographic changes	48.8% (41/84)	32.1% (27/84)	24.1% (20/83)	35.1% (88/251)	12.96	0.20×10^{-2}
Arthalgia	39.3% (33/84)	45.2% (38/84)	48.2% (40/83)	44.2% (111/251)	1.34	0.49
Impotence	57.3% (43/75)	30.7% (23/75)	20.3% (15/74)	36.2% (81/224)	23.6	0.74×10^{-5}
Asymptomatic patients	4.8% (4/84)	22.6% (19/84)	30.1% (25/83)	19.1% (48/251)	18.4	0.10×10^{-3}
Mobilizable iron (g)	25.5 ± 1.8	20.7 ± 1.7	17.1 ± 1.4	21.2 ± 1.1	t-test	<0.01

Source: These data are adapted from ref.[2]. Mobilizable iron is expressed as mean grams of elemental ± 1.5.[2]

Improvement in survival during recent decades due to earlier diagnosis

A recent survival analysis[2] suggests that the prognosis of patients diagnosed in the last decade may be almost normal, even for the total group of hemochromatosis patients. This is probably due to earlier diagnosis. This result supports the value of early diagnosis and prophylactic iron removal in patients with hemochromatosis. The latter cohort study followed 251 patients with hemochromatosis for as long as 33 years (mean 14.1 years). Mean survival was 21.0 years and thus approximately 2 years longer than that in our previous report[1]. Survival gradually increased during the long-term follow-up of the most recent cohort study[2]. Patients diagnosed between 1947–1969 ($n=83$) had reduced survival as compared with patients diagnosed between 1970 and 1981 ($n=84$). Patients diagnosed in the interval 1982–1991 had a better survival than the latter subgroups (Fig. 34.7). Correspondingly, survival in the subgroup of patients diagnosed in the interval 1982–1991 did not differ significantly from the rates expected in a matched normal population for that period, whereas survival of patients diagnosed during the two earlier periods was significantly reduced (compare Figs. 34.1 and 34.7). Further analysis of the three subgroups showed that the percentage of patients without symptoms or cirrhosis at entry into the study significantly increased during the study period (Table 34.3). The percentage of early diagnoses (patients without cirrhosis and those without symptoms) increased more than four-fold from the period 1947–1969 to that of 1970–1981, whereas there was only a further 20–25% increase from the period 1970–1981 to that of 1982–1991. In contrast, the mean amount of mobilizable iron decreased significantly during the three intervals of follow-up (Table 34.3). Corresponding to the increase of patients diagnosed with less severe iron overload, the percentage of patients with hepatic cirrhosis, diabetes mellitus, electrocardiographic changes, and impotence decreased significantly (Table 34.3). The percentage of patients with arthralgia did not change.

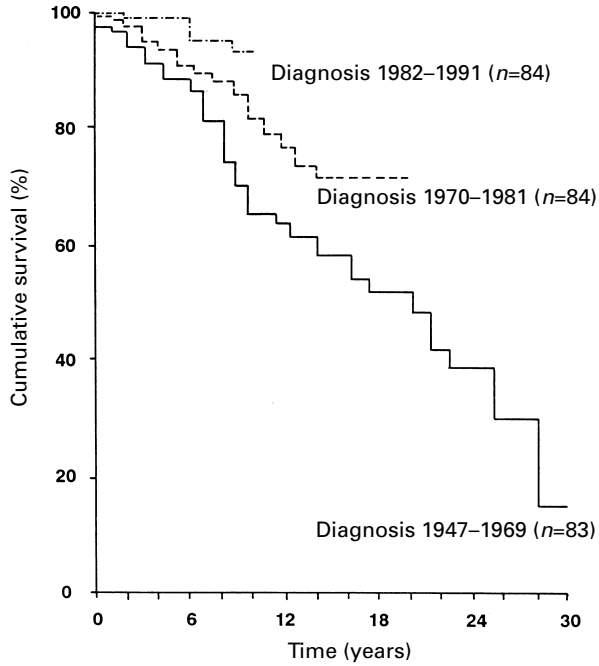

Fig. 34.6. Cumulative survival in three subgroups of patients diagnosed in three different time periods. Patients diagnosed in 1947–1969 ($n=83$) had deceased survival as compared with patients diagnosed in 1970–1981 ($n=84$) and patients diagnosed in 1982–1991 had a better survival than the subgroups diagnosed earlier ($p \leq 0.05$ log-rank test). Adapted from ref.[2].)

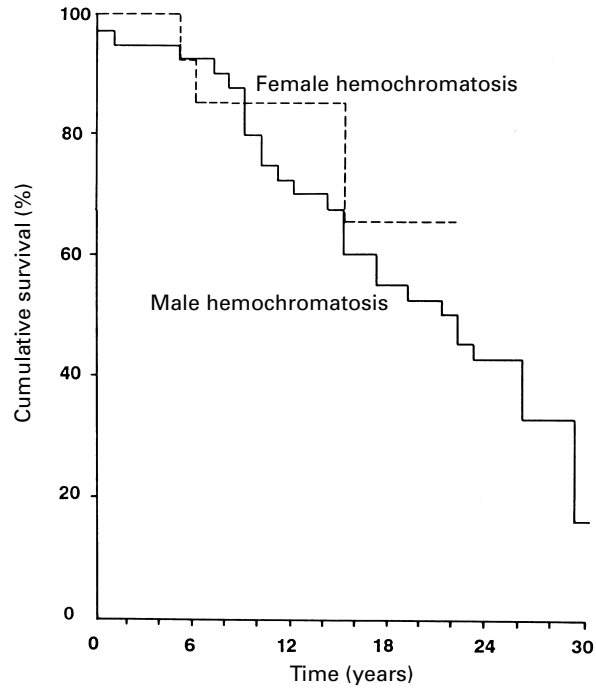

Fig. 34.7. Cumulative survival in 224 male patients with hemochromatosis and 27 female patients. Survival was similar for both ($p>0.2$, log-rank test).

Patients diagnosed in the previous decade could only be followed for ≤ 10 years. Thus, it is unknown whether long-term survival in this subgroup will remain normal after 20 or 30 years. In addition, several patients were diagnosed with hepatic cirrhosis and insulin-dependent diabetes mellitus in the interval 1982–1991[2]. Thus, there was still significant morbidity at diagnosis in some cases, supporting the need to improve the rate of early diagnoses.

The increase in survival during the last decades is best explained by a corresponding increase in the percentage of asymptomatic patients and by a decrease in the severity and duration of iron overload. Our recent data also confirm that the amount of mobilizable iron is closely related to the hepatic iron concentration[2]. Because the reduced frequency of patients with severe iron overload was closely related to a reduced frequency of patients who had hepatic cirrhosis, diabetes mellitus, electrocardiographic changes and impotence, iron overload in hemochromatosis occurs gradually and leads to complications as iron overload increases.

The analysis of cases with early diagnosis shows that the rate of early diagnosis has decreased in the last decade. Early diagnosis was defined as the identification of patients who had hemochromatosis proved after liver biopsy, but who were asymptomatic and non-cirrhotic. There was more than a four-fold increase in early diagnoses from the period 1947–1969 to that of 1970–1981. This marked improvement is best explained by advances in the understanding of the inheritance of the disorder and by introduction of the serum ferritin concentration as a non-invasive test for quantitative assessment of iron stores[15, 16]. Although there was some further increase in the percentage of patients with asymptomatic and non-cirrhotic disease during the interval 1982–1991, this increase was much smaller than that of preceding decades[2]. Several non-cirrhotic patients were detected in rheumatology clinics that routinely measure serum ferritin concentrations in patients with arthralgia. Most patients without symptoms were diagnosed by screening for hemochromatosis in families with a hemochromatosis proband. We believe it is important to support further 'screening' in subgroups of persons who have symptoms and findings often found in hemochromatosis, such as arthralgia or diabetes mellitus[17–19]. Some of these patients will be detected before the development of cirrhosis. Screening of siblings of a proband is mandatory and may yield an even higher

percentage of affected persons who are asymptomatic[20, 21]. These two 'screening' approaches, however, will result in limited improvement in survival statistics, because they either rely on the development of symptoms, or identification of a family member with symptomatic disease. Further improvement in early diagnosis and long-term outcome will be possible when screening of non-selected subjects is done in the general population[2].

Impact of length and severity of iron overload on prognosis

The present findings suggest that prognosis and complications of hemochromatosis, probably including the development of primary hepatic cancer, depend on the amount and duration of iron overload and the point at which iron accumulation is interrupted by phlebotomy therapy. Patients who died during our study had much more mobilizable iron than surviving patients. Furthermore, survival was markedly reduced in patients who could not be depleted of iron after >80 phlebotomies (i.e., large) iron excess vs. those who could be depleted by <80 phlebotomies (less severe) iron excess[2]. Previously we had shown that patients who could be depleted of iron by phlebotomy during the initial 18 months of treatment had a significantly better survival than patients who could not be depleted of iron during this period, and who thus probably had greater iron stores[1]. These data are supported by the analyses of Adams et al.[9] and another German cohort study[2]. Our data show, in addition, that cirrhotic and diabetic patients had a markedly greater iron excess than patients who did not have these complications at diagnosis (Table 34.2). Correspondingly, prognosis in non-cirrhotic patients was markedly better than that in cirrhotic patients and could not be distinguished from that expected for a matched normal population. Patients who died during the period of the present study had significantly more mobilizable iron than patients who were living at the end of the study (Table 34.2). It is yet unknown whether early diagnosis and treatment may also prevent further development of complications of iron overload, in particular the late and fatal development of primary hepatic cancer.

Recent data strongly suggest that early diagnosis and removal of excess iron can improve the patient's prognosis to normal life expectancy and largely prevent most complications of iron overload. The prognosis mainly depends on the duration and degree of iron overload that also determines the development of the major complications such as hepatic cirrhosis, diabetes mellitus, and primary hepatic cancer (Table 34.4). Other factors such as alcohol abuse and viral hepatitis may reduce overall survival statistics only if they are present at a high rate among study subjects. The increase in the rate of early diagnosis has slowed down during recent years[2] suggesting that further improvement will require screening of non-selected subjects in the general population[22].

Table 34.4. Factors that affect survival in hemochromatosis

Major factors
Length and severity of iron overload
Hepatic cirrhosis
Diabetes mellitus
Cardiomyopathy (especially young subjects)
Hepatic cancer (even many years after iron depletion)
Minor or controversial factors
Additional infection with hepatitis B or C
Alcohol abuse (probably significant if exceeding 80 g/day)
Factors without significant effect on survival
Sex
Arthropathy

Effect of therapy on symptoms and clinical findings

The development of the two major complications of hemochromatosis – hepatic cirrhosis and diabetes mellitus – are closely related. Most patients with cirrhosis at entry also have diabetes mellitus whereas only a few non-cirrhotic patients do[2]. Cirrhotic patients are more likely to be symptomatic than non-cirrhotic patients; the latter relationship is similar for diabetic and non-diabetic patients. Patients with diabetes or cirrhosis also had more severe iron overload than patients without these complications (Table 34.2). Thus, the presence of both hepatic cirrhosis and diabetes mellitus were closely associated with the amount of iron mobilizable by phlebotomy.

The stage of hepatic fibrosis is correlated with the amount of mobilizable iron and with hepatic iron concentration[2] (Table 34.5). Mobilizable iron was also closely related to the hepatic iron concentration in patients in whom quantitative measurements were done. The threshold value of ≥ 500 μM iron/g of liver is necessary to induce fibrosis[23]. The degree of fibrosis is significantly reduced after removal of iron[2] (Table 34.6). Reversal of cirrhosis has previously been documented by repeat laparoscopy in some patients[24, 25]. Because laparoscopy is not routinely

Table 34.5. Mobilizable iron, hepatic iron concentration at diagnosis, and stages of hepatic fibrosis in hemochromatosis

	Mobilizable iron		Hepatic iron concentration	
Stage of hepatic fibrosis	Number of patients	Mobilizable iron (g)	Number of patients	Liver iron (mg/g dry weight)
0	11	10.1 ± 0.9	7	11.6 ± 1.8
1	32	13.7 ± 1.3	10	13.9 ± 1.1
2	39	17.4 ± 1.5	9	16.9 ± 1.4
3	93	25.7 ± 1.7	15	22.4 ± 2.0
All patients	185	21.1 ± 1.1	41	16.1 ± 5.6

The mobilizable iron (expresssed as mean grams of elemental iron ± 1 S.E.) was calculated from the number of phlebotomies patients in whom a repeat hepatic biopsy revealed complete iron depletion (see Table 34.2). Quantitative liver iron concentrations were available only for patients diagnosed after 1984. By t-tests, there was a step-wise, significant increase in the amount of mobilizable iron and the hepatic iron concentration with progression of the stages of fibrosis ($p < 0.05$ for all comparisons). Adapted from ref.[2].

Table 34.6. Changes in fibrosis stage due to iron removal in hemochromatosis

Fibrosis stage	Improved	Worsened	Unchanged	Sum
0	0	1	20	21
1	10	1	21	32
2	20	0	19	39
3	12	0	81	93
Sum	42	2	141	185

These data are compiled from 185 patients in whom a repeated hepatic biopsy showed complete iron depletion. $\chi^2 = 33.19$; $p = 0.000009625$. Adapted from ref.[2].

performed for re-evaluation of patients in most centers, the latter data cannot be compared directly to those of other series. When judged by hepatic histology, advanced hepatic cirrhosis does not regress to a normal liver architecture.

Causes of death

Today there are two major causes of death in the large series of patients with hemochromatosis: hepatic disease and cardiomyopathy[1, 2, 9, 10].

Hepatic cirrhosis and cardiomyopathy

Patients who present with major complications of cirrhosis or cardiomyopathy have the worst prognosis; many of these patients die before completion of iron depletion therapy. Patients who present with major complications of cirrhosis, or with a combination of decompensated cirrhosis and cardiac failure, have a bad early prognosis. In our German patients, 9 of 13 persons who presented with ascites, splenomegaly, esophageal varices, and impaired hepatic function died before iron depletion could be completed. The risk to die from a direct consequence of hepatic cirrhosis was markedly increased in both of our case series[1, 2](Table 34.7). Although cardiomyopathy and diabetes mellitus are not frequent causes of death, the risk of death from cardiomyopathy or diabetes mellitus was still significantly greater than that expected for the normal population[1,2].

Primary hepatic cancer

The second major cause of death is cancer of the liver. In our cohort of 251 German patients, 19 died of cancer of the liver (16 with hepatocellular carcinoma and 3 with carcinoma of intrahepatic bile ducts). In addition, the present cohort includes two additional men with biopsy-proven cases of hepatocellular carcinoma who were still alive at the end of the observation period. In most recent series, hepatic neoplasms accounted for 10–30% of all deaths[26]. Hepatocellular carcinoma occurred in 18.5% of 649 cirrhotic patients with hemochromatosis[28]. This percentage decreases when the percentage of cirrhotic patients is low in the total cohort[29]. In the most recent German series, the risk of death in a patient with hemochromatosis primary hepatic cancer is more than 100-fold greater than that in the general population (Table 34.6). Iron depletion had been documented in 17 of 21 patients with hepatic cancer. The mean interval between iron depletion and diagnosis of hepatic cancer was 9.4 ± 4.8 years (range 3–19 years) in these patients. Thus, the great majority of hepatic cancers developed in persons who were depleted of iron for many years. As in our previous report all patients with liver

Table 34.7. Death rates in 251 patients with hemochromatosis and expected rates in the normal population (Federal Republic of Germany)[a]

Cause of death	Deaths expected	Deaths observed	95% confidence limits for observed deaths	Mortality ratio (observed/expected)
All causes	24.7	69	53.7–86.2	2.77
Neoplasms	8.21	27	17.8–38.1	3.29
of liver	0.16	19	11.4–28.4	118.75
of other sites	8.05	8	3.4–14.4	0.99
Diabetes mellitus	0.29	4	1.1–8.8	13.79
Diseases of circulatory system	7.41	14	7.6–22.4	1.97
Cardiomyopathy	0.36	5	1.6–10.2	13.9
Myocardial infarction	3.21	4	1.1–8.8	1.27
Others	3.84	5	1.6–10.2	1.40
Diseases of the digestive system	2.50	18	10.7–27.2	7.2
Hepatic cirrhosis	1.40	14	7.6–22.4	10.00
Others	1.10	4	1.1–8.8	3.64
Trauma	1.15	3	0.6–7.3	2.61
Others	5.14	3	0.6–7.3	0.58

Note:
[a] Adapted from [2].

cancer had cirrhosis[1, 2]. Although there are a few case reports about development of cancer in a non-cirrhotic liver in hemochromatosis[27, 28], the risk is mainly restricted to cirrhotic patients. Development of hepatic cancer may also depend on the amount and duration of iron overload, because patients who died from hepatic cancer had significantly greater iron stores than patients who died from other causes. In 15 of 19 patients who died of hepatic cancer, iron depletion had been achieved. These 15 patients had markedly increased amounts of mobilizable iron in comparison with patients who survived and with patients who died from other causes (Table 34.2)[2].

Our experience does not support recent hypotheses that the hepatitis B or C virus[10, 29, 30] plays an important role in the increased risk of developing hepatic cancer. Hepatitis B surface antigen was not detected in 21 German patients who died of hepatic cancer[2]. Hepatitis B core antibodies were detected in five patients. Serum was analyzed for hepatitis C antibodies in 11 of the 21 subjects; none of these had hepatitis C antibodies[2].

Other causes of death

Cardiac failure due to cardiomyopathy was an infrequent cause of death in our series, although it was more common than expected. Similar to our previous analysis[1] but in contrast to an older report, in [31], the frequency of extrahepatic carcinomas was virtually identical with the expected rate. A cohort of Australian patients with hemochromatosis also failed to show an increased risk for extrahepatic malignancies[32].

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Part VII

Infections and immunity in hemochromatosis

35

Role of iron in infections and immunity

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Introduction

Maintenance of immune function and defenses against infection requires careful physiological control of various metabolic events. The homeostatic regulation of iron metabolism exemplifies this requirement. The body is faced with the fundamental problem of ensuring that mechanisms exist to allow acquisition of iron by host tissues, while at the same time preventing its becoming readily available to invading microorganisms. For this to occur, two conditions have to be met:

(i) Specific mechanisms must exist to allow iron to be available for a regulated uptake and utilization by host tissues, but at the same time:

(ii) Iron must not be present in forms that are readily utilized by microorganisms, or could cause potentially toxic reactions.

In hemochromatosis, the regulation implied by the former condition is disrupted, with a consequent danger that the latter condition will also not be met. This chapter reviews the role played by iron in normal immune function, the potential for iron overload to cause dysregulation of the immune system, and the extent to which this is realized in hemochromatosis. More specific topics, such as the role of iron in microbial pathogenicity, its effect on viral hepatitis, and abnormalities in lymphocyte subsets are addressed in other chapters.

The immune system

The immune system consists of a complex network of leukocytes (lymphocytes, macrophages/monocytes, neutrophils, dendritic cells, etc.) and soluble mediators (immunoglobulins, cytokines) the activities of which are tightly controlled and extensively interlinked. Although some functional redundancy may occur (e.g., among cytokines), most individual components have crucial functions that, if unable to operate normally, may lead to immunological defects. Specific immunity is traditionally split into humoral immunity, mediated by antibodies, and cell-mediated immunity, mediated by T-lymphocytes. However, such a division is now regarded as artificial. For example, T-lymphocytes are usually required for B-cell activation and thus for antibody production, and B-lymphocytes can activate T-cells by acting as antigen-presenting cells.

Activation of the immune system initially involves recognition and processing of an antigen by antigen-presenting cells such as macrophages, dendritic cells, or B-lymphocytes. This leads to activation of T- and/or B-lymphocytes that results in cell division, phenotypic changes, and synthesis and secretion of various proteins. These include not only antibodies, but other molecules such as cytokines that act as intercellular messengers. Cytokines activate lymphocytes, and also act upon other cells such as phagocytic cells, leading to increased microbicidal and cytotoxic activity.

Because immunological activation involves up-regulation of biosynthetic activity, and iron is required for many metabolic events, it is reasonable to postulate that iron availability can influence immune function. Evidence for this has come mainly from two sources. Firstly, studies of immune responses in man and experimental animals have revealed defects associated with abnormalities of iron metabolism. These may occur in iron deficiency and in iron overload. Secondly, specific events in the immune system are iron-dependent in vitro, or highly sensitive to excessive amounts or abnormal forms of iron. However, much of this area remains poorly defined, and, to some extent, controversial.

The major pathways of the immune system, together

with the possible effect of iron on the various steps are shown in Fig. 35.1.

Iron and immune function

The role of iron in lymphocyte function

A key event in activation of the immune system is the clonal expansion of T- or B-lymphocytes after antigen stimulation. Evidence that iron is important for normal lymphocyte activation comes from studies demonstrating impaired cell-mediated immunity in iron-deficient animals or patients, and a failure of T-cells to undergo a normal proliferative response to polyclonal mitogens ex vivo[1, 2]. Clonal expansion requires a period of rapid cell division, a process for which iron is essential, particularly as a co-factor for ribonucleotide reductase[3], and possibly other enzymes. Lymphocyte activation is thus accompanied by a marked up-regulation of transferrin receptor expression[4] and increased uptake of transferrin-bound iron[5]. In the absence of transferrin-bound iron, lymphocyte proliferation is usually decreased by 50–90%[6, 7], although T-cells may utilize intracellular iron in the later stages of activation[8]. It is controversial whether the transferrin receptor has any function beyond mediating iron uptake. Lymphocyte proliferation does not occur in the presence of transferrin unless it contains iron[9]. Nevertheless, ligand-binding to the transferrin receptor can cause activation of transmembrane signaling molecules such as ZAP-70[10], suggesting a direct effect on cell signaling. The issue is further complicated by the fact that signaling through p33 (cdk2) kinase, important in cell cycling, is an iron-sensitive mechanism[11].

Studies that dissected the sensitization step from the activation step in T-cell responses in vivo have concluded that the latter, but not the former, is sensitive to iron deficiency[12, 13]. These observations support the critical role for iron in the proliferative step. Indeed, iron deprivation also leads to apoptosis of both B- and T-lymphocytes[14, 15]. Nevertheless, a role for iron in other stages of lymphocyte activation cannot be excluded, and iron deficiency reduces activation of protein kinase C in T-lymphocytes by approximately 50%[16].

The requirement for iron is a late event in the activation-proliferation sequence after stimulation of T-cells with lectin-type mitogens such as phytohemagglutinin, and is usually dependent on the induction of transferrin receptor expression via the interaction of interleukin-2 (IL-2) with its receptor[17, 18]. The most obvious explanation for this is that IL-2 induces metabolic activity that rapidly depletes the iron reserves of the resting T-cell, resulting in a post-transcriptional up-regulation of the transferrin receptor through the iron-regulatory protein/iron-responsive element (IRP/IRE) system. However, if other mitogenic stimuli such as phorbol esters are employed, transferrin receptor expression is induced much earlier and without a requirement for IL-2[19, 20]. Iron may also influence other aspects of T-cell function. Maturation and differentiation may be iron-dependent, because blockage of iron uptake by thymocytes with an anti-transferrin receptor monoclonal antibody impaired maturation of $\alpha\beta$ T-cells[21]. However, development of $\gamma\delta$ T-cells was not affected, suggesting that this latter subset might possess alternative iron uptake mechanisms.

Early expression of transferrin receptors also occurs in stimulated B-cells[22, 23], and this is detectable 3–4 h after activation. B-cells, like T-cells, require delivery of transferrin-bound iron for proliferation[18, 24]. However, B-cells stimulated with staphylococcal protein A synthesized immunoglobulin prior to transferrin receptor expression[25], suggesting that antibody production might be relatively independent of iron supply. There is evidence that B-cells can utilize alternative forms of iron more readily[26]. This might explain why clinical studies have tended to find that iron deficiency has less effect on antibody production than on T-cell function. Nevertheless, transferrin was essential for in vitro production of immunoglobulin E, and promoted a 40% increase in immunoglobulin M synthesis by blood mononuclear cells[27]. Inhibition by transforming growth factor-β (TGFβ) of immunoglobulin G production by mouse B-cells was associated with cell cycle arrest in the early G_1 phase and with blockage of transferrin receptor expression[28]. Iron may also be involved in lymphocyte cytotoxicity, because iron deficiency reduces the cytotoxic activity of lymphocytes[29]. However, the defect persists after correction of iron deficiency and, in the case of natural killer cells[30], may be an indirect effect due to reduced production of interferons by macrophages or T-cells[31,32].

Iron and macrophage function

Defects of macrophage function observed during clinical and experimental studies in iron deficiency suggest that iron may be important for cytokine synthesis[31, 33], although this may simply reflect a general reduction in protein synthesis. The tumoricidal activity of splenic macrophages in iron-deficient mice was also impaired by $\geq 50\%$[34]. This may be more specifically related to lack of iron, because this metal is required for cytotoxic mechanisms mediated by reactive oxygen and nitrogen compounds. Iron-

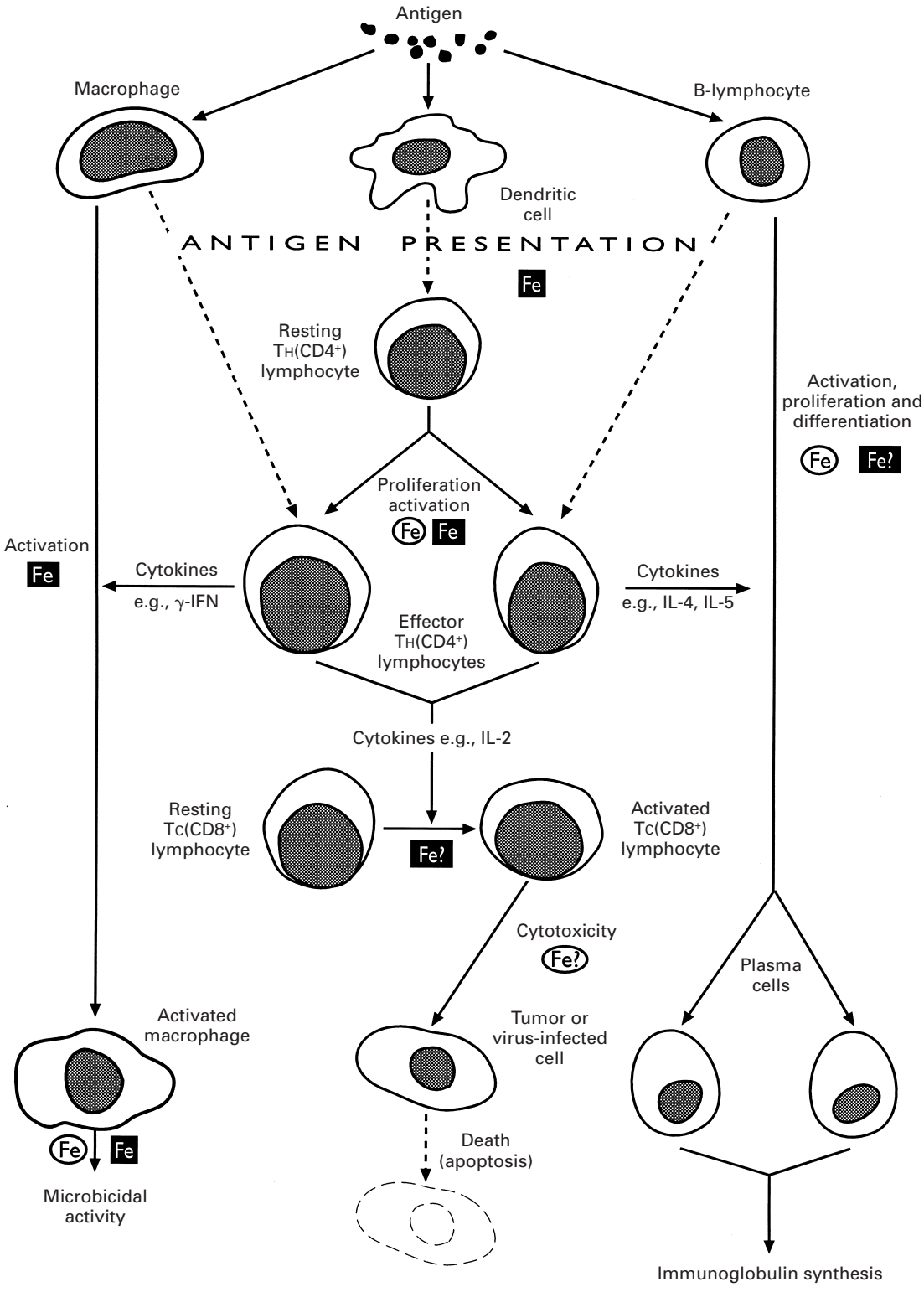

Fig. 35.1. An overview of the immune system and possible influences of iron. In processes marked Fe (encircled), iron may be necessary for proper function. For those marked Fe (in a black square), excess iron may be inhibitory.

depleted macrophages have reduced hydrogen peroxide release upon stimulation[35]. However, iron may also promote survival of intracellular pathogens. Given these opposing effects of iron, it is not surprising that the role of iron in the microbicidal activity of monocytes and macrophages is complex. In some situations, iron enhances activity. For example, the killing of trypanosomes by macrophages is increased by iron-lactoferrin, but not apolactoferrin[36], and iron-loaded *Staphylococcus aureus*[37] and *Borrelia burgdorferi*[38] were more susceptible to killing by hydrogen peroxide and by monocytes than iron-poor organisms. In contrast, *Histoplasma capsulatum*[39, 40], *Paracoccidioides brasiliensis*[41] and *Legionella pneumophila*[42, 43] were more readily killed when macrophage intracellular iron levels were low. Thus intracellular iron may need to be carefully regulated for optimal microbicidal activity and, indeed, killing of *Listeria* by macrophages is positively correlated with iron content at low macrophage iron levels, but is inhibited by the higher levels found in thioglycolate-elicited macrophages[44]. Overall, the evidence suggests that iron enhances killing of micro-organisms that are normally extracellular, but inhibits killing of intracellular pathogens, perhaps by permitting more rapid intracellular multiplication. Recent work suggests that *Nramp1* (natural resistance-associated macrophage protein 1), the product of a gene associated with resistance to infection by intracellular microorganisms, is a transporter of metal ions, including iron (II)[45]. *Nramp1* is associated with phagolysosome membranes and may act by pumping iron out of the phagolysosome and preventing microbial growth. A similar protein (*Nramp2* or DCT1) has been identified in the plasma membrane of mucosal cells and may play a role in iron absorption[46].

Reactive nitrogen compounds are important in macrophage-mediated killing of many microorganisms[47] and in the tumoricidal action of activated macrophages. One mechanism by which these compounds mediate their effect is by destroying iron-sulfur clusters in enzymes of the target cell, and perhaps by inactivating other types of iron-proteins[48, 49]. Nitric oxide production is thus an important component of macrophage cytotoxicity and its activity is clearly linked to cellular iron metabolism, a link that has been reinforced by the demonstration that iron down-regulates inducible nitric oxide (iNOS) gene expression[50, 51]. Furthermore, nitric oxide can regulate cellular iron metabolism via iron responsive elements in transferrin receptor and ferritin mRNA[52]. Iron is reported to be important for activation of the transcription factor NFkB that plays a key role in up-regulating expression of various macrophage-derived inflammatory response cytokines such as tumor necrosis factor-alpha and interleukin-6[53]. This effect is probably linked to iron-mediated generation of reactive oxygen compounds[54].

Iron and neutrophil function

Clinical and experimental studies of neutrophil function in iron deficiency indicate that phagocytic uptake is generally normal, but that bactericidal activity is impaired[55, 56]. The latter appears to be due to impairment of the myeloperoxidase-halide system[57, 58]. Why iron is important for the myeloperoxidase system is not established, but it is likely due to the fact that myeloperoxidase is an iron-containing enzyme.

The effect of iron overload on immune function

Hemochromatosis can potentially affect the immune system in two ways. Firstly, the associated iron overload exposes the system to higher levels and/or different chemical forms of iron to those encountered under normal conditions, and this may lead to altered function. Secondly, iron overload may facilitate iron availability to microorganisms, the growth of which under normal circumstances is limited by the host iron-withholding mechanisms. However, the identification of the hemochromatosis gene product as a mutation in a β_2-microglobulin-binding HLA-like molecule, called initially HLA-H and subsequently *HFE*[59], opens up other possibilities. HLA molecules play key roles in many immune functions such as antigen presentation. Immune functions have not yet been specifically ascribed to *HFE*, the molecule associated with hemochromatosis. However, it seems likely that such functions may be reported, especially because the C282Y mutation of *HFE* present in hemochromatosis patients abrogates binding of β_2-microglobulin and trafficking of the molecule to the cell surface[60]. A key feature of hemochromatosis is the relative sparing of macrophages from iron overload, suggesting that they fail to regulate iron release. This might be a consequence of high-level expression of defective *HFE*. However, there is scant evidence from clinical studies for any immunological (as opposed to iron-handling) abnormality of macrophage function in patients with hemochromatosis.

Assessment of the effect of iron overload on immune function comes not only from studies of hemochromatosis, but also from those of secondary iron overload (e.g., in thalassemia) and from animal studies. However, caution is needed in extrapolating from thalassemia to hemochromatosis: patients with thalassemia often receive chelation therapy, and it is important to distinguish between

Table 35.1. Effect of iron overload on immune function

Effect	Possible mechanisms	References
Inhibition of lymphocyte proliferation	Membrane lipid peroxidation	66
	Polymerized iron on cell membrane	74
	Increased ferritin binding	86
Alterations of lymphocyte cell-surface markers	See Chapter 38	
Impaired natural killer cell activity	Not known, possibly a secondary effect	68
Decreased T-cell cytotoxicity	Impaired interleukin-2 production	64
Antigen presentation	Lipid peroxidation	79
Neutrophil intracellular killing	Uptake of non-transferrin-bound iron	106
Macrophage antimicrobial and antitumour activity	Inhibition of H_2O_2 production	94
	Interference with nitric oxide activity	98
	Impairment of γ-interferon activity	100
	Iron binding to cell membranes	103

genuine effects of iron overload and epiphenomena such as the effects of splenectomy and repeated blood transfusions. Animal models of iron overload have also been developed, but these have been used mostly for testing the efficacy of iron chelators, rather than the effect of iron overload on immune function. The main effects of iron overload on parameters of immune function, derived from both clinical and animal studies, are summarized in Table 35.1.

Studies of the effect of iron overload on the immune system have focused particularly on its effect on lymphocyte subpopulations. Clinical studies of immune function have often yielded inconsistent results. Both normal and depressed in vitro responses to mitogens by B- and T-cells from hemochromatosis patients have been reported by the same investigators, although spontaneous immunoglobulin production by B-cells was increased[61, 62]. Reduced p56lck kinase activity associated with CD8+ T-cells, but not CD4+ T-cells, has been reported in hemochromatosis, and was not corrected by therapeutic phlebotomy[63]. This suggests a direct effect of the mutation of the *HFE* gene and alteration of the *HFE* protein. In mice with experimental iron overload, T-cell responses to concanavalin-A were impaired by 15–50%, though IL-2 production was examined, rather than the proliferative response[64]. Contact sensitivity responses were also impaired[65]. Similar findings in iron-overloaded rats have been ascribed to increased lipid peroxidation[66].

Impaired natural killer cell activity has also been reported in iron-overload disease, but only in β-thalassemia[67–69], and not hemochromatosis[70]. Furthermore, the defect in thalassemia was not corrected by chelation therapy[68], and decreased cytotoxic T-cell activity reported in spleen cells from iron-overloaded mice[64] was due to impaired production of IL-2, suggesting an indirect effect. Thus evidence that iron overload or hemochromatosis impair natural killer activity is inconclusive.

The impairment of lymphocyte function reported in iron overload may be due to the inhibitory effect of non-transferrin-bound iron that is often present in the serum of these patients. Iron salts or hydrophilic iron chelates such as iron nitrilotriacetate or iron citrate do not stimulate lymphocyte proliferation and may be inhibitory[71, 72]. This is possibly due to polymerization and binding of these compounds to the cell membrane[73, 74], with resulting toxic effects. Alternatively, excess uptake of iron might be mediated via *Nramp2*/DCT1[46]. Moreover, the inhibitory effect of excess iron on T-cell cloning efficiency was more pronounced on murine CD4+ memory cells and CD8+ cytotoxic cells than on CD8− helper cells[75], due in part to an increase in T-suppressor function.

In humans, non-transferrin-bound iron preferentially reduced the in vitro cloning efficiency[76] and proliferation[7] of CD4+ T-cells. These observations may explain the consistent reduction in CD4+ T-helper cells in patients with iron overload. In addition, iron may influence the relative development of Th1 and Th2 CD4+ cells, though its effects are not clear. On the one hand, iron deprivation inhibited proliferation of cloned murine Th1 helper cells more than Th2 clones[77], but, in an animal model, iron overload depressed Th1-mediated anti-fungal activity[78]. A further mechanism by which iron overload may depress lymphocyte activation is impairment of antigen presentation, inhibitable by iron or heme compounds through a lipid peroxidation-mediated effect[79].

Lymphocytes, particularly when activated, can bind ferritin[80, 81]. This appears to involve specific receptors[82–84] that preferentially bind the heavy (H) subunit. In contrast to the

stimulatory effect of transferrin, ferritin has an inhibitory effect on mitogen-stimulated T-cell proliferation[85, 86], probably due to its iron content[87]. Ferritin may have an immunosuppressive effect in vivo[86]. Thus ferritin, particularly molecules containing predominantly H subunits, may have an immunoregulatory role, and high concentrations of serum ferritin could contribute to impaired lymphocyte activity in iron overload. In unstimulated peripheral blood lymphocytes, ferritin binding occurs mainly to B-cells rather than to T-cells[80], though no clear effect of ferritin on antibody production has been reported.

H-rich ferritin has been implicated as an inhibitor of myelopoiesis[88, 89]. Acidic (i.e., H-rich) ferritin released by leukemia cells[88] or monocytes[90] inhibits in vitro colony formation of myeloid precursors. Involvement of the ferroxidase activity of ferritin[91] and stimulation of production of an endogenous cytokine-like molecule that acts in an autocrine manner on the precursors themselves[92] have been suggested as possible mechanisms. However, it is unclear whether these effects are important in iron overload, as opposed to neoplasia, and the anti-myelopoietic effect of ferritin is controversial[93].

The monocyte/macrophage system is also susceptible to excess iron. Ingestion of erythrocytes or iron loading of macrophages impairs in vitro hydrogen peroxide release[94, 95] and tumoricidal activity[96, 97]. In addition, excess iron reverses the microbicidal activity of reactive nitrogen compounds[98]. The ability of nitric oxide to inhibit viral replication is likewise reversed by iron[99]. Excess iron reduces the activity of gamma-interferon[100] and may impair phagolysosomal fusion[101]. Thus iron overload has the potential to impair protective functions of the monocyte–macrophage system seriously. However, the apparent sparing of the monocyte/macrophage system from iron loading in hemochromatosis suggests that these in vitro effects might not occur in vivo. Indeed hemochromatosis monocytes have unusually high iron-regulatory protein activity[102], suggestive of relative iron deficiency. Defective macrophage antimicrobial function has nevertheless been reported in hemochromatosis patients. Impaired in vitro phagocytosis of *Staphylococcus aureus* by monocytes from persons with iron overload[103, 104] and was corrected after phlebotomy therapy[103]. However, it is also possible that the effect was extracellular and mediated by non-transferrin-bound plasma iron.

Abnormalities in neutrophil function associated with iron overload seem to occur mainly as a result of hypertransfusion. In transfused renal failure patients undergoing hemodialysis, intracellular killing mechanisms of neutrophils were impaired[105, 106]. This is probably due to non-transferrin-bound iron, because the defect was abolished by adding desferrioxamine to the patients' sera[107]. Despite the fact that such an effect could operate in iron-overloaded hemochromatosis patients, their neutrophil function appears to be normal[103, 104].

Hemochromatosis, immune function and infection: clinical findings

Despite the overwhelming evidence that iron plays a role in immune function and, more specifically, that iron overload can impair various mechanisms, surveys have not generally reported infections to be a major cause of morbidity and mortality in hemochromatosis[108, 109]. Iron overload may sometimes be a risk factor for zygomycosis[110], and occasional cases of septic shock have been reported in patients with hemochromatosis[111, 112]. Infections by *Vibrio vulnificus* have also been reported in hemochromatosis patients[113], as has infection with *Yersinia enterocolitica*, an opportunist organism often associated with iron overload[114]. There have also been occasional reports of malaria in non-endemic areas associated with hemochromatosis[115, 116]. A reevaluation of the seminal work of Strachan[117] on the causes of death of iron-overloaded black South Africans revealed that tuberculosis was a significant cause of death[118], though there appears to be no such association with 'classical' hemochromatosis in Caucasians.

The effects of iron overload on the immune system and resistance to infection may be indirect. For example, depressed cell-mediated immunity might contribute to the high incidence of hepatocellular carcinoma in hemochromatosis patients, and increased susceptibility to minor, non-life-threatening infections may go unreported. Indeed, it has been suggested that septicemia is often an unrecognized terminal event in persons with iron overload[119]. Because iron affects both immune function and microbial growth, the possibility that hemochromatosis patients are immunocompromised should always be borne in mind.

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Bacterial infections in hemochromatosis

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Introduction

The availability of iron for pathogenic organisms plays a critical role in infection. In normal tissue, iron is not readily available because plasma and other tissue fluids contain unsaturated iron-binding proteins. These are transferrin in plasma and lymph, and lactoferrin in external secretions such as milk and mucous. These proteins ensure that the concentration of free iron in these fluids is virtually zero. This is essential for the bactericidal and bacteriostatic properties of plasma and other tissue fluids, and has a marked influence on overall resistance to infection.

Hemochromatosis is one of many disorders in which susceptibility to bacterial infection is increased by the presence of freely available iron, and this may occur for a number of reasons. Iron overload that develops in many persons with hemochromatosis is one reason. Other sources of freely available iron include iron from hemoglobin freed by the lysis of erythrocytes, and iron released from traumatised tissue. Each of these circumstances leads to the same result: iron-replete bacteria are no longer susceptible to the bactericidal or bacteriostatic mechanisms present in tissue fluids. This allows rapid extracellular bacterial growth with a great increase in bacterial virulence[1].

The importance of iron-binding proteins

The iron-binding protein transferrin present in normal human plasma and lymph is ~30% saturated with ferric iron. Its high association constant for Fe^{3+} ($\sim 10^{36}$) ensures that the concentration of free ferric iron in tissue fluids is $\sim 10^{-18}$ M, which may be regarded as virtually zero[2]. Complement, specific antibody, and unsaturated transferrin comprise the bactericidal and bacteriostatic systems in plasma. These components, acting in concert, are often extremely effective, particularly against Gram-negative bacteria, but can also operate against some Gram-positive bacteria and fungi[2]. The killing or inhibitory process involves direct interference with the organism's metabolism, and can occur rapidly. For example, net synthesis of bacterial RNA was depressed within 20 minutes after the addition of specific antiserum to *P. multocida* growing in fresh serum. Bacterial RNA synthesis was blocked completely after 40–50 minutes, followed eventually by the cessation of protein synthesis. The iron-binding capacity of unsaturated transferrin is essential for this process, because the bacterial killing can be reversed by adding enough ferric iron to saturate transferrin, or by adding a heme compound such as hematin that is not bound by transferrin[3,4]. Furthermore, similar inhibition of macromolecular synthesis occurs in *Escherichia coli* after exposure to serum[5,6]. This demonstrates that similar antibacterial mechanisms can affect the growth of more than one species of bacteria.

Bacterial virulence and iron availability

In the normal, iron-restricted environment in vivo, pathogenic bacteria employ a wide variety of mechanisms for acquiring essential iron. These include soluble iron chelators that can remove iron from unsaturated transferrin such as enterobactin secreted by *E. coli, Klebsiella pneumoniae,* and *Salmonella typhimurium.* Other bacteria, e.g., *Neisseria meningitidis,* that do not produce siderophores can remove iron directly from transferrin. In addition, some bacteria, such as *Yersinia pestis,* can utilise the iron from heme compounds even when these are present in minute concentrations[7]. Others, like *E. coli,* may be able to utilize freely available heme iron, such as that from modified hemoglobin intended as a blood substitute[8].

Thus, bacteria have a wide variety of devices available for the acquisition of iron in vivo, and there are detailed reviews on this topic[9, 10].

In pathological conditions, the presence of freely available iron does not appear to interfere directly with the power of individual phagocytic cells to ingest and kill bacteria. However, the presence of freely available iron does abolish bactericidal and bacteriostatic systems in plasma and fresh serum. This is true for many organisms, including *E. coli, K. pneumoniae, N. gonorrhoea, Yersinia enterocolitica, Y. pestis, Pseudomonas aeruginosa, P. multocida, Vibrio vulnificus, Clostridium perfringens, Candida albicans,* and others[1, 10].

The virulence-enhancing effect of freely available iron can be very large. For example, the lethal intraperitoneal dose (LD_{100}) of *E. coli* O111 for guinea-pigs was approximately 1×10^8 bacteria. The lethal dose after injecting hemoglobin intraperitoneally with the bacteria was 3×10^4 bacteria, approximately a 10000-fold increase in virulence. Saturation of circulating transferrin with ferric iron resulted in a lethal dose of 3×10^3 bacteria, an increase in virulence of ~100000-fold; only 300 organisms caused deaths of 80% of the test animals. Thus, injection of Fe^{3+} almost completely overcame the usually high resistance of normal guinea-pigs to *E. coli* infection[11]. There are other examples, including abolition of the protective power of specific antiserum against *P. multocida* and *C. perfringens*[1, 10].

When iron is freely available, the normal destruction or inhibition of bacteria in extracellular fluid such as plasma can be reversed suddenly[12]. In these circumstances, the generation time in vivo of organisms such as *E. coli*[8, 11], *P. multocida*[13], or *C. perfringens*[14] may be $\leq$30 minutes, and results in rapid bacterial invasion. Both ferric iron and heme compounds are effective. These observations suggest that once the rate of bacterial growth in extracellular fluid exceeds the rate of overall phagocytosis, resistance to infection inevitably fails[1]. If this hypothesis is correct, it would account for the very large increase in bacterial virulence seen in many experimental infections when iron is freely available, and for the sudden development of septicemia due to organisms such as *V. vulnificus* and *Y. enterocolitica* observed in some clinical infections.

Clinical infections in hemochromatosis

Vibrio vulnificus

Iron overload in hemochromatosis is characterised by increased saturation of the plasma transferrin with iron. In addition, there is often a pool of freely available low-molecular-mass iron in the plasma that can be detected by the 'bleomycin' assay at pH 7.4 (non-transferrin-bound iron)[15]. 'Bleomycin' iron is not detectable in normal persons. In persons with hemochromatosis, iron also becomes more available as the pH of plasma or other body fluids is lowered, suggesting that some of the iron is less tightly bound to transferrin than in normal persons. For example, five patients with hemochromatosis undergoing therapeutic phlebotomy had transferrin saturation values of 49–95% (mean 83%). In plasma samples, one had 0.48 μmol 'bleomycin' iron/l released at pH 7.4, two had 0.21 and 0.68 μmol/l 'bleomycin' iron released at pH 6.3, respectively, and all five released 0.63–17.4 μmol 'bleomycin' iron/l at pH 5.3. This does not occur in normal persons[15, 16].

In vitro cultures of *V. vulnificus* added to clotted blood from normal persons were rapidly destroyed, but rapid growth occurred in the blood of all five hemochromatosis patients. In addition, *V. vulnificus* was destroyed by normal plasma, but grew readily in three out of four of the hemochromatosis plasma samples (the fifth could not be tested). The hemochromatosis plasma sample that killed the bacteria had the iron bound tightly to transferrin, as measured by the 'bleomycin' test[16]. *V. vulnificus* does not have a high-affinity iron uptake system and is unable to acquire iron from normal tissue fluids such as plasma[17]. It can be highly virulent when free iron becomes available. With one strain, the median lethal dose, (LD_{50}), for 6–8-week-old mice was reduced from 6×10^6 to 1×10^0 after intraperitoneal injection of 80 μg of ferric ammonium citrate, an increase in virulence of $>$100000-fold[18]. This confirmed that a relatively high degree of resistance to bacterial infection in normal animals could be eliminated by the injection of iron.

Clinical infections with *V. vulnificus* occur in two essentially different forms: primary septicemia, and local wound infections. In 24 persons with primary *V. vulnificus* septicemia, abrupt onset of chills, fever, and prostration occurred singly or in combination[19]. Eighteen patients developed secondary cutaneous lesions, usually within 36 h. These lesions consisted of ecchymotic areas, vesicles, and necrotic ulcers. *V. vulnificus* was isolated from the blood of 20 patients, and from 10 cutaneous lesions. Eleven patients died within two weeks of the onset of illness or hospitalisation. Eighteen patients (75%) had pre-existing hepatic disease. This included four persons with hemochromatosis, and fourteen patients with varying degrees of hepatitis, including some with hepatic cirrhosis. Of the remaining six patients, three had histories of alcohol abuse, one had thalassaemia, and one had diabetes mellitus. One had no apparent underlying disease.

In a review of 30 cases of *V. vulnificus* infection, 18

patients had primary septicemia, and *V. vulnificus* was isolated from the blood of each patient[20]. Symptoms and predisposing causes were similar to those previously described[19]. Thirteen patients (72%) had a history of liver disease (nine due to alcohol abuse, two due to chronic hepatitis, one due to hemochromatosis, and one due to alcoholic liver disease and the presence of serum hepatitis B surface antigen). In two patients with alcoholic cirrhosis who developed sudden peritonitis and sepsis with *V. vulnificus*, the organism was isolated from skin lesions and ascitic fluid[21].

In 15 persons who developed wound infections with *V. vulnificus* associated with contact with sea water, five patients had serious underlying disease: two had diabetes mellitus, one was an alcoholic, one had congestive heart failure, and one had chronic lymphocytic leukemia and diabetes mellitus[19]. In 12 other cases of *V. vulnificus* infection, three developed a bacteraemia and had cirrhosis and hemochromatosis[22]. Nine had wound infections associated with contact with contaminated sea water[22]. Two of these patients had cirrhosis, one hemochromatosis, one leukemia, one diabetes mellitus, and one had steroid-dependent asthma, three with no underlying disease. In nine similar patients, eight reported exposure of the skin to sea water or shellfish[20]. In this report, there was no statistically significant relationship of the infections with underlying disease.

How are these infections related to the availability of iron? *V. vulnificus* does not grow in human plasma unless iron is readily available[16, 17]. For example, a virulent strain of *V. vulnificus* grew in the fresh serum of an alcoholic patient who had transferrin saturation of 98%, but failed to grow in normal serum unless the transferrin saturation was raised to $\geq 74\%$, even though serum complement had been inactivated[23]. Thus, both experimental and clinical evidence strongly suggest that readily available iron is a crucial factor in the development of primary septicemia.

With wound infections, the presence of underlying disease sometimes appeared to be important, but there was a close correlation between infection and contamination of local injuries with sea water. Iron in hemoglobin from lysed red blood cells could be responsible for initiating infection after trauma, especially if a wound were contaminated with sea water. In addition, *V. vulnificus* produces a cytotoxic hemolysin[24] that could make hemoglobin iron more readily available in a wound. In experiments with mice given intraperitoneal injections of *V. vulnificus,* lethality was directly related to the concentration of hemoglobin in the plasma, although some increase in susceptibility lasted beyond the time when hemoglobin levels had returned to normal[25].

In many of these human cases, the source of *V. vulnificus* infection appeared to be shellfish, particularly oysters. *V. vulnificus* has been isolated from warm coastal waters in the USA, and may be widespread in sea water and oysters[26]. Blake et al. recorded that all 19 patients with primary *V. vulnificus* septicemia often ate raw oysters[19], and 13 of 15 other persons with primary septicemia had eaten raw oysters in the two weeks before illness[20].

Other *Vibrio* species

Several other *Vibrio* species can be pathogenic for man. In addition to *V. cholerae* O1 which is of major public health significance, other pathogenic *Vibrio* species include *V. parahaemolyticus* frequently associated with gastroenteritis. *V. cholerae* non-O1 is associated with gastroenteritis and occasional extraintestinal infections in patients with underlying diseases such as hepatic cirrhosis or diabetes mellitus. *V. alginolyticus* has been described as a cause of septicemia after severe burns, and *V. damsela* has been associated with wound infections in persons in whom there was no evidence of underlying disease[27]. In hemochromatosis, no *Vibrio* species appears to be as potentially pathogenic as *V. vulnificus*.

Yersinia enterocolitica

Y. enterocolitica has all the attributes of virulence except the ability to acquire iron readily from normal tissues. It does not appear to possess a high-affinity iron uptake system[28], and cannot grow in normal human serum[29] or in the presence of unsaturated transferrin[30]. Its virulence is increased by the availability of free iron, particularly if the iron chelator desferrioxamine is also present. For example, the LD_{50} for mice of one strain of *Y. enterocolitica* was $>10^8$ bacteria. Five mg of iron as iron dextran reduced the LD_{50} ten-fold. With 5 mg of desferrioxamine, the LD_{50} was reduced $>100\,000$ fold, and with iron and desferrioxamine the LD_{50} was <10 organisms[30]. Thus, the injection of iron compounds virtually eliminated normal resistance to *Y. enterocolitica* infection.

In normal persons, *Y. enterocolitica* appears to be relatively avirulent, and infection usually takes the form of self-limited gastroenteritis. Marks et al. described 181 cases of *Y. enterocolitica* enteritis among 6346 children (2.8%) whose mean age was 24 months[31]. Their symptoms included diarrhea, fever, abdominal pain, and vomiting. The organism was isolated from all 118 patients. Similar circumstances occurred in 14 infants who were probably infected by eating pork products; there were no deaths[32].

Generalized infections with *Y. enterocolitica* are almost

invariably associated with iron overload or increased iron availability. Some of the most striking examples of septicemia have occurred when desferrioxamine was used to treat acute or chronic iron overload. For example, a 15-month old boy was given desferrioxamine intramuscularly after eating approximately 30 tablets of ferrous sulphate. Severe fever and bloody diarrhea developed on the second day. *Y. enterocolitica* was cultured from the blood and feces on the fourth day. The patient's twin sister, who had not ingested iron and was not treated, also harbored *Y. enterocolitica* in her intestinal tract but suffered only mild diarrhea[33]. This particular case closely parallels the results of experimental infection in mice described above.

Hemochromatosis and hepatic disease are frequent predisposing causes of *Y. enterocolitica* infection. Rabson, Hallett, and Koornhof described seven cases of acute septicemia, and six of subacute local infections with hepatic or splenic abscesses[34]. Of those who developed septicemia, three had hepatic cirrhosis, one possibly had siderosis, one had thalassaemia, one had kwashiorkor, and one had hyperplastic kidneys. Although all were very ill, only one succumbed to infection. Among the six subacute cases, all had hepatic cirrhosis and four also had hemochromatosis. There were five deaths among these patients.

Sometimes the diagnosis of spontaneous *Y. enterocolitica* infection results in the recognition of associated hemochromatosis. For example, a patient presented with a two-month old history of abdominal pain, diarrhea, and fever. *Y. enterocolitica* was isolated from the patient's ascitic fluid, and a hepatic biopsy specimen contained large deposits of hemosiderin in the hepatocytes and fibrous tissue[35]. The patient's plasma transferrin was 89% saturated with iron, and the hepatic iron concentration was 51.7 μmol/100 mg dry weight of liver (normal $<$3.6 μmol/100 mg dry weight)[35]. In three similar cases, one patient with acute abdominal symptoms had multiple liver abscesses from which *Y. enterocolitica* was isolated in pure culture. The serum transferrin was 97% saturated, and hepatic biopsy specimens revealed hemochromatosis[36]. Two other patients had a history of fever with cultures of *Y. enterocolitica* isolated from hepatic abscesses. A liver biopsy specimen indicated the diagnosis of hemochromatosis in one patient, and the same diagnosis was confirmed two years later in the second patient when the transferrin saturation was 87%[36]. In an unusual case, a 42-year-old diabetic woman with fever, headache, polydipsia, and confusion developed a subcutaneous abscess of the skull from which *Y. entercolitica* was isolated; blood cultures were also positive for *Y. entercolitica*. The diagnosis of hemochromatosis was confirmed by hepatic biopsy[37].

Hepatitis and cirrhosis

In alcoholic cirrhosis there are varying degrees of iron overload in the liver, although this rarely approaches the levels found in hemochromatosis[38]. Among 42 alcoholic patients, there was an increase in stainable liver iron in 70% of cirrhotic patients, and in 32% of those whose livers had histologic features of alcoholic hepatitis[39]. Serum ferritin concentrations were invariably elevated, but were not related to stainable iron in the bone marrow[39]. Iron and alcohol may have a synergistic effect that could explain the early onset of hepatic cirrhosis in patients who have iron overload and an excessive alcohol intake[40]. Among 12 patients who consumed $>$80 g of alcohol daily for $\geq$3 months, seven had high levels of iron saturation of their plasma transferrin (47–98%; mean 60%)[23].

Although persons with hemochromatosis are predisposed to have infections, all forms of hepatic disease have a similar effect. Wyke records that bacterial infection was responsible for one-quarter of all deaths in patients with hepatic disease; persons with alcoholic cirrhosis were the most susceptible[41]. The occurrence of spontaneous bacterial peritonitis appears to be confined largely to patients with hepatic cirrhosis[42, 43]. This form of infection accounts for 5–30% of all infections in patients with hepatic cirrhosis, and 60–75% of all serious infections[44]. The death rate can be high. Of 43 patients with spontaneous bacterial peritonitis, 17 died within 7 days of diagnosis, and 13 died between 10 and 102 days[45]. The disease is often a recurrent process, because 51% of 75 patients had more than one episode of infection[46]. The majority of infections were caused by Gram-negative bacteria, especially *E. coli*. Other organisms included *Streptococcus pneumoniae, Staphylococcus* sp., *Bacteroides* sp., and *C. albicans*[41, 44, 47, 48].

Diabetes mellitus is another condition associated with increased susceptibility to infection, but more study is needed to understand the mechanisms involved in infection in diabetic patients (with or without hemochromatosis). The blood pH of patients with diabetic ketoacidosis is subnormal, and this may favor the proliferation of *Rhizopus oryzae* in plasma by making iron more readily available[49].

Conclusions

Hemochromatosis is but one disorder in which the abnormal availability of iron has a critical influence on the occurrence of infection. The increased availability of iron need not be associated with iron overload. For example, fatal

E. coli septicemia can occur when the infecting organisms acquire iron from hemoglobin released from red blood cells during peritonitis[8]. Another is the effect of a fall in Eh and pH in human plasma that can make normal transferrin iron readily available to *E. coli* and *K. pneumonia*e, and result in rapid bacterial growth. This could be important in trauma[12]. Other examples are not confined to bacteria. Patients with acute myeloid leukemia frequently have increased serum iron concentrations that are associated with their increased susceptibility to *C. albicans* infections. This fungus grows readily in patient's sera that are saturated with iron, but it will not grow in normal serum unless the transferrin is almost or completely saturated with iron[50]. Other examples include unexpected effects of therapy. For example, chemotherapy can increase iron availability. High doses of methotrexate for the treatment of acute lymphoblastic leukemia in children can lead to the release of iron into the plasma, raising the transferrin saturation to 100%[51]. In thalassaemia, splenectomy has the unexpected effect of raising already elevated values of transferrin saturation[52]. In conclusion, increased availability of iron is responsible for loss of resistance to certain infections in persons with hemochromatosis.

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Chronic viral hepatitis and hemochromatosis

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Introduction

The liver synthesizes transferrin and ferritin, and is the major tissue storage site of iron[1]. Consequently, iron metabolism may be altered in chronic hepatitis. Stored ferritin may be released into the circulation due to lysis of damaged hepatocytes, leading to elevated serum ferritin concentrations. Transferrin receptor expression by hepatocytes may be up-regulated as a consequence of chronic inflammation, and lead to increased iron absorption and hepatic iron. Chronic viral hepatitis may up-regulate hepatic production of transferrin receptor or ferritin directly and effect increased hepatic iron deposition. Thus, chronic inflammatory processes in the liver such as viral hepatitis may cause increased plasma and hepatic iron concentrations by a variety of mechanisms[1–3]. Ferrous iron promotes free radical formation and lipid peroxidation that mediates acute and chronic tissue damage[2]. Because iron status influences the immune response, primary iron overload disorders and hepatic iron overload have been linked to an increased risk of bacterial infections due to *Vibrio*, *Neisseria*, and *Yersinia*[2, 4]. Iron may act as a substrate for viral replication, and therefore patients with chronic viral hepatitis and iron overload may have increased viral replication. The iron-binding proteins lactoferrin and transferrin play an important role in the immune response to infection. Although the effect of lactoferrin on hepatitis C virus replication is unknown, lactoferrin inhibits replication of herpes simplex virus, cytomegalovirus, and human immunodeficiency virus in vitro[2]. Therefore, iron status in patients with chronic viral hepatitis may be important clinically due to its effects on hepatic inflammation and viral replication, and reducing iron stores may decrease hepatic injury in patients with chronic hepatitis. This chapter surveys the available data on the associations of hemochromatosis (and secondary iron overload) and chronic viral hepatitis, examines the degree, type, and clinical significance of iron overload in patients with hepatitis C, and reviews the role of iron reduction therapy in patients with chronic hepatitis. The relationship between hepatitis C infection and porphyria cutanea tarda is also reviewed.

Hemochromatosis and chronic hepatitis C

Several studies have suggested that there is a high prevalence of hepatitis C infection in patients with hemochromatosis. Piperno et al. studied the presence of antibodies to hepatitis B and C viruses, serum alanine aminotransferase (ALT) concentrations, hepatic histology, and total body iron stores (assessed by phlebotomy) in 78 Italian patients with hemochromatosis, none of whom had an obvious risk factor for hepatitis C infection[5]. All patients met traditional criteria for homozygous hemochromatosis by quantitative phlebotomy: stored iron exceeded 5 g in men and 3 g in women. 21% of patients had serum anti-hepatitis C antibody, a significantly greater frequency than in Italian blood donors[6, 7]. Evidence of chronic hepatitis was observed on hepatic biopsy in 10 of 16 (63%) hemochromatosis patients who had hepatitis C antibody. Furthermore, hemochromatosis patients with chronic hepatitis without hepatic fibrosis had a higher mean serum ALT concentrations than those with fibrosis or cirrhosis and those with normal hepatic histology (123 ± 63 U/l (S.D.) vs. 63 ± 42 U/l and 42 ± 28 U/l, respectively). All patients with histologic evidence of hepatitis and detectable hepatitis C antibody had hepatitis C virus (HCV) RNA in their sera. Hepatic iron loading and serum ferritin concentrations were greatest in hemochromatosis patients with fibrosis or cirrhosis, intermediate in those with hepatitis C without significant fibrosis, and lowest in

hemochromatosis patients with normal hepatic histology. Further, patients with chronic hepatitis had fibrosis at much lower concentrations of hepatic iron. Taken together, these observations suggest that chronic hepatitis and hepatic iron have a synergistic effect in causing hepatic fibrosis. Serum ALT concentrations became normal after iron depletion in all patients without chronic hepatitis, but in only two of the 16 (12.5%) of those who had chronic hepatitis. Although the prevalence of serum hepatitis B surface antigen in patients in this study was only 5%, hemochromatosis patients who were hepatitis C antibody-positive were more likely to have hepatitis B surface antibody .

The frequency of hepatitis B infection may also be increased in patients with hemochromatosis. In 1979, Felton et al. noted that iron promoted hepatitis B virus replication[6], and Lustbader et al. reported that there was a greater risk of chronic hepatitis B in persons with elevated serum ferritin concentrations[7]. Deugnier et al. found that the prevalence of anti-hepatitis B core antibody was significantly higher in 272 hemochromatosis patients (13%) than in 255 029 control blood donors[8]. However, the apparent associations between exposure to hepatitis B and C and hemochromatosis are difficult to explain, because parenteral exposure (unknown in most hemochromatosis patients) is generally necessary for acquisition of these viral infections.

A basis for the increased susceptibility of patients with iron overload to develop chronic viral infection has been sought. The increased prevalence rates may reflect a lower rate of spontaneous clearance of these viruses in iron-overloaded individuals. Iron overload may be associated with a deficient cellular immune response[9]. In hemochromatosis, levels of $CD8^+$ lymphocytes are decreased and $CD4^+/CD8^+$ ratios are increased[10]. Patients with hemochromatosis sometimes have decreased levels of antioxidant vitamins and evidence of increased lipid peroxidation, and these factors could also increase the risk of developing chronic viral hepatitis.

Iron overload in chronic hepatitis C

Mild or moderate iron overload is frequently observed in patients with chronic hepatitis C[2]. However, their hepatic iron concentrations are usually normal or minimally elevated, and the mechanism by which iron overload occurs is not well understood. Hepatic uptake of iron occurs through up-regulation of transferrin receptors or via non-receptor-mediated membrane transport (non-transferrin-bound iron)[11]. Hepatic transferrin receptor expression is normally down-regulated in persons with iron overload, including those with hemochromatosis[12, 13]. Up-regulation of transferrin receptor may occur in chronic viral hepatitis as a consequence of cellular proliferation or altered differentiation of cells involved in transferrin synthesis. Iron-responsive elements (IREs) alter expression of ferritin and transferrin receptor in accordance with cellular and body iron status. Hepatitis C virus could affect the structure or function of IREs or alter their biochemical behavior, although this is unproven.

Iron stores in patients with chronic viral hepatitis

Elevated serum ferritin concentrations in chronic hepatitis may reflect either increased total body iron stores, or increased release of ferritin from injured hepatocytes into the circulation[1]. Furthermore, ferritin is an acute phase reactant, and serum concentrations of ferritin may be increased as a non-specific marker of inflammation. Some authors have suggested that the ratio of the serum ferritin concentration: serum ALT concentration provides a more accurate assessment of total body iron stores in chronic hepatitis.

Arber et al. measured serum iron parameters and hepatic iron staining in 63 Israeli patients with chronic hepatitis C and in 14 with chronic hepatitis B[14]. 59% of hepatitis C patients had elevated serum iron concentrations, but serum ferritin concentrations were not significantly higher in the hepatitis C group. The mean transferrin saturation value was 50 ± 18 (S.D.)% in the hepatitis C group, 48 ± 16% in the hepatitis B group, and 28 ± 10% in control subjects with non-viral chronic hepatic diseases. Stainable hepatic iron was commonly observed in the hepatitis C group. Di Bisceglie et al. retrospectively reviewed serum iron studies in 80 patients with non-A, non-B hepatitis who had participated in α-interferon treatment trials (most of whom were subsequently found to have hepatitis C)[15]. They also prospectively examined biochemical and histologic markers of iron overload in 28 patients. Twenty-nine of 80 (36%) patients had elevated serum iron concentrations; 18% had serum transferrin saturation values >50%. Serum ferritin concentrations were elevated in almost half the men, but in less than 10% of women. There was stainable iron in hepatocytes and Kupffer cells in all but two patients. However, only 4 of 28 patients had an elevated hepatic iron concentration, and only two were in the range associated with homozygous hemochromatosis with a hepatic iron index (expressed as μmol Fe/g dry weight of liver $\div$ age in years) >2.0. Serum ferritin concentrations were directly correlated with serum aspartate aminotransferase (AST) and ALT concentrations ($p<0.00001$ and $p<0.02$, respectively). There was a correlation between the

ratio of the serum ferritin and AST concentrations, and the hepatic iron concentration. However, there was no significant correlation between hepatic iron concentration and serum ferritin concentration or transferrin saturation. Patients with more active necro-inflammatory disease on hepatic biopsy specimens showed a trend towards more iron loading. Similarly, Beinker et al. studied 30 patients with chronic hepatitis B and C. Among those with hepatitis C, there was minimal hepatic fibrosis in 77% of those with no stainable hepatic iron and in 24% with stainable iron; marked fibrosis was present in 56% with stainable iron and in 15% without stainable iron ($p<0.01$)[16]. Taken together, these data suggest that the presence of iron in the liver may exacerbate inflammation or fibrosis in patients with chronic hepatitis B and C.

Patients with chronic hepatitis C may have mild to moderate iron overload, but generally have normal hepatic iron concentration. Kowdley et al. studied serum and hepatic iron status in a large cohort of patients referred for evaluation of hepatic disease, including possible iron overload. Many patients with hepatitis C had elevated serum ferritin concentrations, and elevated serum transferrin saturation values were common. However, the mean hepatic iron concentration was normal in the patients with chronic hepatitis C ($n=228$)[17]. A 'threshold' hepatic iron concentration of 4000 mg Fe/g dry weight of liver distinguished patients with hemochromatosis from the others with one exception (Fig. 37.1). In contrast, Piperno et al. examined hepatic iron concentration in a large cohort of patients with chronic hepatitis B and C, and observed that their average hepatic iron concentration was significantly higher than that of control subjects[18]. In patients with chronic hepatitis, 'minor' elevation in hepatic iron concentration was present in 36%, and 'major' elevation (>3000 mg Fe/g) was detected in 9%. Five of 26 patients with 'minor' iron overload and four of nine who had 'major' iron overload had HLA-A3, a higher prevalence than would be expected in the general population[18]. However, HLA-identical siblings of two of these patients had no evidence of iron overload. The authors concluded that the mechanism for iron overload in hepatitis C is unknown, but may be related to heterozygosity for the hemochromatosis gene.

The putative gene associated with hemochromatosis[19], known as *HFE*, codes for an HLA class I-like molecule that is mutated in 80–85% of patients with phenotypic features of hemochromatosis. The 'major' mutation results in a cysteine to tyrosine substitution at amino acid 282 (C282Y), and 'minor' mutation is characterized by a histidine to aspartate substitution at amino acid 63 (H63D)[19]. Accordingly, the role of the C282Y mutation in modulating iron status in patients with chronic hepatitis C has been evaluated. Hezode et al. studied histologic evidence of

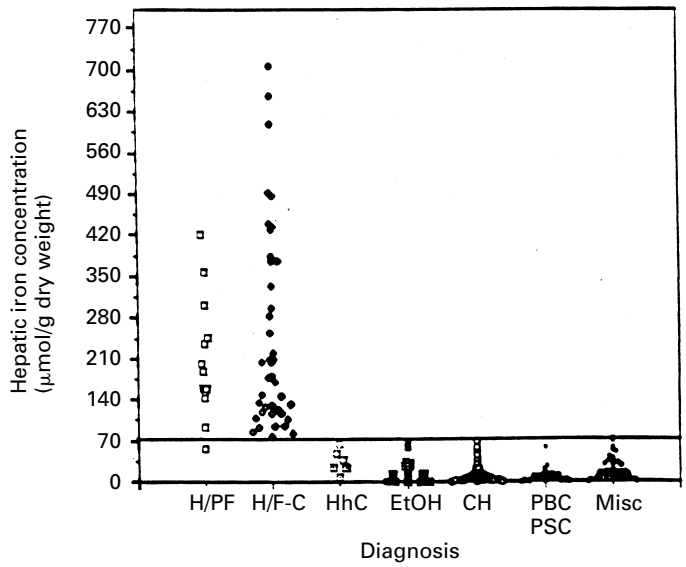

Fig. 37.1. Distribution of hepatic iron concentration among the various patient groups studied. H, hemochromatosis; PF, H without fibrosis on liver biopsy; H/F-C, H with fibrosis or cirrhosis on biopsy; HhC, heterozygous; EtOH, alcoholic liver disease; CH, chronic hepatitis; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; PBC and PSC; Misc, miscellaneous, histological diagnosis other than any of the above. (Adapted from ref.[17] with permission of the authors and publisher.)

hepatitis activity and hepatic iron staining in 133 patients with chronic hepatitis C[20]. Hepatic iron stained with Perls' technique was graded 0–3, and iron overload was defined as the presence of stainable liver iron. Although iron staining was found in 46% of patients, the C282Y mutation was not detected in 90% of patients. There was a trend towards a greater prevalence of heterozygosity for C282Y among patients with grade 1 staining than in those without stainable iron, but no data regarding serum iron parameters or quantification of hepatic iron was provided. In another study, the C282Y mutation was detected in nine of 76 patients with chronic hepatitis C. The prevalence of C282Y was slightly higher among women (14% vs. 11%), but the difference was not statistically different. There was no apparent relationship of histologic severity of hepatitis and the presence of the C282Y mutation. However, the serum ferritin concentrations in patients who were C282Y-positive had a trend towards higher values (279 mg/l vs. 146 mg/l); serum iron parameters and hepatic iron concentrations were not reported. The authors speculate that the presence of C282Y may be associated with some iron overload in persons with chronic hepatitis C[21].

Thus a significant proportion of patients with chronic hepatitis C, particularly men, have elevated serum iron

concentrations, serum ferritin concentrations, and transferrin saturation. Larson et al. examined the natural history of hepatic iron accumulation in chronic hepatitis C in 14 patients who had undergone paired hepatic biopsies; no patient was treated with α-interferon[22]. There was a trend towards a decrease in hepatic iron concentration over time, but no relationship between histological severity and hepatic iron concentration was apparent. Although inflammation and fibrosis in the liver progressed among the group between performance of the first and second hepatic biopsies, there was no accompanying increase in hepatic iron concentration. This demonstrates that iron accumulation in the liver in chronic hepatitis C is not secondary to necro-inflammatory changes or fibrosis alone.

Hepatic iron stores in end-stage hepatic disease due to chronic viral hepatitis

The hepatic iron concentration in most patients with chronic viral hepatitis is within the normal range, although a few patients have an increased amount of stainable and measurable iron in the liver. In contrast, hepatic iron loading may be significantly increased in patients with chronic hepatitis C who have end-stage hepatic disease, particularly when this is accompanied by alcoholic disease of the liver. Cotler et al. measured the serum transferrin saturation, hepatic iron concentration, and hepatic iron index in approximately 100 patients with end-stage hepatic disease[23]. Those with chronic hepatitis C had higher serum iron concentrations and higher transferrin saturation values than patients with non-viral diseases. Infrequently, the hepatic iron concentration and hepatic iron index were in the range typically associated with hemochromatosis, suggesting that these parameters may be non-specific, and therefore cannot distinguish hemochromatosis from end-stage chronic hepatitis C and alcoholic disease of the liver[23]. Patchy, variable iron deposition in the liver is frequently observed in patients with end-stage hepatic disease due to hepatitis C and alcoholism, and is probably the explanation for the occasional high values of hepatic iron concentration or hepatic iron index observed in such patients[24–26] (Fig. 37.1). However, the mechanism by which increased hepatic iron deposition occurs in end-stage hepatic disease is unknown.

Hepatic iron and response to α-interferon therapy in chronic hepatitis C

α-Interferon treatment is effective in 40–50% of patients with chronic hepatitis C, although sustained responses are uncommon with 24-week regimens[27]. Responses to α-interferon therapy may be related to hepatic iron stores. Van Thiel et al. first observed that the hepatic iron concentration may be a predictor of response to α-interferon therapy of chronic viral hepatitis[27], and subsequently studied 79 patients with chronic viral hepatitis treated with α-interferon (5 million units three times weekly). Hepatic iron concentration was significantly higher in non-responders than responders (1252 μg Fe/g dry weight of liver vs. 828 μg Fe/g dry weight of liver, respectively)[28]. However, all these values are normal (<2000 μg Fe/g dry weight of liver)[29]. There was no difference in transferrin saturation or serum ferritin concentration in patients who responded and in those who did not. Hepatic iron concentration was positively correlated with serum total iron-binding capacity and ferritin concentration, but no other significant differences between responders and non-responders were reported. Olynyk et al. examined hepatic iron concentration and response to α-interferon therapy in 58 patients with chronic hepatitis C[30]. Hepatic iron concentration was greater in non-responders than responders (860 ± 100 (S.D.) μg Fe/g dry weight of livers vs. 548 ± 85 μg Fe/g dry weight of liver, respectively), but all patients had an hepatic iron index < 2.0. Age, total α-interferon dose, or prevalence of hepatic cirrhosis did not differ significantly between the two groups. Tung et al. also observed that hepatic iron concentration was a predictor of response to higher doses of α-interferon therapy in patients with chronic hepatitis C who had high viral titers[31].

Other studies have found similar relationships between hepatic iron concentration and response to α-interferon therapy. Perez et al. performed a multivariate analysis of risk factors for non-response to α-interferon therapy in 48 patients with chronic hepatitis C[32]. Independent variables that predicted unresponsiveness to α-interferon therapy were elevated serum iron and ferritin concentrations. In 40 patients with chronic hepatitis C treated with α-interferon, Piperno et al. observed that non-responders had statistically higher serum ferritin concentrations (436 μg/l vs. 126 μg/l, respectively) and hepatic iron concentrations (1620 μg Fe/g dry weight of liver vs. 550 μg Fe/g dry weight of liver, respectively) than non-responders[33]. Fourteen of 15 (93%) of patients with elevated hepatic iron concentrations did not respond to α-interferon therapy. Some studies have not found a relationship between hepatic iron concentration and response to α-interferon therapy in chronic hepatitis C[34, 35]. The cumulative data on hepatic iron and response to α-interferon therapy have been reviewed[2] (Table 37.1).

The pattern of hepatic iron staining may be a predictive factor for response to α-interferon therapy in chronic

Table 37.1. Iron status and response to interferon in patients wth chronic viral hepatitis

		Number of subjects		Mean serum ferritin (ng/ml)			Mean HIC (μg/g dry wt)		
References	Disease	CR	NCR	CR	NCR	*p*	CR	NCR	*p*
Zein	HCV	10	29	187	283	NR	NR	NR	NR
van Thiel	HBV, HCV	32	47	329	376	>0.05	638	1156	<0.05
Olynyk	HCV	24	34	NR	NR	NR	548	860	<0.05
Izumi	HCV	65	NR	NR	NR	NR	429	875	<0.05
Clemente	HCV[a]	21	30	1812	2463	<0.015	11.8[b]	21.8[b]	<0.009
Barton	HCV	6	8	150	387	0.09	624	782	0.60
Banner	HCV	20	15	152	359	0.05	1248	548	0.24
Girelli	HCV	37	37	168	439	0.003	NR	NR	NR
Murakami	HCV	39	47	NR	NR	—	333 596[c]	753	<0.05
Ikura[d]	HCV	20	43	160	170	<0.5	1350[e]	1430[e]	>0.4

Notes:

Adapted from Bonkowsky[2] with permission of the authors and publisher.

Abbreviations: CR, complete remission; NCR, no complete remission; NR, not reported; HCV, hepatitis C; HBV, hepatitis B; HIC, hepatic iron concentration.

[a] All patients in this study also had transfusion-dependent thalassemia major.

[b] These values appear to be falsely low; perhaps the units should have been μmol Fe/g dry weight, in which case, the correct values (μg/g) would be 659 (CR) and 1 218 (NCR).

[c] HIC was 333 in sustained complete responders and 596 in complete responders who relapsed after therapy was stopped. Both were significantly lower than the mean value for non-responders.

[d] The degree of portal iron deposits correlated directly with hepatic inflammatory activity and fibrosis, and inversel with the response to interferon.

[e] HICs were given as median values in this study.

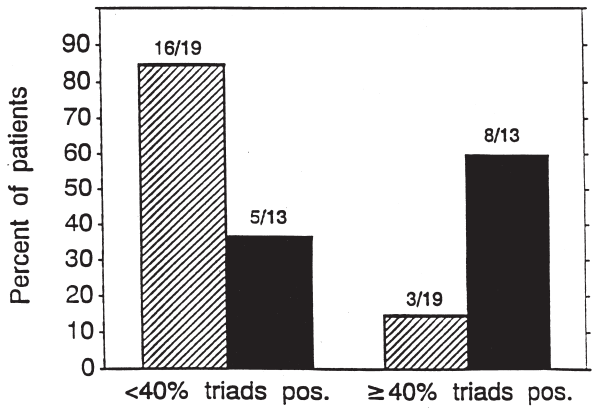

Fig. 37.2. The inverse association between the percentage of portal triads positive for stainable iron in liver biopsies and the response to IFN-α therapy in subjects with chronic hepatitis C. Biopsies with stainable iron in 40% or more of the triads were more frequent in the no complete remission (NCR) group to IFN-α, while biopsies with less than 40% of the triads positive were more frequent in the complete remission (CR) group ($p < .05$). Adapted from ref.[35] with permission of the authors and publisher. (Shading from A/W), CR; and (■), NCR.

hepatitis C (Fig. 37.2). Banner et al. studied 35 unselected patients who underwent α-interferon therapy and performed detailed histological assessments, including that of hepatic iron distribution[35]. Complete responders had fewer portal triads that contained stainable iron, and there was a positive correlation between the percentage of iron-positive triads and the presence of hepatic cirrhosis. Most of the iron was present in Kupffer cells. In contrast to the results of previous studies, there was no significant difference in hepatic iron concentrations among responders and non-responders, suggesting that lack of portal iron deposition was a good predictor of response to α-interferon therapy. Increased iron in the Kupffer cells could impair phagocytic defense mechanisms[33]. However, more aggressive viral hepatitis may lead to greater iron deposition in Kupffer cells. Other studies have supported the findings of Banner et al.[36].

Increased iron staining in portal triads of the liver in patients with chronic hepatitis C is probably a marker for more severe disease and thus lower response rates to α-interferon therapy. In contrast, one report suggests that there is a positive relationship between portal iron staining and response to α-interferon therapy, independent of viral titer. In 75 patients with chronic hepatitis C who had serum HCV RNA levels <1 million genome equivalents/ml, Akiyoshi et al. measured serum iron, ferritin, and hepatic iron concentrations and a quantitative histologic iron score[37]. There was a direct correlation between serum iron, ferritin, hepatic iron concentrations and the iron score, and a direct relationship between the iron score and response to α-interferon therapy.

Iron reduction therapy in chronic hepatitis C

Phlebotomy is used experimentally to treat chronic hepatitis patients[38–40]. Hayashi et al. studied ten Japanese patients who had chronic active hepatitis C with serum ALT >100 IU/l and evidence of hepatic iron deposition[38]. Serum ferritin concentrations were mildly elevated in five patients (>200 ng/ml), and moderately elevated in three (>300 ng/ml). Phlebotomy was performed to reduce the serum ferritin <10 ng/ml. The average quantity of iron removed over 2–9 months was 1.4 g (range 0.4–1.8 g). Serum concentrations of aminotransferases decreased in all patients; AST concentrations became normal in 50%, and ALT and AST concentrations became normal in 40%. Stainable iron was not observed in a second hepatic biopsy specimen obtained in seven patients. Inadequate information was provided to determine whether there was an effect of phlebotomy therapy on virologic response. Twenty patients with chronic hepatitis C who had previously failed a course of α-interferon therapy were studied by Tsai et al.[39]. An average of six phlebotomies of 500 ml each was required to render the patients iron depleted (range 1–14 phlebotomies). There was a significant reduction in mean serum ALT concentration. With α-interferon re-treatment after iron depletion, 15% of patients had a sustained response determined by persistent absence of HCV RNA in the serum[40].

Van Thiel et al. studied 30 patients with chronic hepatitis C who were randomized to receive α-interferon therapy (5 million units daily for 6 months), with or without weekly phlebotomy to maintain hemoglobin >11 g/dl[41]. There was greater histological improvement in the group treated with phlebotomy, and a greater proportion of patients in the phlebotomy group became negative for HCV RNA (79% vs. 59%, respectively). More patients who did not receive phlebotomy therapy relapsed after treatment was discontinued (59% vs. 25%). Deugnier et al. reported that there was a significant reduction in hepatic iron concentration in patients with chronic hepatitis C after treatment with α-interferon, regardless of virologic response[42]. The decrease was apparently due to a reduction in iron in Kupffer and endothelial cells of the liver, but elevated hepatic iron concentrations did not predict response to α-interferon therapy.

Iron reduction ameliorates inflammation in the liver in patients with chronic hepatitis C, and may be beneficial

due to its effect on lipid peroxidation and free radical-mediated injury. Hepatitis C patients had higher hepatic iron and serum ferritin concentrations, and higher hepatic levels of malondialdehyde and more evidence of increased glutathione turnover, suggesting increased free-radical mediated lipid peroxidation[43]. Whether α-interferon therapy has a role in the long-term management of patients with chronic viral hepatitis must await completion of long-term prospective trials examining the influence of iron reduction therapy on symptoms and hepatic fibrosis.

Hepatitis C and thalassemia

Homozygous β-thalassemia is associated with significant iron overload due to multiple erythrocyte transfusions and increased iron absorption. Thalassemia patients sometimes develop systemic manifestations of iron overload, including cardiomyopathy, diabetes mellitus, and hepatic cirrhosis. Among these patients, the risk to develop hepatitis C infection due to multiple erythrocyte transfusions is increased, and chronic viral hepatitis is prevalent. Angelucci et al. evaluated 556 thalassemia patients at a bone marrow transplant center, of whom 35% had antibody to hepatitis B[44]. Sixty percent of 156 patients tested using a second-generation assay had antibody to hepatitis C. Fifty-eight percent of 503 hepatic biopsies demonstrated some degree of chronic hepatitis classified as 'persistent' in 30%, 'mild active' in 27%, and 'severe active' in 1%[44]. More than 75% of the patients >11 years of age had fibrosis on hepatic biopsy specimens. Among patients >15 years of age who had undergone intermittent iron chelation therapy, the prevalence of fibrosis was ≥ 90%.

α-Interferon has been used to treat thalassemia patients with hepatitis C. Clemente et al. evaluated 65 children with chronic active hepatitis C treated with α-interferon (3 million units three times weekly for 6 months), including 14 children who were untreated[45]. The mean age of treated patients was 15 years. 41% of treated patients responded (normal values of serum ALT concentrations and disappearance of HCV RNA from serum), and 19 of 21 (90%) responders had significant histologic improvement in hepatic biopsy specimens. Hepatic iron and serum ferritin concentrations were significantly higher in non-responders than responders. Patients who had a transient reduction or disappearance of hepatitis C virus RNA from serum had lower hepatic iron concentrations than non-responders. Among iron-depleted subjects, a response rate of 76% was observed. Only two responders relapsed during one-year period of follow-up.

Di Marco et al. treated 70 patients (mean age 12.6 years) with β-thalassemia and chronic hepatitis C (90% HCV RNA-positive) with α-interferon for 12 months, and followed these patients off-treatment for 24 months[46]. 90% had detectable HCV RNA prior to therapy. HCV genotype 1b was detected in 41 patients (65%). Hepatic biopsy specimens revealed cirrhosis in 11 patients (16%) and grade 3–4 iron staining in 24 patients (34%)[46]. Serum concentrations of aminotransferases returned to normal and HCV RNA was cleared from the serum at the end of follow-up (mean 36.5 months; range 25–49 months) in 28 patients (40%). Nine additional patients become negative for HCV RNA despite having elevated serum concentrations of aminotransferases. Multivariate analysis revealed that the absence of cirrhosis, low hepatic iron concentration, and non-1b HCV genotype were independent predictors of a sustained response.

That high response rates to α-interferon therapy occurred in thalassemia patients with chronic viral hepatitis despite significant iron loading is encouraging, but this is difficult to reconcile with previous reports in which non-thalassemia patients with viral hepatitis and mild hepatic iron overload did not respond to α-interferon therapy. It is possible that age and the concomitant use of iron chelation therapy promotes responses to antihepatitis therapy in patients with thalassemia, particularly because chelation therapy mobilizes hepatic iron that causes cytotoxicity from cytoplasmic to other subcellular compartments.

Porphyria cutanea tarda (PCT) and hepatitis C

PCT is caused by decreased activity of hepatic uroporphyrinogen decarboxylase, and is associated with cutaneous photosensitivity, hypertrichosis, and hepatic disease, including mild or moderate liver iron overload[47]. It has been proposed that the enzymatic defect is necessary for the development of the disease, but that extrinsic or environmental factors are also important for disease expression, particularly in the sporadic form of PCT. There is a strong association between chronic hepatitis C and PCT. Fargion et al. studied 74 Italian patients with PCT, of whom 76% were seropositive for anti-HCV by an enzyme-linked immunosorbent assay and 66% had hepatitis C viremia[47]; a majority had chronic active hepatitis. Patients with PCT and chronic hepatitis C had no apparent risk factors for hepatitis C infection.

The prevalence of hepatitis B and C in patients with PCT may be even higher if more sensitive methods to detect virus are used. Navas et al. studied 34 patients with

sporadic PCT and used the polymerase chain reaction (PCR) to examine the prevalence of hepatitis B virus (HBV) DNA and HCV RNA in sera, liver, and peripheral blood mononuclear cells[48]. Ninety-one percent of the patients had antibody against hepatitis C by a second-generation assay; 21 of 32 patients (65%) had blood HCV RNA by detected by PCR technique. Each of eleven PCT patients studied had HCV RNA in the liver and peripheral blood mononuclear cells[48]. Forty-one percent had antibodies against hepatitis B surface antigen, and 40% had HBV DNA in their sera. The high prevalence of hepatitis B infection could have been due to exposure to the virus during phlebotomy treatment, although no mechanism for the high rate of hepatitis C infection was proposed by the authors[41]. In contrast, only 8% of 106 German PCT patients were seropositive for antiHCV[49]. It is possible that the low prevalence of viral hepatitis in these patients was attributable primarily to avoidance of phlebotomy and reduced iatrogenic transmission. Thus, hepatitis C infection and subsequent hepatic iron overload may modulate disease expression in patients with a predisposition to develop sporadic PCT and in patients with hereditary PCT.

Conclusions

Iron overload is associated with an increased risk of chronic viral hepatitis B and C. Mild or moderate iron overload is common in patients with hepatitis C, and appears to be correlated with more severe disease. Greater degrees of iron overload may be observed in patients with end-stage hepatic disease associated with hepatitis C, especially when alcoholism is also present. The degree of iron overload occasionally suggests the diagnosis of hemochromatosis. Phlebotomy is a promising treatment for chronic hepatitis and appears to reduce hepatic inflammation and improve serum concentrations of hepatic enzymes. PCT and hepatitis C may lead to phenotypic expression of otherwise silent PCT. Further understanding of the mechanism of iron overload, and the effect of iron reduction on the natural history and viral replication of viral hepatitis is needed. Studies to detect mutations in the putative hemochromatosis gene and their interactions with viral hepatitis will play an important role in answering some of these questions.

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38

T-lymphocyte expression and function in hemochromatosis

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Introduction

In 1978, it was postulated that the immunological system could have a role in monitoring tissue iron toxicity as part of its surveillance function. Such a function was thought to be expressed in the sequence of cell migration to an inflammatory site: neutrophils, followed by macrophages and lymphocytes[1]. In 1981, the concept was reiterated more extensively and focussed on the question of why there is a circulation of lymphocytes[2]. It has been updated with regularity until 1992[3–7]. Studies of lymphocyte expression in hemochromatosis strongly supported the initial concept, and were aided by concomitant progress in the development of new tools in immunology and molecular biology that led to the discovery of a new major histocompatibility (MHC) class I gene *HFE*, the hemochromatosis-associated gene[8]. This strengthened the postulate that there is a role of lymphocytes in the regulation of iron load. The central role of macrophages in erythrophagocytosis and iron recycling has long been established[4]. This chapter reviews three main topics: (i) the emergence of the new tools in immunology used in the study of lymphocyte populations in hemochromatosis; (ii) lymphocyte expression and function in hemochromatosis; and (iii) the relevance of the findings in the light of current dogma and of the new evidence. This chapter concludes with a caveat to the clinician, because the ultimate value of experimental evidence must be tested in clinical practice.

New tools in immunology used in the study of hemochromatosis

Monoclonal antibodies

Although the existence of different functional subsets of lymphocytes was suspected for many years, only with the development of the hybridoma technology in 1975[9] was it possible to produce different monoclonal antibodies (moAbs). These were first produced against rat T-lymphoid cells, and that enabled two major subsets of T-lymphocytes in peripheral blood to be characterized: $CD8^+$ and $CD4^+$ T-cells[10]. Shortly afterwards, equivalent moAbs were raised against human and mouse T-cells[11, 12]. Characterization of these T-cell surface markers and the antigen-specific T-cell receptor and its associated molecules (TcR/CD3 complex)[13–17] were invaluable tools for studying T-cell function. With rare exceptions, expression of these surface markers is mutually exclusive. It is now generally accepted that $CD8^+$ and $CD4^+$ T-cells constitute separate lineages of thymus-derived cells, and that the CD8 and CD4 molecules act themselves as co-receptors in the T-cell signaling machine[17].

CD8, CD4, the T-cell receptor, and MHC restriction

CD8 is a surface molecule that occurs as a heterodimer or homodimer in human peripheral blood T-lymphocytes[18, 19]. It defines a subset of T-lymphocytes with cytotoxic/suppressor function that is restricted by MHC class I molecules[20]. In contrast, CD4 is a surface molecule constituted by a single chain[21]. It defines a subset of T-lymphocytes with helper/inducer function that is restricted by MHC-class II molecules[20]. The TcR/CD3 complex is constituted by a clonotypic structure α and β chains of the TcR) and several non-covalent associated molecules (the CD3 complex)[22, 23]. MHC-class I molecules are dimers of a transmembrane glycoprotein (heavy chain) and a soluble protein, β_2-microglobulin[24]. MHC class II molecules are heterodimers of two transmembrane glycoproteins α and β heavy chains)[25]. TcR interacts with the polymorphic regions of MHC class I and II molecules[26–28], whereas the CD8 and CD4 molecules interact with the non-polymorphic regions of MHC class I α3

domain) and MHC class II (α2 domain) molecules, respectively[17, 29, 30].

Signaling

The TcR/CD3/CD8 and TcR/CD3/CD4 oligomeric complexes thus constitute the basic structural units involved in T-cell recognition. The characterization of intracellular tyrosine kinases associated with both CD8 and CD4 (i.e., p56lck)[31, 32] and with components of the TcR/CD3 complex (i.e., p59fyn and ZAP70)[33, 34] provided the molecular basis for the functional role of these oligomeric complexes in regulating T-cell activation after T-cell recognition[35–37]. However, the signal transduction events that occur after triggering of the TcR/CD3/CD8 or TcR/CD3/CD4 complexes are not sufficient to induce optimal T-lymphocyte proliferation. Co-stimulatory signals provided by antigen-presenting cells (APC) are required to achieve complete T-lymphocyte activation capable of driving resting T-lymphocytes into cell division and differentiation.

Co-stimulatory molecules

Among the receptor–ligand interactions involved in such co-stimulatory T-cell signals CD2 and CD28 constituted the most likely candidates[38–41]. The CD28 molecule was one of the first T-cell surface molecules to be characterized by the moAb technology. Further, CD28 is a key co-stimulatory molecule that triggers an independent intracellular signal transduction cascade that is synergistic with that of TcR/CD3/CD8 or CD4 oligomeric complexes in inducing T-cell proliferation after engagement of counter-receptors (the B7 molecules) present on APC[42] (Fig. 38.1). However, not all T-lymphocytes express CD28; in the peripheral $CD8^+$ T-cell pool, as many as 30% of cells in healthy individuals lack CD28 expression[42]. This percentage, however, can represent up to 50–70% of $CD8^+$ T-lymphocytes in certain chronic disorders, although the role of these cells in health and disease is otherwise undefined.

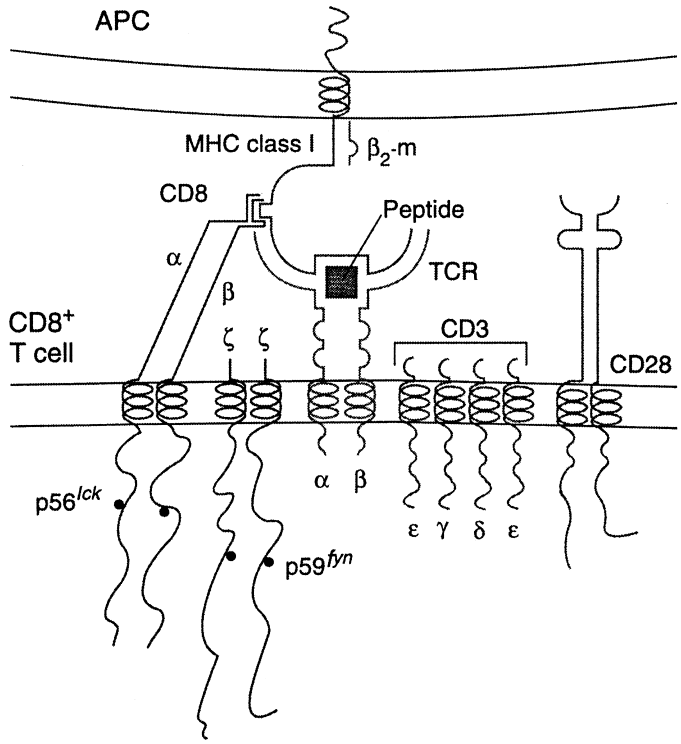

Figure 38.1. Model of the structures (MHC-class I/β_2m, TcR/CD3 complex and its associated kinase p59fyn, CD8 and its associated kinase p56lck, and CD28) involved in antigen recognition by a CD8+ T-cell. This model illustrates some of the findings that permitted the characterization of the abnormalities found in hemochromatosis patients. The fact that the most frequent *HFE* mutation in hemochromatosis patients, C282Y, affects binding of *HFE* to β_2m[8, 75] and that β_2m knockout mice have a spontaneous iron overload[71–74], warrants further studies of the role of these molecules in the regulation of intracellular iron metabolism.

Genes altered in animals by gene targeting

The creation of mice in which planned alterations were introduced at a specific locus in the genome has made possible advances in in vivo characterization of the function of $CD8^+$ and $CD4^+$ T-cell populations, in understanding the expression of MHC class I and II molecules in the development of the two main T-cell subpopulations, and in characterizing the role of tyrosine kinases in T-cell development[43]. The planned modification is first introduced into the genome of mouse embryonic stem (ES) cells in tissue culture. This modification is achieved by homologous recombination of the targeted locus and DNA introduced into the ES cells. Cells containing the derived alteration are injected into recipient blastocysts for return to pseudopregnant foster mothers where they can develop into chimeric mice. This allows the altered ES cell genome to be transmitted to future generations. Gene targeting led thus to the breeding of knock-out mice lacking genes thought to be critical in the development and function of the immune system[44]. The wide use of gene knock-out mice permitted definitive establishment of the MHC class I and II restriction of $CD8^+$ and $CD4^+$ T-lymphocytes[45–49], the role played by CD8, CD4, and their associated tyrosine kinase p56lck (but not p59fyn) in T-cell development and activation[50, 51], and the key role of CD28 as a co-stimulatory molecule involved in T-cell activation[52].

MHC class I assembly

The principal structures and molecules involved in the construction of the stable complexes that permit the cell surface expression of the MHC class I molecules are still being analyzed[53]. The structures include the endoplasmic reticulum (ER) where peptide loading of class I occurs[54], and the 26S proteasome complex, a structure primarily responsible for the degradation of cytosolic proteins and the generation of peptides for antigen presentation[55]. The molecules include the heavy chain (H chain) of MHC class I associated with β_2-microglobulin[24], calnexin, calreticulin, TAP1 and TAP2[54], and, more recently, tapasin, a key intermediary molecule in the assembly of calreticulin and the H chain of MHC class I/β_2-microglobulin with TAP[56]. The molecular cloning of tapasin revealed it to be a transmembrane glycoprotein encoded by an MHC-linked gene[57]. These advances have all been used within the dominant concept of function of the immunological system as a system essential to the maintenance of self-tolerance and effective response to infectious agents. They opened a new world of experimentation that served to reveal unexpected roles for MHC class I molecules[58] and the TcR[59] and also to assist the new concept driven studies of lymphocyte expression and function in hemochromatosis, described in the next section.

Lymphocyte expression and function in hemochromatosis

Hemochromatosis, a disorder of iron overload uncomplicated by the immunological effects of multiple blood transfusions like those that occur, for example, in transfusional iron overload of thalassemia major patients[60] constitutes a unique model in which to study the physiological interdependence between the immunological system and the metabolism of iron in humans. If lymphocyte abnormalities occurred in hemochromatosis as a consequence of progressive iron overload, treatment by repeated phlebotomies until correction of the iron balance should correct those abnormalities. Alternatively, if abnormalities were independent of or possibly preceded iron overload, they would be detectable in persons with hemochromatosis who were diagnosed before the development of clinical manifestations of the iron overload, and would not likely be corrected by iron depletion therapy.

Anomalies of the relative proportions of CD4/CD8 lymphocytes

Follow-up studies of $CD4^+$ and $CD8^+$ peripheral blood cells in hemochromatosis patients were initiated in the late 1980s, and were continued weekly until iron overload in these patients was corrected by therapeutic phlebotomy. These provided the first demonstration of the stability of the relative numbers of the two major peripheral T-cell pools in humans[61] (Fig. 38.2). Some hemochromatosis patients have abnormally high CD4:CD8 ratios (>3.0; normal 0.61–1.77). Furthermore, studies of the two major T-cell populations long after completion of the intensive phlebotomy treatment indicated that high CD4:CD8 ratios persisted in the absence of overload and were associated with a faster re-entry of iron into the transferrin pool[61]. Subsequent studies confirmed and extended these early findings by demonstrating that the percentage of peripheral blood $CD8^+$, but not $CD4^+$, T-cells correlated significantly with the correction of iron balance by repeated phlebotomy, and that low $CD8^+$ cell numbers in combination with the presence of the MHC-class I molecule HLA-A3 were associated with greater iron stores measured by the amount of iron removed by phlebotomy[62, 63]. These studies excluded an effect of iron overload *per se* as the cause of the low percentages of $CD8^+$ T-cells[61–63]. However, the frequency of CD4:CD8 ratios in a normal population and in relatives of hemochromatosis patients, and their relationship to transferrin saturation and serum ferritin concentration had not been determined. Finally, because of the documented role of classical MHC class I molecules in peptide presentation to $CD8^+$ cells and regulation of expansion of this T-cell population, it was important to determine whether the anomalies found earlier in hemochromatosis patients are associated with the mutations found in *HFE*.

Existing data on CD4:CD8 ratios, transferrin saturation, and ferritin concentration were analyzed in groups of persons genotyped for the two hemochromatosis-associated mutations. These consisted of 33 patients selected according to stringent criteria (including hepatic biopsy and iron stores >5 g of iron measured by phlebotomy), 54 control subjects from the same geographical regions as the patients (in the north of Portugal), and 80 relatives. As summarized in Table 38.1, the frequency of higher average CD4:CD8 ratios was related to phenotypic expression of the disorder, but unrelated to the presence of either mutation. Mean CD4:CD8 ratios were divided in subgroups less than or greater than 2.5. Similar frequencies of ratios >2.5 were found in normal control subjects and in relatives of hemochromatosis patients (25.9% and 23.8%, respectively);

these results were independent of the presence or absence of the mutations. In the patients homozygous for the C282Y mutation ($n=27$), the frequency of ratios >2.5 was 40.7% (11/27). Of these 11 patients, seven had ratios >3.0, confirming the earlier finding of abnormally high ratios in hemochromatosis patients[63]. Only four patients diagnosed to have hemochromatosis were heterozygous for the C282Y mutation. Of these, two patients (from different families) had ratios >3.0: a man 43 years old (CD4:CD8 ratio 4.0, transferrin saturation 100%, serum ferritin concentration 3600 ng/ml) and a female 40 years old (CD4:CD8 ratio 8.8, transferrin saturation 142%, serum ferritin concentration 2417 ng/ml). Both had the 'classical' clinical phenotype of hemochromatosis. The other two were asymptomatic males ages 33 and 37 years who had normal ratios, and were diagnosed by family screening studies. They had transferrin saturations of 94% and 98% and serum ferritin concentrations of 592 ng/ml and 880 ng/ml, respectively. From these observations and those of others, it is clear that the analysis of transferrin saturation and serum ferritin concentrations of groups of individuals with high or normal CD4:CD8 ratios may not be fruitful. Analysis of ratios in individual cases can, however, be fruitful in the follow-up of patients homozygous for the C282Y mutation, in C282Y heterozygotes, and in the detection of risk of expression of disease in young relatives. This latter point is illustrated by the finding of two healthy daughters of hemochromatosis patients who were heterozygous for the H63D mutation. At ages 14 and 20 years, they had CD4:CD8 ratios of 3.17 and 3.18, transferrin saturation values of 54.2% and 52.9%, and serum ferritin concentrations of 198 ng/ml and 37 ng/ml, respectively. Follow-up of these two young females permitted the detection five years later of their respective transferrin saturation values of 66% and 73%.

These observations suggest that a high CD4:CD8 ratio may be among the factors that predispose to the development of a heavier iron load. This conclusion is strengthened by the results of an on-going study of hepatic iron content and $CD8^+$ cell numbers in the blood and in sections of hepatic biopsy specimens from Swedish hemochromatosis patients. This study is providing the evidence that, in general, the low percentages of $CD8^+$ cells in the blood correspond with low numbers of the $CD8^+$ cells in the liver (Cardoso E.; personal communication). More importantly, the low numbers of $CD8^+$ cells in the liver correspond with greater hepatic iron concentration. Another current study of lymphocyte populations in 57 Dutch patients with hemochromatosis includes iron retention analysis (Moura E, personal communication). In this study, a statistical correlation between high CD4:CD8 ratios (>3.0) and iron overload measured by transferrin satura-

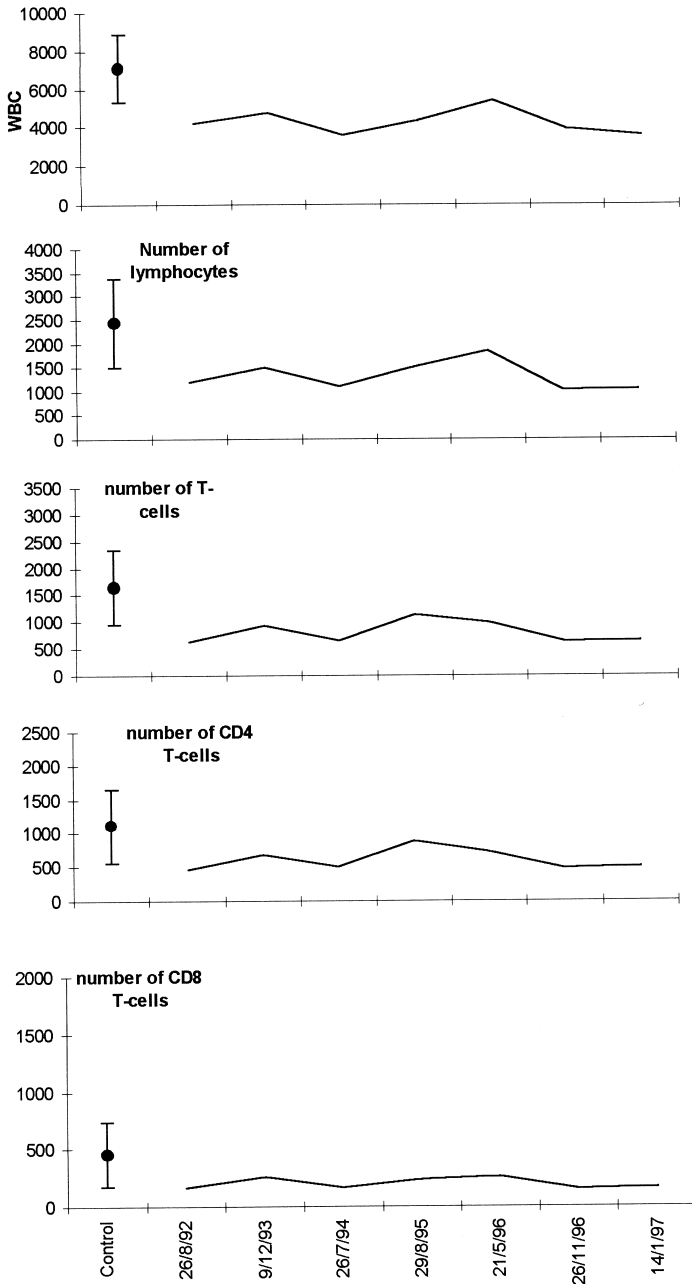

Fig. 38.2. Follow-up studies of leukocytes, peripheral blood total and T-lymphocyte absolute numbers of a patient (male, born in 1920) with hemochromatosis over a period of 5 years of maintenance therapy showing the stability of the absolute numbers of the two major T-cell subsets. Contl: mean ± 1SD of corresponding values of a population of 55 individuals from the same region space as the proband.

Table 38.1. Distribution of CD4:CD8 ratios in groups genotyped for the *HFE* mutation

Group (*n*)	Males/females	Age range (years)	CD4: CD8 ratio <2.5	CD4: CD8 ratio ≥2.5
Control (*n*=54)	13/41	20–76	40 (74.1%)	14 (25.9%)
C282Y−/− H63D−/− (*n*=3 3)	8/25	26–64	24 (72.7%)	9 (27.3%)
C282Y+/− H63D−/− (*n*=5)	1/4	25–64	4 (80.0%)	1 (20.0%)
C282Y−/− H63D+/− (*n*=14)	2/12	20–62	11 (78.6%)	3 (21.4%)
C282Y−/− H63D+/+ (*n*=2)	2/0	53 and 76	1	1
Relatives (*n*=80)	33/47	10–82	61 (76.25%)	19 (23.75%)
C282Y−/− H63D−/− (*n*=14)	11/3	14–61	10 (71.4%)	4 (28.6%)
C282Y+/− H63D−/− (*n*=33)	7/26	10–82	26 (78.8%)	7 (21.2%)
C282Y−/− H63D+/− (*n*=20)	10/10	10–68	15 (75.0%)	5(25.0%)
C282Y−/− H63D+/+ (*n*=3)	1/2	34–63	3	0
C282Y+/− H63D+/− (*n*=10)	4/6	18–60	7 (70.0%)	3 (30.0%)
Patients (*n*=33)	24/9	22–70	20 (60.6%)	13 (34.3%)
C282Y+/+ H63D−/− (*n*=27)	20/7	22–70	16 (59.3%)	11 (40.7%)
C282Y+/− H63D−/− (*n*=4)	3/1	33–40	2 (50.0%)	2 (50.0%)
C282Y+/− H63D+/− (*n*=2)	1/1	47 and 61	2	0

tion and serum ferritin concentration could not be ascertained. In 51 patients with available T-cell phenotyping and measures of iron retention, the lowest percentages of $CD8^+$ cells (<17%) were seen in patients with the highest iron retention values (>75%) (Moura E, personal communication). Because $CD8^+$ T-lymphocytes are activated and clonally expanded in the context of MHC-class I molecules[45, 46], a long-term characterization of the molecules involved in this process (TcR, CD8, and their associated kinase p56lck, and MHC-class I molecules) has been initiated in ex vivo peripheral blood samples obtained from HLA-typed hemochromatosis patients, although the results are incomplete.

p56lck enzyme activity

In 16 of 18 (87%) hemochromatosis patients, the in vitro activity of the CD4- and CD8-associated tyrosine kinase p56lck, a key enzyme involved in human T-cell activation, was defective in $CD8^+$, but not $CD4^+$, T-cells[64] (Fig. 38.3). Subsequent studies that reproduced in vitro iron overload conditions in cultures of peripheral blood T-lymphocytes demonstrated that the defective CD8-associated p56lck activity found in hemochromatosis patients could not be attributed to iron overload[65]. In vitro, $CD4^+$ cells, but not $CD8^+$ cells, appear to be negatively affected by exposure to iron.

The TcR repertoire

Characterization of the variable region of the β chains of the T-cell receptor in peripheral blood T-lymphocytes with a panel of six moAbs revealed abnormally low percentages of $CD8^+$ $V6.7^+$ cells in hemochromatosis patients, particularly those with hepatic cirrhosis[66, 67]. Serial studies of the $CD4^+$ and $CD8^+$ TcR Vβ repertoire during intensive phlebotomy treatment in each patient demonstrated the stability of the individual values studied[67]. In the case of the TcR repertoire, additional evidence of the importance of the role of MHC-class I molecules was drawn from the analysis of control data obtained in HLA-typed control subjects. This indicated that normal persons that express $HLA\text{-}A3^+$ and low percentages of $CD8^+$ cells do not have compensatory increases in the relative proportions of $V\beta6.7^+$ cells within the $CD8^+$ cell population[67]. This is in contrast to normal persons who are $HLA\text{-}A3^-$. Hemochromatosis patients, whether $HLA\text{-}A3^+$ or $\text{-}A3^-$, 'failed' to expand the $V\beta\ 6.7^+$ cell population within the $CD8^+$ pool. The significance of these findings is not fully understood and they need confirmation from studies of other patient populations. This may constitute additional evidence for the importance of MHC class I antigens in this disorder, both in the pathogenesis of iron overload and in expression of differences in discrete $CD8^+$ T-cell subsets.

Activated T-cells

$HLA\text{-}DR^+$ cells were identified among the total T-cell population in a study of $CD3^+$ T-lymphocytes in which HLA-DR, a marker of lymphocyte activation, was used to study hemochromatosis patients (Moura E, personal communication). Moreover, the lowest percentages of activated T-cells in the blood were found in patients with the highest

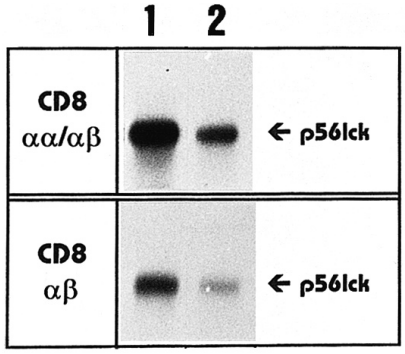

Fig. 38.3. p56lck kinase activity in CD8α and CD8β immunoprecipitates. Equal amounts of freshly collected peripheral bood lymphocytes were lysed in lysis buffer and duplicates of the lysates immunoprecipitated with either anti-CD8α (21Thy 2D3) or anti-CD8β (2ST8 5H7) monoclonal antibodies. Immunoprecipitates were subjected to in vitro kinase assay as described by Arosa et al.[64] and separated in SDS/PAGE. Autoradiograph shows the CD8αα/αβ and CD8αβ-associated p56lck activity in a healthy control (lane 1) and a patient with hemochromatosis (lane 2).

iron retention values (75%). Characterization of the same marker of activation (HLA-DR) in a study of Arosa et al.[68] revealed that the relative percentages of HLA-DR$^+$ among CD8$^+$ T-cells were significantly higher in hemochromatosis patients than in controls (23.1% vs. 9.8%, respectively; $p<0.002$). The detection in these two studies of activated T-cells in hemochromatosis patients, probably within the CD8$^+$ population[68], may reflect a response of a selected population of T-cells to increased iron load, with a possible effect on the level of iron retention.

CD8$^+$ CD28$^+$ Cells and defective cytotoxicity

Other studies of key molecules involved in T-cell activation in hemochromatosis patients have demonstrated further anomalies in the expression of the co-stimulatory molecule CD28 in peripheral blood CD8$^+$ but not CD4$^+$ T-lymphocytes. The percentage of CD4$^+$ CD28$^+$ and CD4$^+$ CD28$^-$ T-cells was similar in patients and controls (Table 38.2). In contrast, lower percentages of CD28$^+$ CD28$^+$ cells and greater percentages of CD8$^+$ CD28$^-$ cells were observed in the peripheral blood of patients than in controls. These anomalies were observed both in iron-loaded and iron-depleted patients. Serial studies over two years showed that these abnormalities were due to an increase in numbers of CD8$^+$ CD28$^-$ cells and were persistent[68]. Further dissection of the anomalies of the CD8$^+$ CD28$^+$ cells in HLA-A3$^+$ and -A3$^-$ patients showed the inability of CD8$^+$ CD28$^+$ (but not CD8$^+$ CD28$^-$) T-cells to accompany the increase in the CD8$^+$ T-cell pool in HLA-A3$^+$ patients[68]. Low percentages of CD8$^+$ CD28$^+$ cells, however, were found predominantly in patients with hepatic cirrhosis, regardless of HLA phenotype.

Table 38.2. Percentage (mean ± S.D.) of CD28+ and CD28- T-lymphocyte subsets in controls and hemochromatosis patients[a]

T-cell subset	Healthy controls ($n=30$)	Hemochromatosis patients ($n=21$)	Value of p [b]
% CD4$^+$	47.4 ± 8.3	50.6 ± 9.8	ns
% CD4$^+$ CD28$^+$	45.0 ± 8.9	47.9 ± 9.4	ns
% CD4$^+$ CD28$^-$	2.4 ± 2.7	2.7 ± 1.9	ns
% CD8$^+$	22.6 ± 6.6	22.7 ± 11.0	ns
% CD8$^+$ CD28$^+$	15.4 ± 4.0	10.3 ± 4.10	<0.0005
% CD8$^+$ CD28$^-$	7.2 ± 5.0	12.5 ± 11.0	<0.03

Notes:

[a] Peripheral blood mononuclear cells were stained with CD8-FITC/CD28-PE and CD4-PerCP and analyzed in a FACscan. Values represent the mean ± S.D/ of the percentage of each population gated on total peripheral blood lymphocytes (modified from ref. [68]).

[b] Differences between groups (Student's *t*-test) are indicated. ns, not significant.

CD8$^+$ CD28$^+$ are precursors of MHC class I restricted cytotoxic cells. Accordingly, Arosa et al. established six-day mixed lymphocyte cultures (MLC) from hemochromatosis patients and control subjects, generated CD8$^+$ cytotoxic T-lymphocytes (CTL), and measured their cytotoxic activity against allogeneic Cr^{51}-labeled target blast cells[68]. CD8$^+$ CTL from hemochromatosis patients had a significantly lower cytotoxic activity than CTL from control subjects (Fig. 38.4). At all effector:target ratios tested, cells from hemochromatosis patients had an approximately two-fold lower cytotoxic activity than control cells. These phenotypic and functional anomalies of CD8$^+$ lymphocyte subpopulations are probably relevant to the more severe clinical expression of hemochromatosis (see below).

Summary of findings and discussion of their relevance

The findings and their clinical correlates are summarized in Table 38.3. Current dogma in immunology dictates that the immunological system has the unique function of defense against foreign pathogens and of maintaining tolerance of self-antigens. Recognition of transformed cells lies at the boundary between two recognitions: the

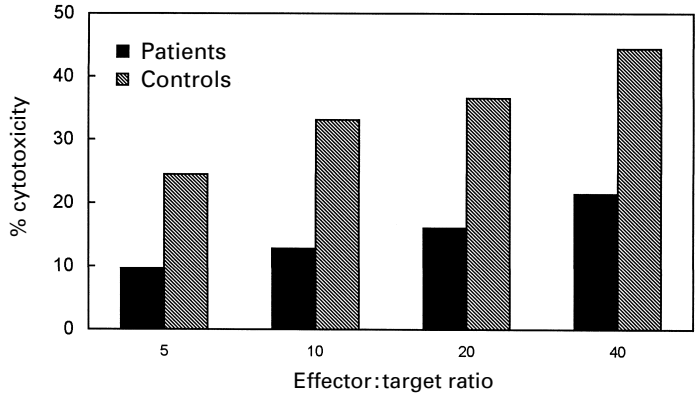

Fig. 38.4. Cytotoxic activity of $CD8^+$ CTL generated after 6-day mixed lymphocyte cultures in hemochromatosis patients and controls. $CD8^+$ CTL were obtained by negative selection using CD4-beads and their cytotoxic activity against allogeneic blast cells was assayed in standard ^{51}Cr-release assays as described by Arosa et al.[68] at the effector : target ratios indicated. Values represent the mean percentage of specific cytotoxicity in controls ($n = 7$) and patients ($n = 7$). S.D. were less than 15%.

recognition of modified self represented in the antigens expressed by tumors, and the defense against cells infected with viruses.

The most significant anomalies known in lymphocytes in hemochromatosis are confined to the $CD8^+$ cells population in association with more severe phenotypic expression of the disorder in patients with particular MHC class I antigens (especially HLA-A3). Thus, an expected consequence of this would be a higher incidence of certain forms of cancer in hemochromatosis. This is the case, because a high percentage of hemochromatosis patients with hepatic cirrhosis develop hepatocellular carcinoma[69]. In this context, the low percentages of circulating $CD8^+$ $CD28^+$ T-lymphocytes that are precursors of MHC class I restricted cytotoxic cells, together with a defective $CD8^+$ CTL population, may contribute to the development and progression of hepatocellular carcinoma in patients who have hepatic cirrhosis. In addition, if the system of circulating cells has a surveillance function, decreases in the relative proportions of functionally significant cells and in total cell numbers should be reflected in more severe clinical manifestations, such as cancer.

This case report illustrates these two points: the possible significance of persistent leukopenia and progressive 'disappearance' of $CD8^+$ $CD28^+$ T-cells from the circulation, preceding a fulminant fatal course of hepatocellular carcinoma (Fig. 38.5). A male born in 1947 first presented at a hemochromatosis clinic in 1992 with ascites, hepatosplenomegaly, altered hepatic function, and skin pigmentation with hepatic cirrhosis; the tentative diagnosis of hemochromatosis was made. Examination of a hepatic biopsy specimen confirmed the diagnosis of hemochromatosis (hepatic iron index 6.1). In August 1993, his treatment with intensive phlebotomy was started; a course of 64 weekly phlebotomies removed a total of 10.9 g of iron. A maintenance regime was started in November 1995. His blood leukocyte count was persistently $< 4.5 \times 10^9$/l (range 2.97×10^9/l – 4.5×10^9/l). His total lymphocyte counts ranged between 0.96×10^9/l and 1.62×10^9/l. The percentages of CD4 and CD8 lymphocytes remained 60–70% and 10–13%, respectively. Measurements of the percentages of $CD8^+$ $CD28^+$ cells were started in May 1994; they remained unchanged for three months (October 1994) (Fig. 38.5). One year later (October 1995), however, a sharp decline in the percentages and total numbers of $CD8^+$ $CD28^+$ was observed. This was accompanied by a similar decline of $CD8^+$ $CD28^-$ cells, and six months later the diagnosis of hepatocellular carcinoma was established; he died in August 1996[70].

In light of the concept that the immunological system has a surveillance function of iron toxicity, anomalies of function and expression of lymphocytes would be expected to precede the development of iron overload. Evidence from the studies reported previously and the identification of *HFE* as a new MHC class I gene provide decisive support for that view. However, the occurrence of spontaneous iron overload in gene knock-out mice that lack lymphocyte populations provides the most implacable evidence in favor of the new physiological function of the immune system. Homozygous β_2m -/- mice do not express MCH class I and, as a consequence, lack $CD4^-$ $CD8^+$ $\alpha\beta$ T-cells[45,46]. Iron overload found in mice could not be attributed to the MHC class I expression or abnormalities of the $CD8^+$ cells. Homozygous β_2-microglobulin knock-out mice were first reported for the occurrence of iron overload in 1994[71]. A study of mutant homozygous β_2m -/- mice and littermate heterozygotes was performed, including examination of liver, kidney, heart, lung, pancreas, and spleen for iron content by light microscopy and atomic absorption. This revealed the occurrence of spontaneous hepatic iron overload in the older homozygous mutant mice with the hepatic iron distribution characteristic of hemochromatosis, i.e., in hepatocytes and not in the Kupffer cells. The development of iron overload in β_2-microglobulin knock-out mice was confirmed by Rothenberg[72].

Santos et al. applied an improved and sensitive method for studying iron absorption in mice[73] and demonstrated the extent of the disturbance of iron homeostasis in the β_2m -/- mutant mice[74]. Most importantly, this study dem-

Table 38.3. Abnormalities of T-lymphocyte expression in hemochromatosis

Abnormality	Clinical correlation	Immunogenetic correlation	References
Abnormally high CD4:CD8 ratio (>3)	High iron stores measured by iron removed	HLA-A3	63
CD4:CD8 ratios	No clinical correlation	No	Moura, personal communication
Low $CD8^+$ cells (<17%)	High iron stores measured by iron removed	HLA-A3/B7	64
Low $CD8^+$ cells (<17%)	High iron retention (>75%)	—	78
Low activity of $CD8^+$ cell p56lck enzyme	No clinical correlation found in 16 of 18 patients studied	—	64
$CD8^+$ Vβ6.7$^+$ cells significantly lower than controls or asymptomatic patients	Patients with cirrhosis	HLA-A3	67
Low $CD8^+$ $CD28^+$ cells	Cirrhosis and possibly of value in hepatocellular carcinoma (see case report)	HLA-A3	68
Activated T/$CD8^+$ cells	Iron retention <25%	HLA-A3	68, 77

onstrated that the reconstitution of the mutant mice with normal hematopoietic cells had a corrective effect of the parenchymal iron overload in the liver[74]. No abnormalities of iron homeostasis could be demonstrated in CD8 gene knock-out mice, emphasizing the importance of the contribution of MHC class I to the development of iron overload[74]. Transfer of fetal liver cells did not correct the defective iron absorption control seen in the β_2m -/- mice[74]. Current experiments with mice lacking all classes of lymphocytes are revealing that these animals develop severe iron overload of the heart, pancreas, and liver similar to that described in the β_2m -/- mice. (Santos M.; Chapter 47).

The discovery of the gene for hemochromatosis as a new MHC class I gene[8], and the subsequent demonstration that the most frequent substitution, C282Y, at a position impairing the assembly of the heavy chain of MHC with β_2-microglobulin[75] concludes this chapter. By providing decisive evidence for the inseparability of the immunological system and regulation of iron overload, these observations also open a new chapter to the clinician treating patients with hemochromatosis. Progress in molecular biology is radically changing the practice of medicine, and is enlarging the scope of responsibility of the clinician beyond the traditional roles of diagnostician and therapist. A patient with hemochromatosis or other disorders may require detection of a specific gene mutation. Finding abnormalities of T-lymphocyte populations in patients with greater iron stores has led to the discovery of experimental models of hemochromatosis in gene knock-out mice with defective lymphocyte populations. In heterozygotes for the C282Y or the H63D mutation, the presence of abnormally high CD4:CD8 ratios may, in some individuals, predict that they will eventually develop a severe clinical phenotype, requiring greater attention from the clinician and more aggressive phlebotomy treatment. The studies of lymphocyte function and expression in hemochromatosis are the first to illustrate that the immunological system regulates the metabolism of iron, an essential element. How this regulatory action is exercised is still not demonstrated or understood. It may depend on the capacity of $CD8^+$ cytotoxic cells to recognize and eliminate epithelial cells modified by an excessive intracellular iron load. It may depend on a more systemic physiological role of T-cell- and macrophage-derived cytokines as indicated by the studies of Gordeuk et al. on tumor necrosis factor production[76] and Moura on iron handling by hemochromatosis macrophages[77]. Nevertheless, the essential role of particular MHC-class I molecules that regulate surface receptors by *cis*-interactions is likely to be important in discerning the regulatory actions of the immunological system. The capability of MHC-class I molecules expressed by T-cells to regulate T-cell function differentially is now well illustrated[78, 79], and has a basis in earlier studies of the function of MHC class I molecules[80]. In clinical practice, however, the importance of the findings reviewed in this chapter are related to the value of incorporating routine immunological tests, particularly CD4:CD8 ratios, $CD4^+$ and $CD8^+$ absolute T-cell counts, and differential leukocyte counts in the evaluation of hemochromatosis patients and the

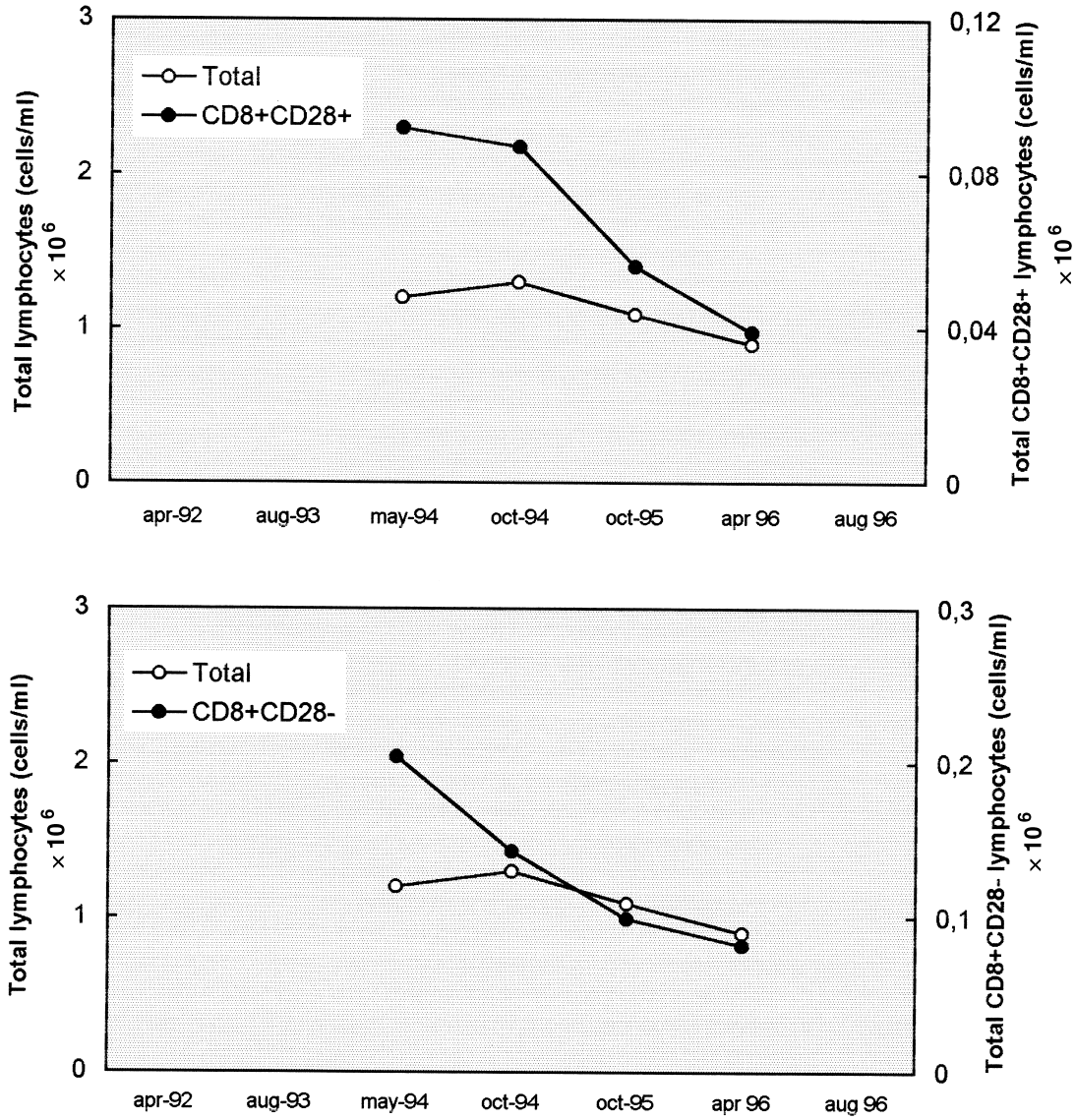

Fig. 38.5. Follow-up studies of $CD8^+$ $CD28^+$ and $CD8^+$ $CD28^-$ T-lymphocytes. Peripheral blood T-lymphocytes from a patient with hemochromatosis were studied during a two-year period. The numbers of total lymphocytes and total $CD8^+$ T-cell subpopulations were determined at the time of study in a FACscan after staining with the proper antibodies as described by Arosa et al.[68]

genetic characterization of this disorder, wherein the immunological system was traditionally not thought to be relevant.

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Part VIII

Hemochromatosis heterozygotes

39

The iron phenotype of hemochromatosis heterozygotes

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Introduction

The issue whether hemochromatosis heterozygotes develop disease-related morbidity is controversial. Because iron is the source of the organ damage in hemochromatosis, heterozygotes would have to become iron loaded for complications to occur. By definition, heterozygotes have one-half of the hemochromatosis genotype, possessing one mutant and one wild-type allele. Does the presence of one-half of the hemochromatosis gene product result in the expression of one-half of the homozygous phenotype? Most importantly, do hemochromatosis heterozygotes develop disease-related morbidity?

Even if heterozygotes do not develop an intermediate hemochromatosis phenotype, they may be at increased risk of morbidity if other disorders, such as porphyria cutanea tarda, hereditary spherocytosis, or beta-thalassemia minor are present. Does excess iron in hemochromatosis heterozygotes confer upon them an increased risk to develop cancer or coronary artery disease? Answers to some of these intriguing questions will be considered in the current chapter and will be considered in additional detail in other chapters of this book.

Identification of heterozygotes

Hemochromatosis is tightly linked to the HLA Class I region on chromosome 6. It is therefore possible to assign a hemochromatosis genotype within a pedigree based on HLA-A serotypes shared with the proband[1–6]. Due to autosomal recessive transmission of hemochromatosis, parents and offspring of a proband are, at least, obligate heterozygotes. This chapter describes the iron phenotype of members of pedigrees who have been assigned the heterozygous genotype based on half-HLA-identity with a homozygous proband.

Population frequency of heterozygosity for hemochromatosis

The frequency of homozygosity for hemochromatosis is about 5 per 1000 among Caucasians of European ancestry. The frequency of heterozygosity in the same population is about 12%[7, 8]. This predicts that approximately 30 000 000 Caucasians in the United States are heterozygotes. If heterozygosity for hemochromatosis leads to an intermediate phenotype, a large number of individuals might be at risk for iron-related organ damage.

Hemochromatosis gene

The hemochromatosis gene (*HFE*) was recently isolated[9, 10], and there are no comprehensive reports as yet of the iron phenotype in individuals with one mutant *HFE* allele. There are a few reports of the iron phenotype in small groups of heterozygotes who were identified as obligate heterozygotes during family studies, or who were identified by half-HLA identity to an affected proband[11–19]. The analysis of heterozygotes is complicated by the fact that penetrance of hemochromatosis is incomplete. Until the basis of this is discovered, we will have to rely on a large sample number to assess the risk associated with heterozygosity properly.

Effect of heterozygosity on iron phenotype

An early study employed multivariate linear analysis of the effects of age, sex, heterozygosity and homozygosity for hemochromatosis on the expected results of tests of iron metabolism, compared to those of normal subjects[13]. The heterozygous state conferred a modest increase in serum iron concentration, transferrin saturation, serum ferritin

Table 39.1. Effects of the heterozygous genotype on iron parameters

Test	Expected normal value	Heterozygous effect on test result (+/−)
Serum iron concentration, μg/dl	87 (144)	+17 (238)
Total iron-binding capacity, μg/dl	356 (144)	−31 (238)
Transferrin saturation, %	24 (144)	+9 (238)
Serum ferritin concentration, μg/ml	104 (135)	+6 (228)
HPCSI,[a] grade 0–4	0.2 (39)	+0.9 (29)

Notes:
[a] Hepatic parenchymal cell.
Adapted from refs. [14,15].
Serum iron concentration, total iron-binding capacity, transferrin saturation, serum ferritin concentration, and hepatic parenchymal cell stainable iron[a], expressed as an increment or decrement in expected arithmetic mean values in normal subjects[14, 15]: results of multivariate linear analysis. The number of subjects tested appears in parentheses below the test result.

concentration, and the grade of hepatic parenchymal cell stainable iron. Heterozygosity was associated with a modest decrement in the total iron-binding capacity, presumably the response of normally-functioning hepatocytes to the presence of adequate transferrin-bound iron. The effect of the heterozygous genotype on the results of tests of iron metabolism appear in Table 39.1.

The iron phenotype in small studies: do heterozygotes accumulate iron?

Between 1979 and 1994, there were several reports of the results of serum iron concentration, transferrin saturation, and serum ferritin concentration in heterozygotes[11–19]. A few studies have included results of blood tests and hepatic biopsy analyses in groups of heterozygotes. The results of tests of iron status in five groups of heterozygotes from three countries were similar (Table 39.2). Most studies did not include adequate detail to allow direct comparison of results among studies from different centers. The results from studies that included enough detail to determine the frequency of elevated results of transferrin saturation and serum ferritin concentration in heterozygotes appear in Table 39.3. Taken together, the results presented in Tables 39.1–39.3 suggest that the presence of one mutant *HFE* allele causes a limited, but not a marked or continuous, increment of iron absorption and accumulation in 9–25% of heterozygotes.

Utah Heterozygote Study: iron phenotype of 1058 heterozygotes and 321 normal subjects

The results of a large study of the iron phenotype of heterozygotes and normal control subjects were published recently[20]. Individuals were classified as heterozygotes based on half-HLA-identity to a homozygous proband. The 505 male and 553 female heterozygotes were identified in 202 pedigrees. To avoid the effect of overlapping values of serum iron concentration, transferrin saturation, and serum ferritin concentration among control and study subjects of different ages and sex, heterozygotes and normal subjects were divided by sex into age groups of 1–30 years, 31–60 years, and 61–90 years. A comparison of the results of serum iron concentration, transferrin saturation, and serum ferritin concentration between the heterozygous and the normal individuals appears in Table 39.4.

Serum iron concentration

The mean serum iron concentration for all heterozygous males was 126 μg/dl (± 44 μg/dl S.D.), 106 μg/dl (± 37) for all normal males, 108 μg/dl (s.d. 44) for all heterozygous females, and 93 μg/dl (s.d. 40) for all normal females. When compared by age and sex, however, heterozygotes had significantly higher serum iron concentration values compared to normal individuals (Table 39.4). These findings were similar to the results in a smaller number of heterozygotes in an earlier publication (Table 39.2).

Transferrin saturation

The mean transferrin saturation among all heterozygous males was 38% (s.d. 13), compared to 29 (s.d. 10) for all normal males, 31 (s.d. 13) for all heterozygous females, and 25 percent (s.d. 11) for all normal females. As with serum iron concentrations, transferrin saturation was significantly higher among all groups of male and female heterozygotes compared to the age- and sex-matched normal subjects (Table 39.4). Male heterozygotes had transferrin saturation values about 20% higher than female

Table 39.2. Mean iron parameters of hemochromatosis heterozygotes in five studies from three countries

Test	The iron phenotype of heterozygotes in five studies									
	USA 1982[14]		France 1979[11]		France 1980[12]	Canada 1983[16]	Canada 1994[18]	Normal values[14]	
	M	F	M	F	M, F	M, F	M, F	M	F
	(116)	(122)	(66)	(75)	(98)[h]	(82)[h]	(255)[h]	(68)	(76)
SI, μg/dl[a]	127	108	125[g]	110[g]	128	128		106	96
	(116)	(122)	(66)	(75)	(98)	(82)		(68)	(76)
TS, %[b]	42	35			40	39	37	31	29
	(116)	(122)			(98)	(82)	(255)	(67)	(76)
SF, ng/ml[c]	155	64	104[g]	50[g]	55[i]	63[i]	112	135	72
	(109)	(119)	(66)	(75)	(98)	(82)	(255)	(63)	(72)
HPCSI, grade 0–4)[d]	1.1	1.0						0.3	0.1
	(23)	(6)						(24)	(15)
HIC,[e] μg/g dry liver;	3885[f]	2664[f]			3069		3013[f]	444[f]	292[f]
	(23)	(6)			(11)		(17)	(24)	(15)
μmol/g dry liver	70	48			55		54	8	5
	(23)	(6)			(11)		(17)	(24)	(15)

Notes:
[a] Serum iron concentration.
[b] Transferrin saturation.
[c] Serum ferritin concentration.
[d] Hepatic parenchymal cell stainable iron.
[e] Hepatic iron concentration.
[f] Not reported in original publication.
[g] Median value, not mean.
[h] Results reported as mean value for all heterozygotes.
[i] Geometric mean.
Where possible results were segregated according to sex (M, F). Number of subjects tested appears in parentheses below test results.

Table 39.3. Frequency of serum iron parameters in hemochromatosis heterozygotes

Country	Percentage of heterozygotes with elevated results		
	Total	TS[a]	SF[b]
France [11]	141	6	6
USA [14]	238	20	10
Canada [16]	82	6	21
Australia [17]	98	16	25
Canada [18]	255	9	11
Denmark [19]	105	18	5
		13%	13%

Notes:
Elevated transferrin saturation[a] or serum ferritin concentration[b] among heterozygotes from five countries in six studies with adequate detail to determine frequency of elevated results.

heterozygotes. Heterozygotes had transferrin saturation values about 22 % higher than the age- and sex-matched normal individuals. The mean transferrin saturation remained constant among all groups of heterozygotes and all groups of normal subjects. These results were slightly higher than the findings in a smaller study (Table 39.2).

Serum ferritin concentration

Serum ferritin concentration values are not normally distributed in heterozygotes. Log transformation is required to achieve a normal distribution. Log transformation allows calculation of a geometric mean and 95% confidence intervals. This is mathematically correct, but it has limited practical value, as clinical laboratories do not routinely report log-transformed serum ferritin values. Geometric mean values may not help a practicing

Table 39.4. The iron phenotype[a] of 1058 Utah hemochromatosis heterozygotes (Hh)[b] and 321 normal individuals (HH)[c]

Sex and age group	Serum iron, μg/dl			Transferrin saturation, %			Serum ferritin, ng/ml		
	Hh[b]	HH[c]	P value	Hh[b]	HH[c]	P value	Hh[b]	HH[c]	P value
Males									
1–30 yrs	132	111	0.005[d]	38	29	<0.001[d]	82	59	0.177[d]
	(47)	(46)		(14)	(11)		(116)	(67)	
31–60	124	108	0.006[d]	37	30	<0.001[e]	181	83	0.0001[d]
	(41)	(35)		(12)	(9)		(154)	(60)	
61–90	117	99	0.009[e]	38	30	<0.001[e]	204	162	0.365[d]
	(38)	(27)		(14)	(8)		(125)	(146)	
Females									
1–30 yrs	118	98	0.01[e]	33	26	0.003[e]	37	46	0.8[d]
	(47)	(37)		(13)	(11)		(37)	(65)	
31–60	103	93	0.02[d]	30	25	0.002[d]	62	40	0.0028[d]
	(42)	(44)		(13)	(12)		(76)	(44)	
61–90	103	90	0.04[e]	31	25	0.008[e]	132	73	0.0118[d]
	(37)	(31)		(13)	(10)		(139)	(65)	

Notes:

[a] Adapted from ref. [20]. These data are presented as arithmetic mean values (+/− s.d.) of serum iron concentration, transferrin saturation, and serum ferritin concentration.

[b] Heterozygotes: total $n=1058$:
Males: age 1–30 ($n=210$); 31–60 ($n=209$); 61–90 ($n=86$); total $n=505$
Females: age 1–30 ($n=201$); 31–60 ($n=260$); 61–90 ($n=92$); total $n=553$.

[c] Normal subjects: total $n=321$:
Males: age 1–30 ($n=38$); 31–60 ($n=66$); 61–90 ($n=28$); total $n=132$
Females: age 1–30 ($n=40$); 31–60 ($n=105$); 61–90 ($n=44$); total $n=189$.

[d] p values calculated by Mann–Whitney U test.

[e] p values calculated by Student's t-test.

physician to decide if a patient's serum ferritin concentration is significantly elevated. In a mathematically rigorous report, the log-transformed values of serum ferritin concentration, with geometric mean values and 95% confidence intervals, were recently presented[20].

In this chapter, arithmetic mean values (and s.d.) of serum ferritin concentration are provided for the purpose of allowing practical application to patient management. The arithmetic mean serum ferritin concentration among all heterozygous males was 144 ng/ml (s.d. 155), compared to 91 ng/ml (s.d. 92) for all normal males, 64 ng/ml (s.d. 85) for all heterozygous females, and 49 ng/ml (s.d. 55) for all normal females. The arithmetic mean of serum ferritin concentration was significantly higher among some, but not all, groups of heterozygotes compared to normal individuals (Table 39.4). In male and female heterozygotes, and in normal males, the serum ferritin concentration increased progressively with age. In normal females, serum ferritin concentration decreased in the age group 31–60 years, then increased modestly in the 61–90 year age group. The mean serum ferritin concentration among male heterozygotes was 15–23% higher than female heterozygotes in the same age group. Serum ferritin values among female heterozygotes were similar to the age-matched normal males. The frequency of an elevated serum ferritin concentration among 1058 heterozygotes appears in Table 39.5.

Frequency of elevation of both transferrin saturation and serum ferritin concentration

Using a threshold value of 60% for transferrin saturation and 325 ng/ml for serum ferritin concentration for males (50% and 125 ng/ml, respectively, for females), only 1% of males and 0.4% of female heterozygotes had an elevation of both transferrin saturation and serum ferritin values (Table 39.5). These results from the study of a large number

Table 39.5. Frequency of elevated results of transferrin saturation and serum ferritin concentration among 1058 Utah hemochromatosis heterozygotes (505 males, 553 females)

	Percentage of heterozygotes with elevated results			
	Transferrin saturation[a]	Serum ferritin[b]	Transferrin saturation and serum ferritin[c]	Transferrin saturation and serum ferritin[d]
Males	6%	10%	4%	1%
Females	8%	12%	1%	0.4%

Notes:
[a] Transferrin saturation: Males >60%; Females >50%.
[b] Serum ferritin concentration: Males >325 ng/ml; Females >125 ng/ml.
[c] Males: transferrin saturation >50% and serum ferritin >325 ng/ml.
Females: transferrin saturation >50% and serum ferritin >125 ng/ml.
[d] Males: transferrin saturation >60% and serum ferritin >325 ng/ml.
Females: transferrin saturation >60% and serum ferritin >125 ng/ml.

of heterozygotes, combined with the results of the small studies that were published earlier (Tables 39.1–39.3), provide evidence that heterozygotes rarely have marked elevation of blood tests of iron metabolism.

Frequency of hepatic iron loading in 39 heterozygotes

Hepatic iron stores were measured chemically by atomic absorption spectrophotometry and microscopically by grading the hepatic parenchymal cell stainable iron[20]. The upper limit of normal for hepatic iron concentration is 1400 μg iron per gram of liver dry weight (25 μmol per gram dry liver). The normal range for hepatic parenchymal cell stainable iron is grade 0–1. Thirty-nine hemochromatosis heterozygotes underwent needle biopsy of the liver (22 males and 17 females). The age range of the heterozygotes who underwent hepatic biopsy was 8–71 years. The occurrence of hepatic iron loading in heterozygotes was uncommon. Massive hepatic iron loading did not occur in any heterozygote[20]. These results are similar to the findings in other studies.

Hepatic iron concentration among 39 heterozygotes

The mean value of hepatic iron concentration among the 39 heterozygotes who underwent hepatic biopsy was 1449 μg of iron per gram of dry liver (26 μmol iron per gram dry liver). These mean values of hepatic iron concentration were only minimally elevated above the upper limit of normal. As expected, the mean value of hepatic iron concentration in the 11 individuals who had an elevated grade of hepatic parenchymal cell stainable iron (grade 2–4 on a scale of 0–4; normal grade 0–1) was 2411 μg iron per gram dry liver (43 μmol iron per gram dry liver). These results are compatible with the findings of other studies in which adequate detail was provided to allow comparison (Table 39.2).

Hepatic parenchymal cell stainable iron

The hepatic biopsy specimens of only 11 heterozygotes had stainable iron that was greater than grade 1. Of these 11 individuals, eight had an iron stain of grade 2, and three subjects had grade 3 stainable iron. Nine of these 11 individuals had an elevated transferrin saturation, or serum ferritin concentration, or both. Seven of these 11 individuals had a serum ferritin value that was >300 ng/ml. Some of the clinical characteristics of the 11 heterozygotes who had increased stainable iron or liver injury appear in Table 39.6.

Frequency of decreased serum ferritin concentration

An iron-deficient serum ferritin value (<12 ng/ml) was present in 21% of female heterozygotes of reproductive age (12–50 years), and in 32% of age-matched normal females. An iron-deficient serum ferritin value was present in 2% of male heterozygotes over age 18, and in 4% of age-matched normal males.

Table 39.6. Clinical features and concurrent disorders in 11 heterozygotes who had elevated hepatic parenchymal cell stainable iron (grade 2–4), or hepatic fibrosis, or cirrhosis

	Age	HPCSI[a]	Fibrosis	Cirrhosis	TS[b]	SF[c]	Hepatitis B or C	PCT[d]	Steato-hepatitis	Alcoholism[e]
Normal		0–1	−	−	<50	M<325 F<125	−/−	−	−	−
Males										
	33	2	−	−	65	174		−	−	−
	34	2	−	−	57	438		−	−	+
	54	2	+	+	42	800	−/+	−	−	+
	65	2	+	−	46	172	−/−	−	−	−
	21	2	−	−	58	998		−	−	+
	51	3	+	−	47	885	+/−	+	−	+
	8[f]	2	−	−	48	217		−	−	−
	59	2	+	−	42	563	−/−	−	−	+
	61	2	−	−	23	552		−	−	+
Females										
	36	0	+	+	22	46	−/−	−	+	−
	32	3	+	−	44	246	−/−	+	−	+

Notes:
[a] Hepatic parenchymal cell stainable iron (grade 0–4).
[b] Transferrin saturation, %.
[c] Serum ferritin concentration, ng/ml.
[d] Porphyria cutanea tarda.
[e] Consumption of >70 grams of alcohol per day.
[f] Hereditary spherocytosis.

Morbidity among hemochromatosis heterozygotes

Does the presence of one mutant *HFE* allele cause disease-related morbidity among heterozygotes? From the data presented in Tables 39.1–39.3, it is apparent that heterozygosity for hemochromatosis does not cause a continuous, progressive accumulation of tissue storage iron. Heterozygotes do not develop heavy iron overload unless they have an additional disease process, such as porphyria cutanea tarda, hereditary spherocytosis, or beta-thalassemia minor[21–25]. Because they do not accumulate massive amounts of organ storage iron, heterozygotes do not develop the disease-related morbidity that occurs among homozygotes[12–20]. Data about hemochromatosis and beta-thalassemia minor, and the possibility of an increased risk for the development of extra-hepatic carcinoma in heterozygotes will be presented in other chapters of this book.

Genotype/phenotype correlations among heterozygotes

Individuals who are heterozygous for the C282Y or the H63D mutation of the *HFE* gene may have an elevated amount or a normal amount of body storage iron[26, 27]. It is not yet known if the H63D mutation is a neutral mutation, or if it worsens the effect of the C282Y mutation in subjects who are compound heterozygotes for these mutations of *HFE*. Most individuals who are compound heterozygotes develop progressive iron loading[26, 27]. Other chapters in this book will present detailed information about the frequency of one or two copies of C282Y, of one or two copies of H63D, the frequency of the compound heterozygous state with both C282Y and H63D, and the iron phenotype among individuals who have these genotypes.

Conclusions and future studies in hemochromatosis heterozygotes

The data presented in this chapter were based on half-HLA-identity to a homozygous relative, prior to the widespread availability of a test for the C282Y mutation of the *HFE* gene. In the future, individuals can be classified as heterozygotes based on the presence of one copy of the C282Y mutation. After a large group of heterozygotes with the C282Y mutation are identified, it will be possible to verify the results of their serum iron concentration, transferrin saturation, and serum ferritin concentration and compare these results to those in the large group of heterozygotes presented in this chapter. It is expected that the results will be similar to the findings presented here. It is likely that studies of mutations of the *HFE* gene heterozygotes or compound heterozygotes will help in the understanding of the function of *HFE*.

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Ascertainment of hemochromatosis heterozygosity

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Introduction

There is now incontrovertible evidence that early diagnosis and therapy of hemochromatosis prevents virtually all manifestations of the disorder and results in normal life expectancy[1, 2]. In contrast, unrecognized and untreated hemochromatosis leads to hepatic cirrhosis, hepatocellular carcinoma, and other lethal complications. A statistically significant increase in risk among heterozygotes for hemochromatosis has been demonstrated: for colorectal polyps in each sex; for colorectal cancer, diabetes mellitus, and hematologic malignancy in men; and for gastric cancer in women[3]. Heterozygotes for hemochromatosis with co-existing hematologic disorders such as idiopathic refractory sideroblastic anemia, hereditary spherocytosis, pyruvate kinase deficiency[4–7] or sporadic porphyria cutanea tarda[8, 9] may develop iron overload sufficient to cause overt organ damage[1, 10]. In addition, the identification of a putative heterozygote provides the opportunity to conduct studies of family members to identify individuals who are homozygous for the disorder. Thus early identification of homozygotes and heterozygotes for hemochromatosis is an important clinical challenge. This chapter provides a review of methods for determination and prevalence estimation of hemochromatosis heterozygosity including kinship and HLA analysis, population surveys, and statistical mixture modeling of transferrin saturation data. Criteria used for hemochromatosis screening studies are reviewed and suggestions for screening based on transferrin saturation are discussed.

Determination of heterozygosity

Kinship and HLA analysis

Hemochromatosis is the most common autosomal recessive disorder in Caucasian populations[11–19]. For pedigree analyses based on HLA typing of index subjects and family members, three different genotypes, determined by a one-locus, two-allele model, have been proposed: homozygous normal; heterozygous for the hemochromatosis allele; and homozygous for the hemochromatosis allele. Pedigree members are assigned genotypes on the basis of having none, one, or two HLA haplotypes in common with the index subject. Subjects sharing one HLA haplotype with the index subject are classified as putative carriers and thus heterozygous for hemochromatosis[20].

The Hardy–Weinberg equilibrium

For an autosomal locus with two alleles, normal gene segregation in a large population is assumed, with equal allele frequencies for men and women, no mutation, selection, or migration, and random mating with respect to genotype. For two alleles with frequencies q and $p = 1 - q$, then genotype frequencies are said to be in Hardy–Weinberg proportions, p^2, $2pq$, and q^2, and represent the proportion of normal homozygotes, heterozygotes, and affected homozygotes in the population, respectively. If allele frequencies achieve equilibrium (tend to remain constant from generation to generation), and if genotype frequencies are in Hardy–Weinberg proportions, then this is known as the Hardy–Weinberg equilibrium[21]. In hemochromatosis population studies the abnormal gene frequency (q), also referred to as the frequency of the allele, is often calculated based on the measured proportions of affected homozygotes and heterozygotes in the population. The expected number of affected homozygotes and heterozygotes is then calculated from the Hardy–Weinberg equation, $p^2 + 2pq + q^2 = 1$. A hypothesis test of the Hardy–Weinberg equilibrium is routinely conducted to compare the observed frequencies to expected frequencies assuming Hardy–Weinberg proportions[22].

Frequency of heterozygosity

Family studies and population surveys

In a review of the epidemiology of hemochromatosis, Neiderau and colleagues reported results of four large prospective family studies and eleven prospective surveys of asymptomatic subjects[23]. Investigators who performed family studies in the United States, Australia, and France have found a gene frequency of 0.5–11.6 per 1000 and a prevalence of heterozygotes from 106 to 160 per 1000 subjects. Prospective surveys based predominantly on the principle of identifying homozygotes in the population were conducted in Australia, Canada, England, Finland, Sweden, South Africa, and the United States. From these surveys, the estimated gene frequencies were 23–107 per 1000 persons, corresponding to a frequency of heterozygotes of 43–191 per 1000. The variability in estimated prevalence of heterozygotes may be explained in part by methodological differences between the studies, including the screening criteria for use of transferrin saturation and serum ferritin concentration values[23]. The fact that all homozygotes do not express the hemochromatosis phenotype also contributes to differences in prevalence estimates.

Distribution of transferrin saturation values

An elevated transferrin saturation value is the earliest phenotypic abnormality in persons with hemochromatosis. Examination of family members of hemochromatosis probands has suggested that mean transferrin saturation values in heterozygotes is higher than among unaffected subjects, but lower than in homozygotes. For example, the mean values for transferrin saturation ranged from 64% to 95% in homozygotes, from 35% to 48% in heterozygotes, and from 29% to 35% in individuals without the hemochromatosis gene in seven studies[11, 13, 24–28]. In contrast, a segregation analysis of family data from Brittany, France that utilized transferrin saturation as a quantitative indicator of hemochromatosis did not find evidence that heterozygotes had significantly higher transferrin saturation values than normal homozygotes. Families in which the segregation pattern was ambiguous were included in the analysis. This may have reduced the distinction between normal homozygotes and heterozygotes with respect to transferrin saturation values[29]. Recently, Bulaj et al.[9] performed a cross-sectional analysis of 1058 genotyped heterozygotes to define the effects of age and sex on the phenotype. They found that mean serum iron concentrations and transferrin saturation values were higher in heterozygotes than in normal subjects, and did not increase with age.

Mixture distribution analysis

Investigators in Utah evaluated 11 065 blood donors who had initial transferrin saturation values that were $>50\%$, and whose repeat transferrin saturation values were $>62\%$[25]. If 12.5% of Caucasians in the United States are heterozygous for the hemochromatosis gene as suggested, then the higher mean transferrin saturation of heterozygotes might affect the distribution of transferrin saturation in the general population. Based on the hypothesis that the distribution of transferrin saturation values in persons in the United States reflects several subpopulations based on individual genotype for hemochromatosis, McLaren and colleagues used a statistical technique to separate finite mixtures of distributions to quantify these groups. They examined the distribution of transferrin saturation in the second National Health and Nutrition Examination Survey (NHANES II) to determine the proportion of hemochromatosis heterozygotes and the proportion of unaffected individuals in the population, and to obtain an independent estimate for the frequency of the hemochromatosis gene in the United States based on these proportions[19].

Methodology for mixture distribution modeling

Statistical mixture modeling is a technique that can be used to separate an observed distribution into the component subpopulations[30, 31]. Because transferrin saturation values are normally distributed in normal homozygotes[19], McLaren and colleagues applied this technique to transferrin saturation data from NHANES II to estimate the parameters of a mixture of normal distributions. The statistical test used to determine the best-fitting model was based upon the likelihood ratio statistic. For the observed distribution, the maximized log-likelihood function for a mixture of normal distributions ($\log L_1$) was evaluated and compared with the maximized log-likelihood function for a single normal distribution ($\log L_0$). Significance of the likelihood ratio statistic, $[-2 \log (L_0/L_1)]$, was assessed using a re-sampling technique[32]. A value of $p<0.05$ indicates a better fit to a mixture of normal distributions. The chi-square statistic was then used to test goodness of fit of the observed distribution to the best-fitting model. For the chi-square test, p values close to 1.0 indicate an excellent fit to the specified model, and values of $p<0.05$ indicate a poor fit. The computer program DISFIT[33], a specialized program that provides parameter estimates and the likelihood ratio statistic, was used for data analysis.

Application to data from NHANES II

For unweighted sample data from NHANES II, analysis of the distribution of transferrin saturation showed a significantly better fit to two normal populations than to a single normal population for men (likelihood ratio statistic: 85.3; $p<0.01$) and women (likelihood ratio statistic: 108.3; $p<0.01$), after removing values for possible homozygotes. The goodness of fit to a mixture of two normal populations was excellent for men ($p=0.813$) and good for women ($p5=0.177$). The observed and fitted distributions for transferrin saturation values according to sex are displayed in Fig. 40.1. When proportions of subjects in corresponding subpopulations were compared for men and women, they were similar ($p=0.124$ for both subpopulations), but the mean transferrin saturations were significantly different ($p<0.0001$).

When weighted to reflect the entire US adult population, the estimated proportion of men in a subpopulation with the lower mean transferrin saturation of 29.7% was 0.85 (95% confidence interval 0.814–0.894) and the estimated proportion of subjects in a subpopulation with the higher mean transferrin saturation of 47% was 0.15 (95% confidence interval 0.106–0.186). The estimated proportion of women in a subpopulation with the lower mean transferrin saturation of 27% was 0.87 (95% confidence interval 0.837–0.901), and the estimated proportion of subjects in a subpopulation with the higher mean transferrin saturation of 44.7% was 0.13 (95% confidence interval 0.100–0.162). The hemochromatosis gene frequencies were estimated to be 0.081 for men and 0.070 for women, corresponding to prevalences of homozygotes of 6.6 and 4.8 per 1000, respectively. The prevalence of heterozygotes was 150 per 1000 for men and 130 per 1000 for women[19]. These estimates of prevalence and gene frequency are similar to those calculated on the basis of the Utah study[25].

Application to data from an Australian population

McLaren and colleagues also applied statistical mixture modeling to population data from asymptomatic Australians[17] and compared the mean transferrin saturation values in subpopulations to those from hemochromatosis heterozygotes and homozygotes identified in pedigree studies. Modeling results support the conclusion that the means for transferrin saturations in male and female heterozygotes differ from those in unaffected subjects (Fig. 40.2). After removal of hemochromatosis homozygotes, two populations of transferrin saturation were identified ($p<0.01$). In analysis of data from men, two populations were detected. One, composed of 88.2% of the values, was characterized by a mean transferrin saturation of 24.1%, and may have included predominantly individuals who are unaffected by hemochromatosis alleles; the second subpopulation (11.8% of the values) had a mean transferrin saturation of 37.3%, and have been composed predominantly of individuals who are heterozygous for the hemochromatosis gene (Fig. 40.2(*a*)). Data from women was similar (Fig. 40.2(*b*)); individuals corresponding to 88.2% of the values had mean transferrin saturation of 22.5%, and may have included women who did not inherit a gene for hemochromatosis; the second subpopulation (11.8% of the values) had an increased mean transferrin saturation of 37.6%, and may have represented predominantly individuals who are heterozygous for the hemochromatosis gene. For comparison, in the Australian population of 343 known heterozygotes, the mean transferrin saturation was 37.1% for males and 32.5% for females[34].

Screening for hemochromatosis

Cost-effectiveness analyses indicate that the cost of screening and treatment is far less than the medical cost of treating chronic disabilities resulting from the secondary disease conditions such as hepatic cirrhosis and diabetes mellitus due to iron overload[35–38]. In population screening, the number and proportion of individuals identified to have hemochromatosis depends, in part, upon the specific initial screening criteria applied. Different screening criteria have been applied in published studies. For example, to establish the diagnosis of hemochromatosis in an Australian study of 1968 employees of two large corporations, subjects who had an initial transferrin saturation $>$ 45% were recalled. Those with a consistently elevated serum ferritin concentration (men >200 μg/l and women >150 μg/l) and transferrin saturation $>45\%$ underwent percutaneous needle biopsy for determination of hepatic iron concentration and hepatic iron index[17]. By comparison, a study of 11 065 Utah blood donors evaluated subjects with a transferrin saturation $>50\%$ initially, and $>$ 62% on repeat testing[25]. To evaluate the feasibility of screening patients for hemochromatosis, Balan and colleagues[30] conducted a pilot study wherein more than 12 000 unselected patients were screened prospectively. Serum iron concentrations were determined in each subject; in samples with serum iron concentrations of ≥ 180 μg/dl, the iron assay was repeated and the total iron-binding capacity was measured to permit derivation of the transferrin saturation. Diagnosis of hemochromatosis was determined by hepatic biopsy performed on patients who

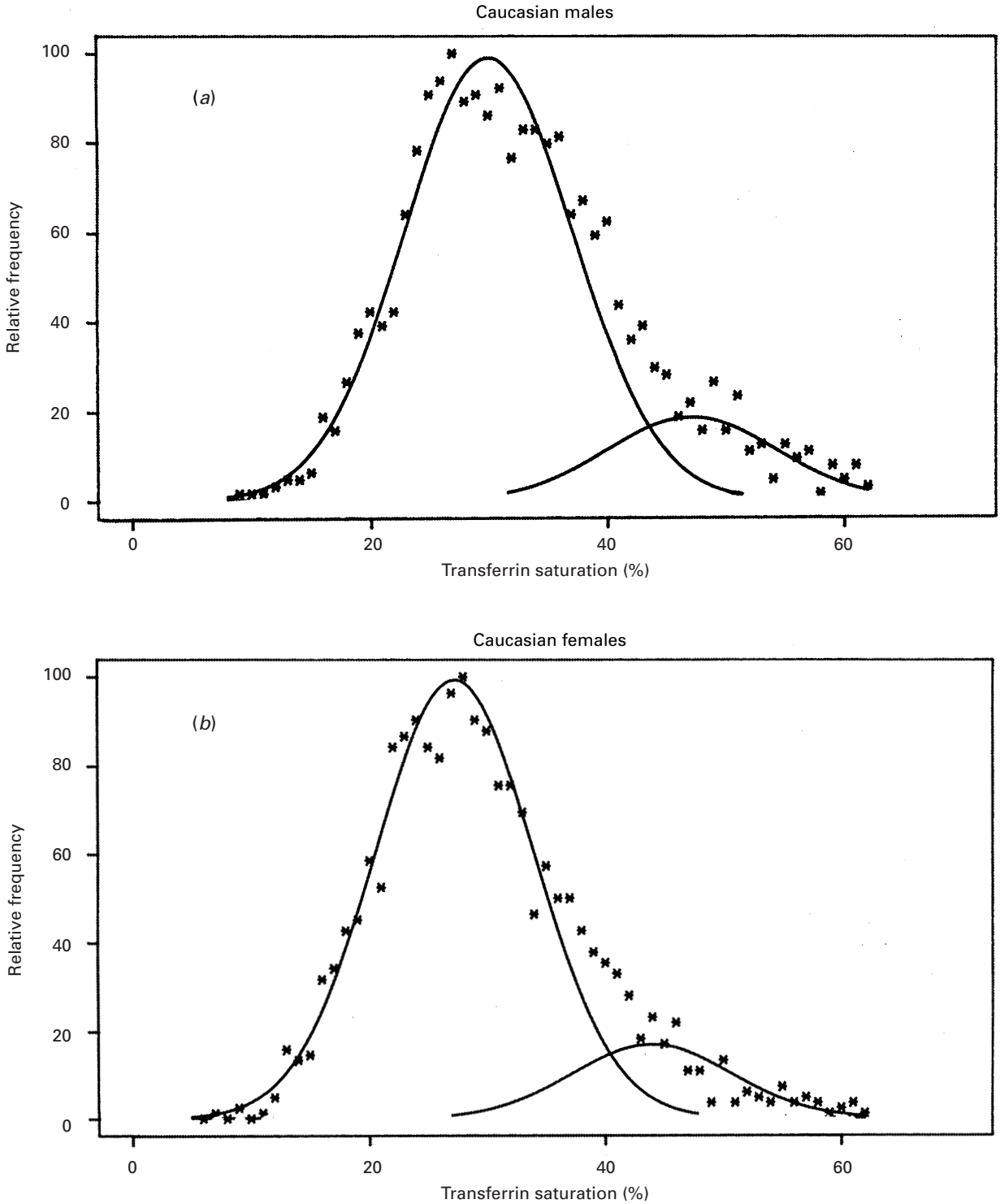

Fig. 40.1. Distribution of transferrin saturation values in (*a*) 1325 Caucasian men and (*b*) 1547 Caucasian women. Observed data are indicated with a solid line. The relative frequency indicates the proportion of subjects having transferrin saturation values within a given interval, relative to that of the interval containing the maximum frequency. For each interval the relative frequency is calculated by (frequency/maximum frequency) $\times 100$.

had ≥62% transferrin saturation and serum ferritin concentration ≥400 μg/l on the second determination. Baer et al.[37] screened for hemochromatosis in more than 3000 asymptomatic ambulatory men ≥30 years of age by measuring transferrin saturation. Subjects with repeated transferrin saturation ≥62% and ferritin concentration ≥500 μg/l were referred for hepatic biopsy. Subjects with iron studies and hepatic histology results consistent with hemochromatosis were treated with phlebotomy until iron deficiency developed, and total iron stores were calculated (200 mg of elemental iron per unit of phlebotomy blood)[37]. For consistent population screening, guidelines are needed for the use of initial non-invasive methods to identify subjects who should undergo further evaluation.

Screening levels based on transferrin saturation

For transferrin saturation values, the upper limit of normal of 50% is frequently quoted[39], although a sustained level of >62% has been proposed to screen for homozygotes[40,41]. A major concern is that applying a threshold value that is too high may not identify some hemochromatosis homozygotes, leading to increased medical care costs from chronic disabilities resulting from the complications of iron overload. To evaluate potential screening levels, McLaren and colleagues examined transferrin saturation data from 485 hemochromatosis heterozygotes and homozygotes[18,42–44]. An objective of the analysis was to identify the most appropriate level of transferrin saturation to maximize identification of hemochromatosis homozygotes, while minimizing mis-identification of hemochromatosis heterozygotes and unaffected normal individuals[34]. They examined successive transferrin saturation thresholds, and calculated the proportion of homozygotes above a given threshold and the corresponding proportion of heterozygotes below that threshold. These results were compared to the population mixture modeling of data from the asymptomatic individuals to evaluate potential transferrin saturation screening levels (Fig.40.2). For a given threshold, the predicted proportion of unaffected individuals below the threshold and the predicted proportion of heterozygotes above the threshold were calculated (Table 40.1). This probability analysis suggests that an upper limit of 45% is the lowest screening value that could be used for identifying individuals at risk for hemochromatosis without unnecessary testing of unaffected normal individuals. Based upon mixture modeling, if a transferrin saturation of 45% were used for screening, none of postulated unaffected individuals would undergo further testing unnecessarily, and approximately 19% of the postulated heterozygotes would be identified. For comparison, 98% of true hemochromatosis homozygotes with phenotypic expression of the disorder and 22% of true hemochromatosis heterozygotes were identified using this transferrin saturation screening level.

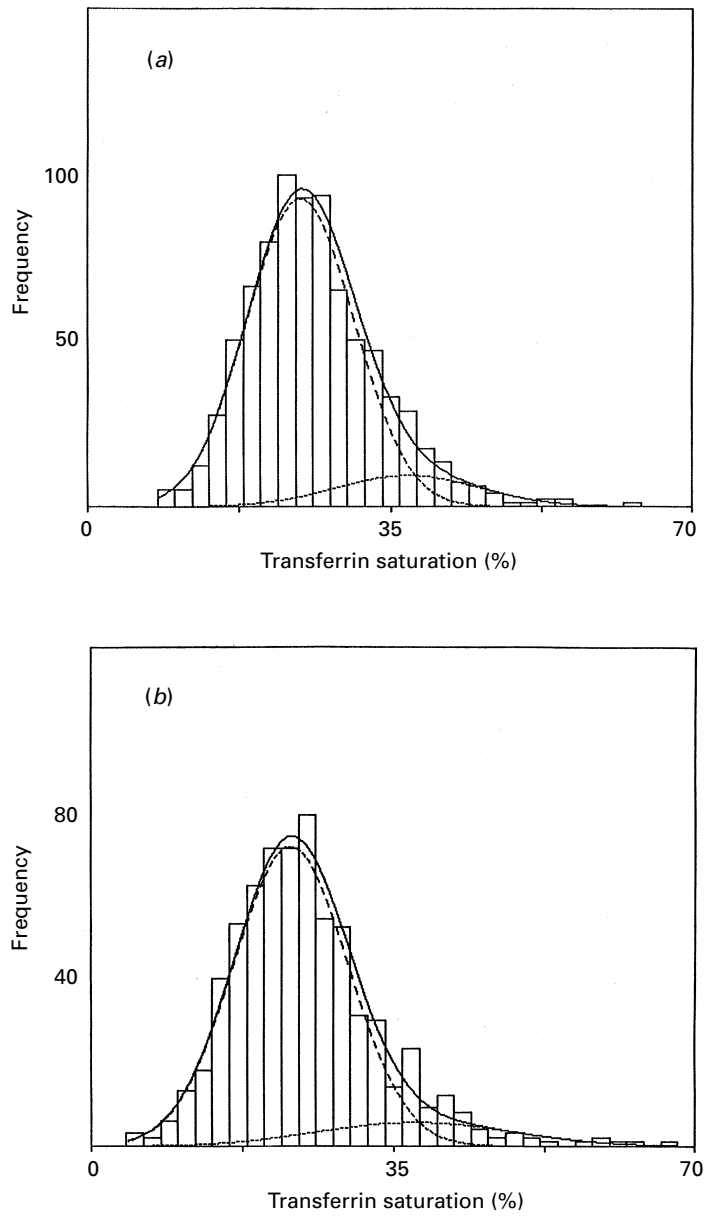

Fig. 40.2. Distribution of transferrin saturation values among asymptomatic Australians: (*a*) 796 men and (*b*) 669 women. The dashed lines represent the fitted curves for individual subpopulations. The overall fitted mixture distribution is shown with a solid line.

These analyses confirm that the sensitivity of the test for identification of homozygotes decreases as the screening

Table 40.1. Transferrin saturation levels in postulated unaffected and heterozygous subjects and in known heterozygous and homozygous individuals[a]

Transferrin saturation, %	Postulated unaffected[b] *n* (% of 1292)	Postulated heterozygotes[c] *n* (% of 173)	Known heterozygotes[c] *n* (% of 343)	Known homozygotes *n* (% of 142)
≥40	5 (0.4)	59 (34.1)	102 (29.7)	141 (99.3)
≥45	0 (0.0)	33 (19.1)	76 (22.2)	139 (97.9)
≥50	0 (0.0)	13 (7.5)	53 (15.5)	135 (95.1)
≥55	0 (0.0)	5 (2.9)	34 (9.9)	131 (92.3)
≥60	0 (0.0)	1 (0.6)	25 (7.3)	122 (85.9)
≥62	0 (0.0)	1 (0.6)	20 (5.8)	120 (84.5)

Notes:

[a] Reproduced from ref. [34].

[b] Derived from the statistical modeling of transferrin saturation data from asymptomatic Australians.

[c] These individuals were identified by pedigree studies and HLA-typing of hemochromatosis probands.

value is raised. For example, a threshold of 60% would identify only 86% of the known homozygotes (Table 40.1). Using a transferrin saturation screening level of 62%, a value previously proposed to screen for homozygotes[40, 41], 85% of homozygotes with expression of the disorder and 6% of heterozygotes would be identified. This is consistent with findings of Bulaj and colleagues[9]. In a study of the distribution of transferrin saturation values in hemochromatosis heterozygotes and normal subjects, they found that 4% of male heterozygotes had an initial transferrin saturation level of >62%[9]. In practice, a fasting transferrin saturation value of ≥ 45% identifies 98% of affected homozygotes[34].

DNA-based diagnostic test

Feder et al.[45] have identified a strong candidate gene for hemochromatosis, highlighting the need for a simple, inexpensive screening test for phenotypic expression of the disorder. Due to expense, a DNA-based diagnostic test for the candidate gene might need to be applied selectively to putatively affected individuals identified by a simple reliable test of disease expression, e.g., transferrin saturation. Cost-effectiveness could be increased by the addition of serum ferritin evaluation, conveniently done at the time of a second, confirmatory transferrin saturation determination.

Acknowledgments

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41

Disease risk in hereditary hemochromatosis heterozygotes

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Introduction

The fitness of heterozygotes with recessive inborn errors of metabolism has been the subject of debate ever since Haldane first hypothesized that the fixation of these genes at high frequency in human populations must be due to some selective advantage of heterozygosity[1]. The field of evolutionary genetics is firmly divided between those who believe this theory and continue (sometimes in vain) to find evidence for it, those who find apparent risk of disease in heterozygotes of most recessive disorders[2], and those who believe that the prevalence of heterozygosity observed is a laboratory artifact of little evolutionary significance[3]. It has, for instance, been hypothesized that females heterozygous for hemochromatosis gene(s) are able to maintain a higher hemoglobin concentration during pregnancy[4] and therefore have healthier babies[5]. One of the more interesting dichotomies in this area of debate is that heterozygotes for mutations that cause phenylketonuria have lower intelligence than persons in the general population[6], but mothers of phenylketonuria homozygotes have larger families than persons who do not carry the phenylketonuria mutation[7]. Other recessive disorders cause serious illness in heterozygotes, e.g., homocystinuria, ornithine transcarbamylase deficiency and Fabry disease[2]. It has been proposed that an '. . . appreciable fraction of "multifactorial" genetic liabilities for common . . . disease might simply be due to heterozygosity for genes whose homozygous effects are already well known'[8].

Disease risk in hemochromatosis heterozygotes

Interest in disease risk in hemochromatosis heterozygotes arose from several perspectives. First, several investigators have found that hemochromatosis heterozygotes in well-characterized groups have higher iron stores than persons without hemochromatosis genes[9–11]. The relationship of mild elevation of iron stores, well below the levels observed in hemochromatosis homozygotes, and the risk of disease was observed in several reports about coronary heart disease[12, 13] and cancer[14, 15]. The strongest association has been observed with benign adenomas of the colon[16]. The risk for adenoma was twice as high in individuals with serum ferritin concentrations above the mean for the population as in those with serum ferritin concentrations below the mean. In a quintile comparison, those with serum ferritin concentrations between 130 ng/ml and 256 ng/ml (the fourth quintile) had more than five times the risk of adenoma as did individuals with the lowest serum ferritin concentrations (Fig. 41.1). These individuals were categorized exclusively by their serum ferritin concentrations, and not by any family history of hemochromatosis[16].

The sex of study subjects and their expression of hemochromatosis gene(s) are the strongest predictors of elevated iron stores among persons in the USA. Therefore, it was hypothesized that hemochromatosis heterozygotes might be at increased risk for heart disease[17] and cancer in general, and for colorectal neoplasia in particular[18]. Though hemochromatosis heterozygotes have been clearly identified through HLA linkage in established hemochromatosis kindreds[9–11], their health histories have not been reported, nor were these individuals old enough or were the kindreds large enough to determine if hemochromatosis heterozygosity was a risk factor for disease.

An alternative method of hemochromatosis heterozygote ascertainment was therefore sought. A cohort was reported in which the exposure variable was heterozygosity for hemochromatosis[18]. The exposed group consisted of the parents of individuals homozygous for hemochromatosis gene(s) who would be predominantly hemochromatosis heterozygotes. An unexposed group in the cohort

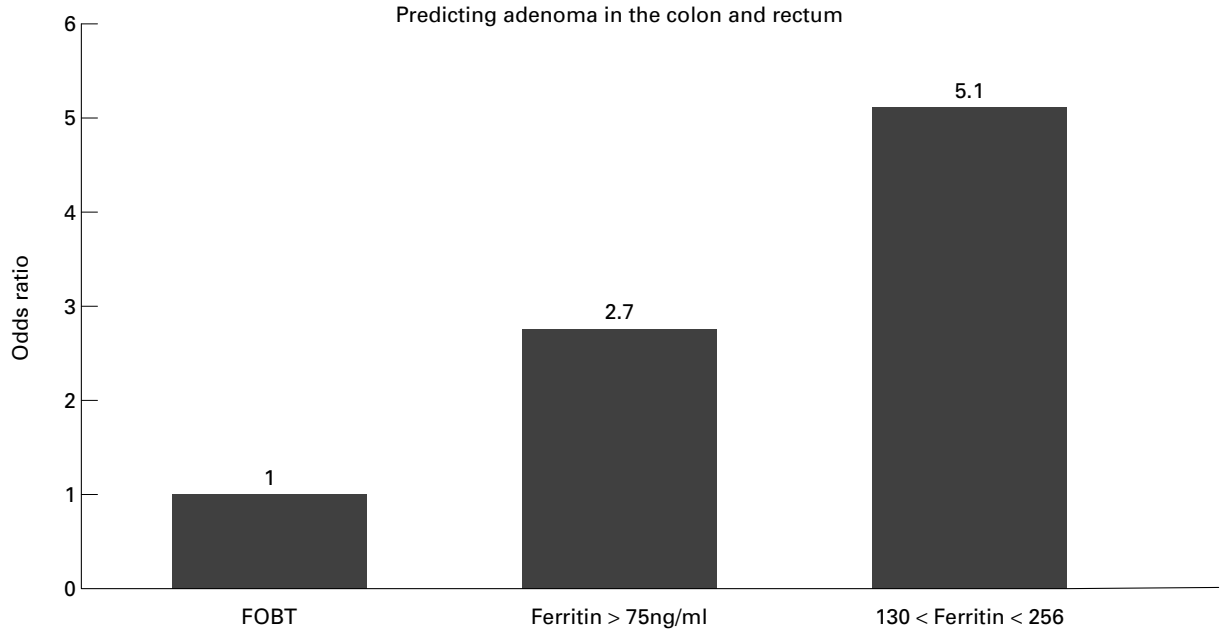

Fig. 41.1. Serum ferritin concentration as a predictor of colonic adenoma. The population mean in the group tested was 75 ng/ml. FOBT = fecal occult blood testing.

consisted of parents of spouses or co-habitants of homozygotes who were far less likely to have a hemochromatosis gene. Persons homozygous for hemochromatosis genes were identified from membership lists of two American hemochromatosis and iron overload awareness and advocacy groups. In a postal survey, hemochromatosis patients were asked to confirm their hemochromatosis diagnosis, provide their current age and date of diagnosis and document the method of diagnosis (hepatic biopsy, serum transferrin saturation, and serum ferritin concentration). Persons in the spouse or co-habitant group were asked if they had any blood relatives with hemochromatosis, and to document their relationship to the homozygous hemochromatosis subject. All subjects were asked to provide disease information about their parents. This included the parents' year of birth, whether the parents were still alive and, if not, whether cancer, heart disease, or stroke were the cause of death for either parent. In addition, the occurrence of specific cancers (breast, cervix, colorectal, stomach, pancreas, lung, blood), colonic polyps, heart attack, hypertension, stroke and diabetes was requested for all parents. Adequate statistical power (0.80) was present to detect a 50% change in risk within persons of the same sex for the most common outcomes: cancers of the lung, colon and rectum, and breast, heart disease, hypertension, diabetes mellitus, stroke, colorectal polyps, and death from cancer, stroke, or heart disease. However, the statistical power was less than 0.8 for the rarer events such as cancers of the pancreas, stomach, cervix, or blood.

Disease information from 975 confirmed hemochromatosis homozygotes reflected 1950 parents heterozygous for hemochromatosis gene(s), and that from the 828 partners reflected 1656 unexposed parents who were unlikely to have hemochromatosis gene(s). At the time of survey, the percent of female parents of hemochromatosis homozygotes alive was 36%; this did not differ significantly from that of the unexposed group (37%). The percent of male parents of hemochromatosis homozygotes alive was 19%; this also did not differ from that of the unexposed group (22%). The mean ages of the exposure groups either at death or at the time of the survey also were not significantly different (70.9 years vs. 71.8 for males, and 75.0 vs. 74.1 years for females). The number and proportion of each cause of death and disease diagnosis in the cohort are displayed in Table 41.1. The age adjusted relative risk and 95% confidence interval for neoplastic diseases between exposure groups of each gender are presented in Table 41.2. A statistically significant increase in risk in the exposed group was observed for colonic polyps in both males and females, for colon and rectal cancer and hematologic malignancy in males, and for gastric cancer in females. Death from cancer occurred with equal frequency in both male and female exposure groups. Data on additional diseases surveyed are shown in Table 41.3. An increase in risk

Table 41.1. Diagnoses in 1950 exposed (hemochromatosis heterozygotes) and unexposed (non-hemochromatosis gene carrier) subjects

	Females				Males			
	Exposed	Unexposed	Total	%[a]	Exposed	Unexposed	Total	%[a]
Cancer death[b]	144	129	273	24	166	138	304	22
Cardiac death[b]	112	88	200	22	210	156	366	32
Stroke death[b]	89	55	144	16	80	57	137	12
Diabetes mellitus[b]	107	72	179	13	93	59	152	11
Heart disease[b]	155	129	284	20	259	213	472	34
Stroke[b]	140	105	245	17	132	100	232	17
Hypertension[b]	232	178	410	29	146	107	253	18
Colon polyp	64	27	91	5	54	33	87	5
Cancer of:								
colon	45	36	88	5	47	26	73	4
pancreas	15	9	24	1	16	11	27	2
stomach	26	12	38	2	21	19	40	2
stomach[c]			25	1			25	1
lung	21	31	52	3	42	54	96	5
lung[d]			44	2			88	5
blood	16	11	27	2	23	12	35	2
breast	66	63	129	7				
cervix	54	44	98	5				

Notes:

RR = age-adjusted relative risk, comparing exposed (hemochromatosis heterozygotes) to unexposed, CI = 95% Cornfield confidence interval.

[a] In the case of cancer, cardiac and stroke death, the denominators in use to calculate proportions are data of those deceased.

[b] Hemochromatosis Research Foundation survey population only (1442 exposed and 1270 unexposed individuals).

[c] Excluding subjects with pancreas or colorectal cancer; some subjects may have confused the word 'stomach' with the word 'abdominal'.

[d] Excluding subjects with gastrointestinal cancer, who may have had pulmonary metastases.

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in the exposed group of borderline statistical significance was seen for diabetes mellitus in males. The risk of heart disease or heart disease as a cause of death did not differ between exposure groups.

These data demonstrate a modest but statistically significant increase in risk among heterozygotes for hemochromatosis for colorectal polyps in each gender, for colon and rectal cancer, diabetes mellitus, and hematologic malignancy in males, and for gastric cancer in females. The elevation of risk among male hemochromatosis heterozygotes for some of the diseases (colon and rectal cancer, blood cancer, diabetes mellitus) is in concordance with previous studies of iron stores and disease risk[14, 15]. This implies that there might be a threshold of iron exposure that must be exceeded before disease risk is increased for some diseases, and that menstruation might protect women heterozygotes for hemochromatosis gene(s). These results differ from those of the NHANES-I[14] and Finnish[15] studies in that the risk of lung cancer was not significantly increased in hemochromatosis heterozygotes of either sex, but was slightly decreased (Table 41.2). These findings regarding hematologic malignancy are also contrary to the Finnish results[15].

Factors causing disease risk among persons with hemochromatosis genes

Iron has long been known to play a role in heart disease risk. For hemochromatosis homozygotes, this is due to iron storage in the myocardium and resulting cardiomyopathy[19]. It has been hypothesized that hemochromatosis heterozygotes are at increased risk for heart disease[17]. Two epidemiologic studies have supported the association of elevated body iron with heart disease[12, 13], though seven subsequent larger studies have shown no association

Table 41.2. Age-adjusted relative risk for neoplasia in hemochromatosis heterozygotes

Disease	RR, females	95% CI	RR, males	95% CI
Cancer as cause of death	0.93	0.81–1.08	1.05	0.93–1.18
Colon polyp	1.29	1.08–1.53	1.24	1.05–1.46
Cancer of:				
colon	1.08	0.87–1.34	1.28	1.07–1.53
pancreas	1.24	0.88–1.75	1.11	0.80–1.55
stomach	1.27	0.98–1.64	1.10	0.82–1.48
stomach[a]	1.37	1.04–1.79	1.04	0.73–1.48
lung	0.74	0.51–1.09	0.91	0.73–1.14
lung[b]	0.68	0.44–1.06	0.92	0.73–1.15
blood	1.04	0.73–1.48	1.30	1.03–1.63
breast	0.98	0.81–1.19		
cervix	1.08	0.89–1.32		

Notes:
RR = age-adjusted relative risk, comparing exposed (hemochromatosis heterozygotes) to unexposed, CI = 95% Cornfield confidence interval.
[a] Excluding subjects with pancreas and colon cancer. Some subjects may have misinterpreted 'stomach' to mean 'abdomen'.
[b] Excluding cases of gastrointestinal cancers that may have metastasized to lung.
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Table 41.3. Age-adjusted relative risk for non-neoplastic disease in hemochromatosis heterozygotes

Disease	RR, females	95% CI	RR, males	95% CI
Fatal heat attack	1.02	0.88–1.18	1.04	0.93–1.16
Fatal stroke	1.14	0.99–1.33	1.09	0.94–1.28
Heart disease	1.01	0.89–1.14	1.01	0.90–1.12
Stroke	1.05	0.93–1.19	1.05	0.92–1.19
Hypertension	1.01	0.90–1.12	1.05	0.92–1.19
Diabetes mellitus	1.05	0.91–1.21	1.16	1.01–1.33

Notes:
These data were compiled from a survey of Hemochromatosis Research Foundation respondents and consisted of 1442 exposed (hemochromatosis heterozygotes) and 1270 unexposed (non-hemochromatosis gene carrier) subjects.
RR = Age Adjusted Relative Risk
CI = Confidence Interval
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between iron repletion and heart disease[20–26]. The hemochromatosis heterozygote cohort data for heart disease as a lethal event and for the prevalence of heart disease (Table 41.3) also show no evidence of increased risk associated with hemochromatosis heterozygotes[18].

This study could be biased towards positive results if responses to the survey were based on family history related to cancer or heart disease. Evidence against this source of bias in the data is found in the specificity of the results. The trends towards diminished risk in lung cancer and the absence of any discernible trend for breast cancer, cervical cancer, heart disease or cancer death suggest that responses were not preferentially received from individuals with relatives with the diseases where significant positive associations were observed. Several factors may have biased this study towards underestimation of risk, particularly for those diseases (e.g., colorectal neoplasia) that were significantly associated with hemochromatosis heterozygosity. Regarding misclassification bias and the accuracy of genotyping, the unexposed group must include some undiagnosed hemochromatosis homozygotes (≤1%) and even more hemochromatosis heterozygotes (15%), due to the prevalence of hemochromatosis homozygosity and heterozygosity in the general population.

Inaccuracy of disease reporting is another potential source of misclassification in this study. Because this effect should be randomly distributed between exposure groups, this would also result in underestimation of risk. An area where this was apparent, and potentially correctable, occurred in reviewing the surveys related to cancers of the 'stomach' and 'lung'. Other abdominal cancers were often reported in the same individual with these diagnoses, implying that the word 'stomach' may have been interpreted to mean 'abdominal' by some respondents and 'lung' was in some cases probably interpreted to mean pulmonary metastases from a primary gastrointestinal cancer (Table 41.2). Further, not all hemochromatosis heterozygotes have elevated body iron stores. 30–35% have been found to have iron stores below the population mean for age and sex; only 36% have iron stores more than one standard deviation above the mean[11]. Therefore, hemochromatosis heterozygotes, though more likely to have iron stores above those measured in persons without hemochromatosis gene(s), exhibit a similarly broad range of iron stores like persons in the general population. It was not possible in this study to determine which hemochromatosis heterozygotes had elevated iron stores. If the risk of colonic neoplasia is mediated by body iron stores, it would be expected that this effect would not be as marked in this study as in a population whose members' body iron stores were measured directly. Thus the observed association of iron stores as a risk factor for all the above diseases would be muted in the study cited above.

Confirmatory data were sought in a second cohort of siblings of hemochromatosis homozygotes and their spouses or co-habitants (R.L. Nelson, F.G. Davis, V. Persky and E. Becker, unpublished data). A postal survey of a portion of

the hemochromatosis homozygotes and their co-habitants described was performed that asked them about the health histories of their siblings. There were three levels of hemochromatosis gene exposure: homozygotes, heterozygotes, and persons without hemochromatosis gene(s). The classification of hemochromatosis homozygote siblings known also to be hemochromatosis homozygotes was assumed to be accurate. Those siblings of hemochromatosis homozygotes not known to be hemochromatosis homozygotes were assumed to be hemochromatosis heterozygotes, an assumption that would be true approximately 67% of the time. It was also assumed that siblings of spouses would not be hemochromatosis gene carriers, an assumption that would be incorrect in approximately 15% of these siblings. The cohort consisted of 279 hemochromatosis homozygotes, 1265 persons categorized as hemochromatosis heterozygotes, and 1338 categorized as persons without hemochromatosis gene(s). Persons in this cohort were younger than those in the parental cohort, and a higher percentage were alive. If the hypothesis that heart disease would occur with greater frequency in younger individuals heterozygous for hemochromatosis gene(s), it should have been apparent. However, heart disease was less prevalent in both hemochromatosis exposure groups when compared to the unexposed group. Colon and rectal cancer, diabetes mellitus, and hepatoma were increased in hemochromatosis homozygotes, but not heterozygotes. Arthritis was increased in hemochromatosis homozygotes and heterozygotes. Both hemochromatosis homozygotes and heterozygotes were more likely to have suffered any serious illness than persons in the unexposed group. Due to the high probability of genetic misclassification in the hemochromatosis heterozygote and unexposed groups, the large variation of iron stores with each genotype, and the younger age of this cohort, negative findings for colon cancer and diabetes mellitus were probable.

Conclusion

Further studies of disease risk in hemochromatosis heterozygotes will be more precise if DNA-based techniques to classify heterozygotes accurately are used, if heterozygotes are followed to old age, and if persons in study groups are stratified based on measures of body iron stores.

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Part IX

Relationship of hemochromatosis to other disorders

42

Thalassemias and their interactions with hemochromatosis

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Thalassemia syndromes

Thalassemia syndromes are very frequent in some parts of the world, especially Mediterranean and Asiatic countries. When the genetic defect underlying these syndromes is severe, iron overload becomes one the major clinical problems and can be life-threatening.

Pathogenesis of iron overload

The pathogenesis of iron overload in patients with thalassemia is multifactorial. Iron overload can develop as a consequence of increased iron absorption from the gastrointestinal tract, similar to that which occurs in persons with hemochromatosis or transfusion-dependent anemias[1, 2]. Under normal conditions, iron homeostasis is maintained by modulation of absorption of dietary iron (the only way iron enters the body). This, in turn, is regulated by a balance between iron needed for the erythron (more than three-fourths of total body iron) and for storage[3]. Thus, all conditions characterized by increased erythropoiesis or by a reduction of iron stores are accompanied by enhanced iron absorption[4–6]. Moreover, patients who have anemia with increased but ineffective erythropoiesis are at greater risk of developing severe iron overload than patients with hemolytic anemias with increased and effective erythropoiesis, e.g., patients with hereditary spherocytosis, who rarely develop iron overload[7–9]. In the presence of severe anemia, intestinal hypoxia also appears to increase iron absorption[10].

Anemia is very mild in β-thalassemia heterozygotes and in persons with α-thalassemia in whom two or three alpha globin genes are functioning, and associated iron overload is rare. In these patients, inefficient erythropoiesis is relatively mild[2]. Iron overload is more prevalent in patients with thalassemia major and intermedia (thalassemia intermedia includes a wide and heterogeneous spectrum of globin defects, including homozygosity for β-mild mutations, double heterozygosity for β-thalassemia/HbE and for β-mild and β-severe mutations, and compound heterozygosity for β-thalassemia plus α or β chain variant such as HbE, HbC, etc.)[11–13]. In patients with thalassemia intermedia who are untransfused or only occasionally transfused, iron overload develops slowly and is mostly related to increased iron absorption due to persistent dyserythropoiesis and erythroid hyperplasia[1, 13]. In regularly transfused patients with thalassemia major, iron absorption is markedly reduced because erythroid hyperplasia is inhibited and iron overload develops at an earlier age, mostly from parenteral entry of iron with transfusions (Fig. 42.1)[14]. The role of ineffective erythropoiesis in the enhancement of iron absorption is emphasized by the evidence that patients with HbH disease, who have one α globin gene working normally and heterogeneous α globin synthesis by the defective genes, do not develop severe iron overload despite the presence of anemia, unless regularly transfused. The effect of hemolytic anemia caused by the excess of β chains precipitated in tetramers (HbH) does not appear to influence the iron status of these patients markedly[15].

The role of splenectomy in iron absorption of thalassemia patients is unclear. Several studies (mostly in subjects from Thailand affected by double heterozygosity for β-thalassemia and HbE) show that increased iron absorption occurs after splenectomy. Shortly after splenectomy, higher values of serum ferritin concentration can be found in these patients, possibly due to the absence of splenic macrophages and to a partial inability of remaining reticuloendothelial cells to retain iron[16–18].

Depending on the severity of ineffective erythropoiesis, intravascular and intramedullary hemolysis, the presence (or absence) of the spleen, and the transfusion regimen,

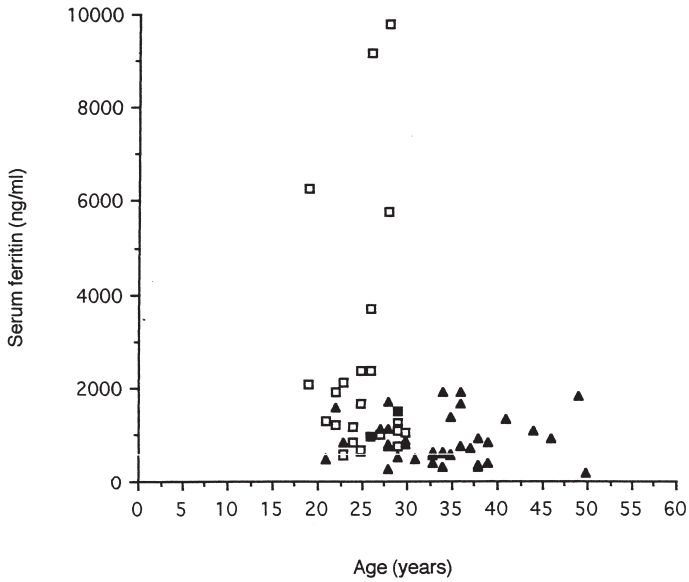

Fig. 42.1. Relationship between serum ferritin concentration and age in patients with thalassemia major (□) and intermedia (▲).

hepatic siderosis of differing characteristics and severity develops. When increased iron absorption predominates, excess iron is stored in hepatocytes where it can exert direct toxic effects. In iron overload that follows parenteral entry of iron or hemolysis, iron is deposited in Kupffer cells where it is less toxic. However, iron recirculates through the different cell compartments with progressive iron overload and, in the presence of severe siderosis, becomes evenly distributed in the liver. The most important and irreversible consequence of the hepatic iron burden is the development of cirrhosis. This occurs through a complex interaction among Kupffer cells, cytokines, and stellate cells, the key effectors in the fibrogenic process[19–21].

Hemochromatosis

Hemochromatosis is the most frequent cause of iron overload in Caucasian countries, where the prevalence of homozygotes is 1 in 400 and of heterozygotes 1 in 10[22]. In homozygotes, iron overload develops slowly but progressively due to poorly regulated absorption of dietary iron that occurs despite increased storage iron. The reasons for the absence of the normal down-regulation of iron absorption in the presence of iron overload are unclear. The recent identification of *HFE*, the candidate gene for hemochromatosis, will permit a better understanding of the mechanisms underlying this process[23]. A possible interaction between hemochromatosis and disorders that interfere with iron metabolism (i.e., hereditary spherocytosis, sideroblastic anemia, porphyria cutanea tarda, and heterozygosity for β-thalassemia) has been suggested to explain the iron overload sometimes found in persons with these conditions[24–31].

Interaction between heterozygous thalassemia and hemochromatosis

The coincidental co-existence of hemochromatosis and thalassemia, whose genes are located on different chromosomes, could result in a synergistic effect on iron absorption, leading to more severe iron overload and possibly to a modification of the natural history of the illnesses of affected patients.

Hemochromatosis heterozygotes

In populations of Caucasian origin where hemochromatosis and thalassemia are prevalent, the coexistence of the two disorders is not so unlikely. For example, in northern Italy where the prevalence of heterozygotes for hemochromatosis is 10%[32] and for β-thalassemia is approximately 3%[33], the expected probability of double heterozygosity is 0.003. A higher prevalence of HLA-A3, considered for its strong association with hemochromatosis as a genetic marker of the disorder[34, 35], was observed in Italian subjects with β-thalassemia trait and iron overload than in those without, and it was hypothesized that these subjects were double heterozygotes for thalassemia and hemochromatosis[27]. The coincidental inheritance of a gene for hemochromatosis and of a hemoglobin variant, Hb Olympia, has been suggested in a family in which increased iron absorption was detected[36], and also in several families in whom β-thalassemia trait and otherwise unexplained severe iron overload coexisted[24, 37]. Thus, although heterozygosity for hemochromatosis alleles rarely causes iron overload[38], a coexistent hemoglobin defect may lead to clinically manifest iron overload. No data are available on the coincidental co-inheritance of hemochromatosis and α-thalassemia.

The recent identification of *HFE* mutations associated with hemochromatosis could allow a more direct approach to the diagnosis of hemochromatosis[23]. However, the genetic heterogeneity of hemochromatosis appears to exist in different countries. In populations of northern European origin, the 'major' mutation of the putative hemochromatosis gene *HFE* (C282Y) is found in 6–10% of controls and in more than 90% of patients with hemochromatosis. In Italy, this mutation is detected in 0.5–1% of controls and in a markedly lower number of patients with

hemochromatosis[39–43]. In a recent multicenter Italian study of *HFE* mutations in a large number of patients with hemochromatosis, 70% of the patients in northern Italy were homozygous for the 'major' C282Y mutation, but only 40% in the southern Italy had C282Y. In addition, homozygotes for the C282Y mutation had a more severe phenotype. These results imply the existence of different genetic determinants, both linked and unlinked to Ch6p, and make a genetic diagnosis of heterozygous linked or unlinked in β-thalassemia patients inconclusive. In addition, the geographic origin of these patients, most of whom are from southern Italy, may increase the possibility that the C282Y mutation is absent.

Hemochromatosis homozygotes

Heterozygous β-thalassemia is present in more than 10% of Italian patients with homozygous hemochromatosis[44]. Analysis of the relation between *HFE* genotypes and β-thalassemia trait indicated that β-thalassemia is significantly more frequent in heterozygotes for the C282Y mutation and in patients carrying the wild-type genotype than in patients homozygous for this mutation. This finding implies that the coexistence of the two defects enhances the clinical manifestations of patients carrying an allele for a milder hemochromatosis-related defect. The influence of heterozygous thalassemia in homozygous hemochromatosis is confirmed by the observation that C282Y homozygotes had a more severe phenotype. These results suggest that thalassemia exerts a synergistic effect with hemochromatosis in inducing iron overload, even in patients with homozygous hemochromatosis.

A small group of African American patients with iron overload was recently described who had a higher incidence of thalassemia and hemoglobinopathy than Caucasian patients with hemochromatosis[45]. These patients had lower values of transferrin saturation, more iron in Kupffer cells, and a normal frequency of HLA-A3, although most of them had hepatic cirrhosis unexplained from other non-iron-related causes. Their clinical picture resembled that of the iron overload syndrome in Saharan Africans, in which there appears to be an unidentified genetic defect unrelated to HLA-A3 and therefore different from that found in Caucasian hemochromatosis[46]. At present, it is impossible to exclude the presence of heterozygous thalassemia or of a hemoglobinopathy associated with a genetic defect of iron absorption in some patients that causes severe iron overload, similar to that of 'classical' hemochromatosis, with a clinical phenotype indistinguishable hemochromatosis from that of 'classical' hemochromatosis.

Interaction between thalassemia intermedia or major and hemochromatosis

When thalassemia is more severe, as in patients with thalassemia intermedia or major, identification of the role of possible coexisting hemochromatosis genes is more difficult.

Thalassemia intermedia

Patients with thalassemia intermedia who are transfused only occasionally have markedly increased iron absorption and may develop severe iron overload that resembles that of patients with hemochromatosis, except for early iron deposition in Kupffer cells due to the hemolysis. Recently, *HFE* mutations have been sought in a group of patients with thalassemia intermedia who, in the majority of cases, were of Asian-African or Middle Eastern origin – populations in which the C282Y mutation is very rare[43, 47]; the C282Y mutation was detected in one of the 81 patients. This patient appeared to have much more severe iron overload than expected, and his clinical presentation resembled that of homozygous hemochromatosis. Based on these findings, it was suggested that the interaction of heterozygosity for hemochromatosis with thalassemia intermedia may lead to a clinical phenotype comparable to that found in homozygous hemochromatosis. However, this patient was diagnosed to have thalassemia intermedia at age 48 years, he underwent splenectomy at age 62, and his clinical presentation was more typical for homozygous hemochromatosis than for thalassemia intermedia. Taken together, these observations make the diagnosis of hemochromatosis doubtful, because it was based only on the presence of heterozygosity for the C282Y mutation. In Italy, 63% of the patients with hemochromatosis are homozygous for the C282Y mutation, despite the presence of HLA-identical siblings with a 'classical' hemochromatosis phenotype[40]. Alternatively, a more complex hematological defect, responsible for severe anemia and iron overload, could explain the hemochromatosis phenotype in a person who is heterozygous for hemochromatosis.

Our preliminary data on the prevalence of *HFE* mutations in Italian patients with thalassemia intermedia and major and control subjects is displayed in Table 42.1. Although *HFE* mutations were slightly more frequent in thalassemia patients than in controls, patients with *HFE* gene mutations do not have more severe iron overload. Interestingly, the older of two sisters who had an identical thalassemia genotype had the C282Y mutation. However, her iron status was similar to that of the younger sister who had the wild-type *HFE* allele (Table 42.2). Thus, heterozygosity for the C282Y

Table 42.1. Prevalence of *HFE* alleles and genotypes in patients with thalassemia intermedia and major

	Thalassemia intermedia ($n=37$)	Thalassemia major ($n=26$)	Controls ($n=139$)
Alleles			
C282Y	3/74 (4.1%)	3/52 (5.8%)	0 (0)
H63D	15/74 (20.3%)	7/52 (13.4%)	33/278 (11.9%)
Genotypes			
C282Y			
+/−	1/37 (2.7%)	3/26 (11.5%)	3/139 (2.2%)
+/+	1/37 (2.7%)	0/26 (0)	0 (0)
H63D			
+/−	7/37 (18.9%)	5/26 (19.2%)	29/139 (20.9%)
+/+	4/37 (10.8%)	1/26 (3.8%)	2/139 (1.4%)

Table 42.2. Characteristics of iron overload in two sisters with thalassemia intermedia with or without C282Y mutation

	ML	MV
Age, years	29	25
Hb, g/dl	9.9	9.8
Thalassemia genotype	cod 39/−87	cod 39/−87
Transferrin saturation, %	63	68
Serum ferritin, ng/ml	621	799
Hepatic iron concentration, μg Fe/mg dry weight	673	771
C282Y	+/−	−/−
H63D	−/−	−/−

mutation does not seem to modify the iron status of thalassemia intermedia patients. In addition, a 27-year-old female with β^0/β^+ genotype who was also homozygous for the C282Y mutation did not have more severe iron overload than other patients with the same thalassemia genotype. However, the youth of the patient could account for this finding. Follow-up of this patient and the family study will define better the interaction between homozygous hemochromatosis and thalassemia intermedia.

The relation between H63D mutation and iron overload in patients with thalassemia intermedia is reported in Table 42.3. Subjects homozygous for this mutation have slightly higher values of transferrin saturation and serum ferritin concentration than those with the wild-type *HFE* allele, suggesting that this 'minor' mutation of the *HFE* gene may also cause an alteration of iron absorption or metabolism when associated with other defects that also affect iron metabolism, e.g., porphyria cutanea tarda[31, 48, 49].

Thalassemia major

In transfusion-dependent patients with thalassemia major, the presence of a coincidental hemochromatosis-associated allele cannot be defined on the basis of the characteristics of iron overload alone. These subjects accumulate 1–2 g of iron monthly, and in a few years they develop an iron burden that causes significant morbidity and mortality. With early and intense subcutaneous chelation therapy, iron overload is usually limited, and the serum ferritin concentrations remain <1000 ng/ml. A preliminary study in Italian subjects with thalassemia major showed that a few patients, despite early treatment and good compliance, had more severe iron overload, although this could not be correlated with the occurrence of coincidental *HFE* mutations. However, the low prevalence of C282Y and H63D mutations in hemochromatosis patients from Southern Italy, the area of origin of most of thalassemia major patients examined, does not allow exclusion of the presence of hemochromatosis. Nevertheless, because the iron overload of subjects with thalassemia major is severe, the coexistence of hemochromatosis is unlikely to influence their iron overload.

Iron overload in 'ex-thalassemic' patients

A new clinical problem is the iron overload of patients with thalassemia major who have undergone successful bone marrow transplantation. These 'ex-thalassemic' patients usually have severe iron overload that can spontaneously decrease after a few years[50]. However, most of them are submitted to chelation and/or to phlebotomy therapy to reduce iron overload-related complications, particularly hepatic damage[51]. At this stage of their clinical history, coexistent hemochromatosis will interfere with their response to iron depletion therapy. Because most patients are transplanted with bone marrow from relatives, many of whom are heterozygous for β-thalassemia, they often have increased iron absorption due to a synergistic effect of hemochromatosis and ineffective erythropoiesis.

Therapy of iron overload in subjects with thalassemia and hemochromatosis

The coexistence of thalassemia with hemochromatosis implies therapeutic consequences that are more evident in

Table 42.3. Iron status and H63D genotype in thalassemia intermedia patients

H63D genotype	*n*	Age, years (mean ± S.D.)	Hb, g/dl	Hb F, %	Transferrin saturation, %	Serum ferritin, ng/ml
−/−	26	33 ± 13	9.5 ± 1.1	40 ± 38	79 ± 32	809 ± 822
+/−	7	32 ± 6	8.9 ± 1.0	41 ± 39	89 ± 21	798 ± 577
+/+	4	39 ± 8	8.3 ± 0.8	58 ± 37	103 ± 9	916 ± 396

the persons with mild thalassemia defects. In persons with thalassemia major, the severity of iron overload is marginally affected by the presence of hemochromatosis gene(s) and it is unnecessary to modify chelation programs[52]. Likewise, the occurrence of hemochromatosis in persons with thalassemia intermedia does not seem to influence the iron status of the patients markedly. Unless managed early and aggressively with chelation therapy, they develop progressive iron overload that causes severe complications, although at an older age than in persons with thalassemia major. However, chelation protocols, strongly recommended in thalassemia intermedia patients in the presence of iron overload, should become mandatory when the two diseases coexist. It is possible that an oral chelator that interferes with dietary iron absorption could be of greater benefit than desferrioxamine in the management of these patients. The evidence of the coexistence of hemochromatosis gene should prompt physicians to recommend that their patients observe more rigid dietary habits with limitation on the consumption of iron-rich foods and vitamin C, and increased consumption of foods rich in inhibitors of iron absorption (e.g., phytates, polyphenols)[4, 6, 53].

In contrast, the coexistence of β-thalassemia trait with homozygous hemochromatosis may interfere with therapy by limiting intensive phlebotomy programs. Despite the fact that most patients readily tolerate weekly therapeutic phlebotomy, some develop rapidly declining blood hemoglobin concentrations such that therapy much be stopped. A novel approach in these patients could be the use of recombinant erythropoietin[54]. A few patients who developed severe anemia after a few phlebotomies were given erythropoietin 150 U/kg three times weekly, and they tolerated therapy much better (Fig. 42.2). It is hypothesized that erythropoietin favors iron depletion in these patients, not only by allowing weekly phlebotomies, but also by a mechanism of direct removal of tissue iron due to erythropoietin-stimulated erythropoiesis. Unfortunately, the high cost of erythropoietin may limit its use in patients with homozygous hemochromatosis and heterozygous thalassemia. In these cases, it may be necessary to use desferrioxamine in association with less frequent phlebotomies.

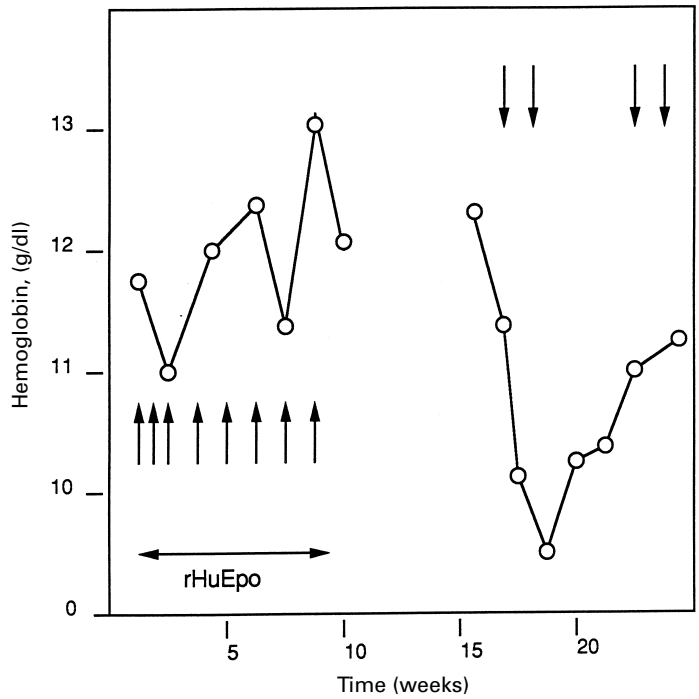

Fig. 42.2. Hemoglobin concentrations during therapeutic phlebotomy in a patient with homozygous hemochromatosis and heterozygous β-thalassemia when treated (or not) with erythropoietin. There was two months between the first and the second series of phlebotomies; phlebotomy treatments are indicated by ↑ [54].

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43

Iron overload in sideroblastic and other non-thalassemic anemias

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Introduction

Iron overload similar to that of hemochromatosis is characteristic of certain forms of anemia. The fundamental mechanisms for accumulation of excess body iron in these disorders appear to be distinct but remain undefined. Chronic anemias characterized by ineffective erythropoiesis are typically associated with iron overload. Rare genetic defects such as atransferrinemia and aceruloplasminemia also lead to unique iron overload patterns of various parenchymal organs and are accompanied by anemia. When hemochromatosis alleles are co-inherited with hereditary hemolytic anemias, the clinical expression of iron overload may be accelerated. Early recognition and treatment of iron accumulation in these disorders prevents irreversible organ damage.

Anemias with ineffective erythropoiesis (iron-loading anemias)

Anemic states characterized by ineffective erythropoiesis are distinguished by defective erythroid maturation and are due to abnormalities in the synthesis of hemoglobin, e.g., thalassemias and sideroblastic anemias, or are due to impaired DNA replication, e.g., megaloblastic and congenital dyserythropoietic anemias. Although increased production of erythropoietin in response to such anemias elicits brisk marrow erythroid hyperplasia, defective erythroblasts are destroyed within the marrow and reticulocytosis is not observed. These features are quantitatively reflected in the ferrokinetic abnormalities of increased plasma iron turnover rate and reduced incorporation of iron into circulating erythrocytes[1], and in an increased erythropoietic component of the 'early-label' bilirubin peak, leading to hyperbilirubinemia and excessive urobilinogen excretion[2]. Erythrocyte survival is normal or slightly reduced, and hemolysis occurs predominantly in the medullary space. In contrast to effective erythropoiesis associated with hemolytic disorders, ineffective erythropoiesis mediates enhanced intestinal absorption of iron in an unknown manner[3]. The *HFE* protein, defective in hemochromatosis[4], associates with the transferrin receptor[5, 6, 87, 88] and thereby may influence regulation of iron absorption. This implicates a role of the very high levels of circulating transferrin receptor found in states of ineffective erythropoiesis[7] in the mechanism by which increased quantities of iron are absorbed. Progressive iron deposition is observed in bone marrow macrophages and also occurs prominently in parenchymal tissues as in hemochromatosis, presumably due to a redistribution of iron in concert with the erythroid hyperplasia and the increased rate of plasma iron turnover[8]. This iron overload is currently referred to as erythropoietic hemochromatosis. The *HFE* protein per se does not seem to be involved (see below).

Sideroblastic anemias

The sideroblastic anemias are a heterogeneous group of disorders (Table 43.1). They are defined by the common morphologic feature of amorphous iron deposits as ferric phosphate and ferric hydroxide in erythroblast mitochondria[9] (Fig. 43.1(A)). These iron-laden mitochondria tend to assume a perinuclear distribution in later stages of erythroblast maturation. On light microscopy, Prussian blue-positive granules form a full or partial 'ring' around the nucleus of the erythroid precursor cells, hence the term 'ring sideroblasts' (Fig. 43.1(B)). Hypochromic and microcytic erythrocytes, the progeny of these cells, provide morphologic evidence of impaired hemoglobin formation and are usually the initial clue to the diagnosis. The basis for the mitochondrial iron accumulation is the apparently

Table 43.1. The sideroblastic anemias

Hereditary
X-linked
Autosomal dominant
Autosomal recessive
Sporadic congenital
Protoporphyria (some cases)
Associated with mitochondrial cytopathy
DIDMOAD syndrome
Acquired
Idiopathic – 'pure sideroblastic anemia'
Associated with myelodysplasia, hematologic malignancies, myeloproliferative disorders, and chemotherapy
Reversible – associated with
Alcoholism
Certain drugs (e.g., isoniazid, chloramphenicol)
Copper deficiency (nutritional, excess zinc ingestion, copper chelation)
Hypothermia

insufficient generation of protoporphyrin to utilize the iron delivered to erythroblasts at a normal rate[10, 11], or the inability to insert iron into protoporphyrin so that the production of heme is compromised (Fig. 43.2). In several forms of sideroblastic anemia, underlying defects in the heme biosynthetic pathway have been uncovered at the molecular level and affect the erythroid 5-aminolevulinate synthase isoform, a putative mitochondrial iron transporter (ABC7), ferrochelatase, or cytochrome oxidase[12–15, 89].

Hereditary sideroblastic anemia

The X-linked pattern of inheritance is probably the most common. In a large proportion of cases, molecular defects in the form of point mutations in the erythroid-specific 5-aminolevulinate synthase (*ALAS2*) gene (located on the X chromosome) have recently been identified[9, 12, 16] that lead to single amino acid replacements and account for reduced activity of the enzyme. Moreover, as many as one-third of probands with mutations in the *ALAS2* gene are females and express the disorder due to unbalanced inactivation of the X chromosomes[16]. In kindreds without identifiable molecular defects in the *ALAS2* gene, another locus on the X chromosome can now be implicated[17, 89]. In a few families, inheritance was consistent with an autosomal dominant or autosomal recessive trait[9]. In numerous sporadic cases of congenital sideroblastic anemia, the pattern of inheritance could not be ascertained and the underlying defect also remains undefined. The clinical phenotype is indistinguishable among the hereditary types. The severity of the anemia varies greatly and not infrequently increases with age[9]. Approximately one-third of patients with X-linked sideroblastic anemia respond variably to pyridoxine supplements; effective doses also vary widely. It is presumed that an increased supply of 5′-pyridoxal phosphate, the essential co-factor for 5-aminolevulinate synthase (Fig. 43.2), enhances its impaired binding to some mutants of the enzyme. As yet, pyridoxine responsiveness cannot be correlated with specific mutations in the *ALAS2* gene.

In patients with hereditary sideroblastic anemia, iron overload is a constant feature[18–21] and is indicated by the usual laboratory parameters of the increased iron saturation of serum transferrin and elevated serum ferritin concentration. The pathologic findings are indistinguishable from those in hemochromatosis (Fig. 43.3). The severity of iron overload is not related to the severity of anemia, and hepatic cirrhosis is sometimes encountered in asymptomatic patients who have mild anemia[22–24]. However, it is closely related to the degree of marrow erythroid hyperplasia and the patient's age[20, 22]. Increased intestinal absorption of iron has been documented in a small number of cases[22, 25]. A role of the *HFE* gene has been excluded in the pathogenesis of iron overload in these disorders. In 50 patients with hereditary sideroblastic anemia, the Cys282Tyr and His63Asp mutations of the *HFE* gene were less common than in normal control populations[21] (Table 43.2). Co-inheritance of a hemochromatosis Cys282Tyr allele may accentuate iron overload in an occasional case[26], as could inappropriate iron therapy or erythrocyte transfusions.

Acquired idiopathic sideroblastic anemia

This category of sideroblastic anemia is a myelodysplastic disorder, and there is evidence that it is clonal[9]. It is frequently mild or moderate in severity, very stable, and refractory to therapy, and as such is called pure acquired idiopathic sideroblastic anemia[27]. Less often, other cell lineages also display morphologic or numerical abnormalities, portending uncertain prognosis and possible evolution to acute leukemia. In contrast to hereditary sideroblastic anemia, the marrow ring sideroblasts encompass all stages of erythroid cell development, and the anemia is not responsive to pyridoxine supplements. The pathogenesis of this erythroid disorder is poorly understood. Although abnormalities in the enzymatic steps of heme biosynthesis have not been demonstrated consistently[9], heteroplasmic mutations of conserved nucleotides in mitochondrial DNA of hematopoietic cells affecting cytochrome c oxidase implicate impaired reduction of iron for its insertion into protoporphyrin by ferrochelatase[15] (Fig.

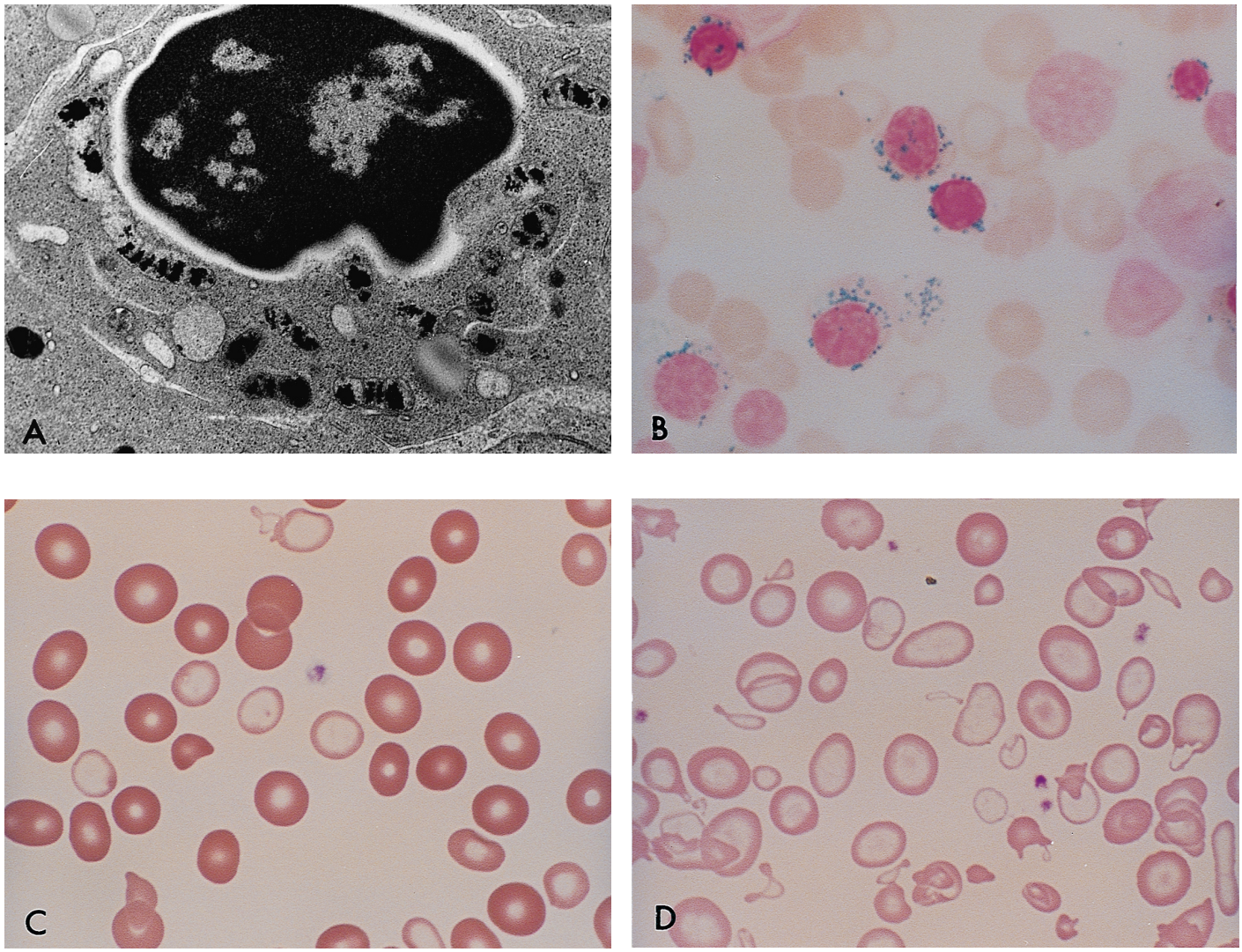

Fig. 43.1. Features of sideroblastic anemia. A. Electron micrograph of an erythroblast with iron-laden mitochondria. B. Bone marrow smear with ring sideroblasts (Prussian blue stain). C. and D. Peripheral blood smears of mild and severe sideroblastic anemia, respectively (Wright's stain).

43.2). Such defects are in accordance with the variable size of marrow ring sideroblast and hypochromic, microcytic erythrocyte populations among patients and with the appearance of the disorder in later life, when higher mutation rates occur in mitochondrial than nuclear DNA.

Iron overload is a common, variable feature[90] that is pathologically indistinguishable from hemochromatosis and can be attributed to excessive absorption of dietary iron in association with ineffective erythropoiesis, like that which occurs in the hereditary sideroblastic anemias[3]. Coexistence of one allele for hemochromatosis had been proposed to account for the iron overload, but this association has not been demonstrable[28, 29]. Recently, DNA analysis of 30 patients for the *HFE* mutations failed to indicate co-inheritance of a hemochromatosis allele beyond the normal incidence (author's unpublished data) (Table 43.2), and is in agreement with the findings in another recent series of patients[30]. Erythrocyte transfusions and inadvertent administration of iron for treatment of the anemia add predictably to the iron overload[90].

Prevention and treatment of iron overload

In patients with hereditary or acquired idiopathic sideroblastic anemia, the degree of iron overload present is best

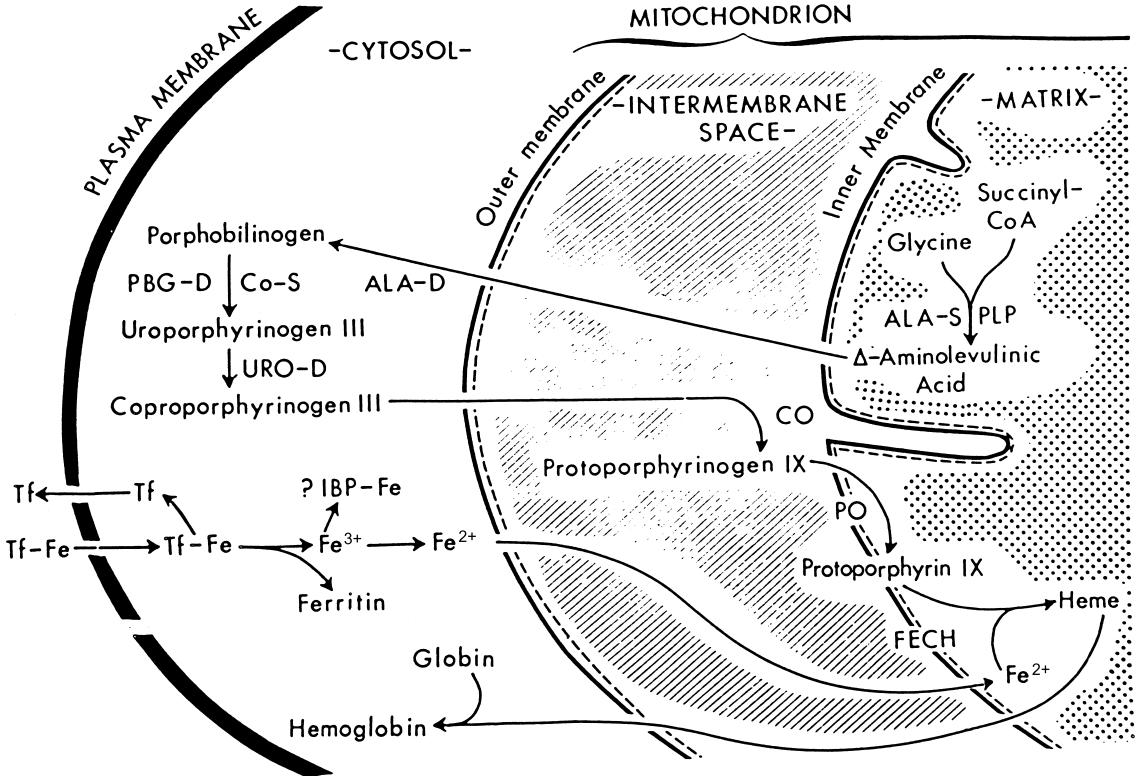

Fig. 43.2. Pathways of hemoglobin synthesis. ALA-S, 5-aminolevulinate synthase; PLP, 5′- pyridoxal phosphate; ALA-D, 5-aminolelvulinate dehydratase; PBG-D, porphobilinogen deaminase; Co-S, uroporphyrinogen III synthase; URO-D, uroporphyrinogen decarboxylase; CO, coproporphyrinogen oxidase; PO, protoporphyrinogen oxidase; FECH, ferrochelatase; Tf, transferrin; IBP, iron-binding protein.

documented by obtaining a hepatic biopsy specimen. In addition, this provides prognostic information, e.g., demonstration of the presence of cirrhosis. Based on the severity of iron overload (e.g., serum ferritin ≥ 500 μg/l), an iron depletion program must be instituted to prevent or stabilize established organ damage. This is accomplished with graded phlebotomies in patients who have responded to pyridoxine supplements and in all others with mild or moderate anemia, when there are no contraindications to therapeutic phlebotomy such as heart disease[11, 31]. Thereafter, phlebotomies should be continued on a regular basis indefinitely to prevent iron reaccumulation. In patients who have more severe anemia and in those who require regular transfusions (and thus become severely iron-loaded), deferoxamine is administered. This siderophore (mwt 657 daltons) is poorly absorbed from the gastrointestinal tract and must be given parenterally. It avidly binds non-protein-bound, non-heme-bound iron that is in a transit phase in cells to form ferrioxamine which is readily excreted in urine and bile. Since its availability in the 1960s, clinical experience with deferoxamine has been extensive, especially in the treatment of the thalassemia syndromes[32]. Continuous infusion of the agent is necessary, and effective iron excretion occurs with daily 12- to 24-hour infusions administered subcutaneously or intravenously. The goal of chelation therapy is to maintain the serum ferritin concentration < 500 μg/l, but the progress of treatment is best assessed with follow-up analysis of hepatic iron content[32]. Adverse effects are limited to local chemical skin reaction and rare hypersensitivity for which desensitization can be performed. Auditory and visual toxicity does not occur when the dose is not excessive. Iron removal with deferoxamine is enhanced by ascorbate. However, large supplements can cause acute cardiac toxicity by facilitating excessive mobilization of ferritin iron, and intake of the vitamin should be limited to 200 mg daily[33]. The oral chelation agent deferiprone has been used in clinical trials with limited success, in that control of hepatic iron levels was not sustained in a substantial proportion of patients[34, 35].

Treatment of iron overload in patients with sideroblastic

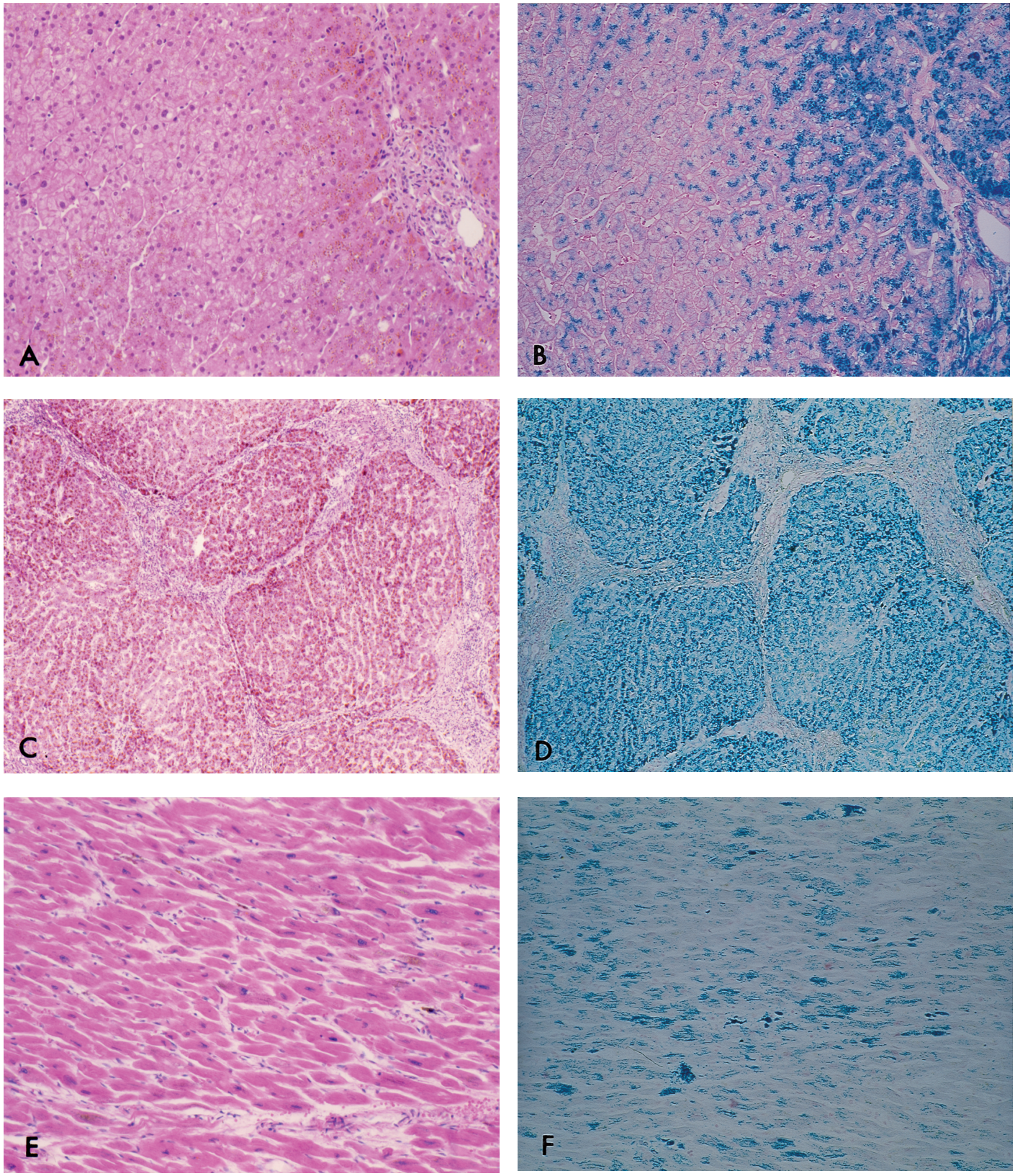

Fig. 43.3. Histopathology of the iron overload in sideroblastic anemia. A. and B. Sections of liver of a 26-year-old man with moderate iron overload (Prussian blue and hematoxylin and eosin stains, respectively). C. and D. Sections of liver obtained at autopsy of a 45-year-old man who had micronodular cirrhosis and iron overload (Prussian blue and hematoxylin and eosin stains, respectively). E. and F. Sections of the heart of the latter patient reveal marked siderosis (Prussian blue and hematoxylin and eosin stains, respectively).

Table 43.2. *HFE* genotypes in patients with sideroblastic anemias

Genotype	Hereditary sideroblastic anemia, $n^{a,b}$	Acquired idiopathic sideroblastic anemia, n^{b}
Cys282Tyr/Cys282Tyr	0	0
Cys282Tyr/His63Asp	1 (2%) [1M]	0
Cys282Tyr/wild-type	3 (6%) [2M, 1F]	2 (7%) [2M]
His63Asp/His63Asp	0	0
His63Asp/wild-type	7 (14%) [2M, 5F]	7 (23%) [5M, 2F]
Wild-type/wild-type	39 (78%) [22M, 17F]	21 (70%) [16M, 5F]

Notes:

[a] Includes 24 patients with X-linked sideroblastic anemia (three sibling pairs) and 26 patients with sporadic congenital hereditary sideroblastic anemia (three sibling pairs).

[b] M, male; F, female.

Table 43.3. Features of the congenital dyserythropoietic anemias

Type, inheritance	Chromosomal location of gene	Chief distinctive hematologic features	References documenting iron overload
Type I, recessive	15q15.1–15.3	Internuclear chromatin bridges, megaloblastic	38, 41
Type II, recessive	20q11.2	Binuclearity, multinuclearity, normoblastic, positive Ham test	20, 38, 42–44
Type III, dominant, recessive, sporadic	15q22	Giant multinuclear erythroblasts, megaloblastic	38

anemia has also reduced the severity of their anemia, presumably by improving erythroblast mitochondrial function and reducing the ineffective erythropoiesis[11, 31, 36, 37] and/or by relieving a component of hypersplenism, and can provide a favorable prognosis or near-normal survival. Although the long-term consequences of persistent transfusion-induced iron overload after an unusual remission of the hematological disorder or after successful bone marrow transplantation are not known, removal of the iron burden by phlebotomy seems advisable. In kinships in which there is a hereditary anemia, early detection of iron overload in asymptomatic relatives with mild anemia is important. This is also facilitated by DNA analysis in kindreds with identified mutations in the *ALAS2* gene.

Congenital dyserythropoietic anemias

The congenital dyserythropoietic anemias (CDAs) are rare disorders characterized by mild or moderate normocytic or macrocytic anemia, various morphologic abnormalities of erythrocytes and their precursors, and prominent ineffective erythropoiesis[38–40]. Three types of CDA are recognized (Table 43.3) and additional variants have been proposed. They are distinguished mainly by mode of inheritance, light and electron microscopic features of erythroid cells, and certain serological findings. The genes for these disorders have been localized on chromosomes using linkage analysis[40, 45]. The most common form, CDA type II, is associated with alpha-mannosidase II deficiency[46, 47] or *N*-acetylglucosaminyl-transferase II deficiency[48], leading to aberrant glycosylation of surface membrane proteins and consequent disruption of the normal cytoskeleton of erythroid cells[39, 44].

Iron overload, a constant finding in these disorders, is attributed to ineffective erythropoiesis and is the major complication[20, 39, 44]. It is independent of the severity of anemia, progresses with time, and may be the presenting feature in later life[42, 43]. The fundamental defect(s) of protein glycosylation in CDA type II may also predispose individuals to hepatic cirrhosis[49] and thus enhance the consequences of iron toxicity. Because anemia is often not severe, phlebotomy therapy is feasible in many cases[50]. Deferoxamine administration is occasionally required[41].

Disorders of iron transport and metabolism

Several rare disorders that represent defects in iron transport are accompanied by anemia and parenchymal iron overload. The associated abnormalities of iron distribution throughout the body point out the important roles of specific components of iron transport in iron homeostasis.

Hereditary atransferrinemia (hypotransferrinemia)

Atransferrinemia is a very rare disorder inherited as an autosomal recessive trait that is characterized by extremely low serum transferrin and serum iron concentrations[51]. Nine unrelated index cases have been reported[52]. Iron absorption is increased, yet little iron is transported to marrow and incorporated into erythrocytes, demonstrating that erythroid bone marrow cells can only obtain iron from transferrin in plasma. Instead, marked siderosis of parenchymal tissues develops as the non-transferrin-bound iron is taken up by non-erythroid tissues. Moderate or severe hypochromic, microcytic anemia is typical and transfusion dependence accentuates the siderosis. Survival beyond childhood has been markedly limited, although one male patient had striking spontaneous improvement at puberty[53]. In several cases, infusions of purified transferrin or plasma have improved or corrected the anemia[52, 53], and permitted subsequent phlebotomies to remove the iron overload in one patient[52]. In heterozygotes for the defect, plasma transferrin concentration is half-normal and causes no ill-effects. Genetic and clinical heterogeneity occur among affected persons and their kin[52], but a molecular defect responsible for the disorder has not been identified.

A spontaneous mutation has been described in inbred mice that causes atransferrinemia with more severe phenotypic features than the human disorder[54]. The mutation causes a splicing defect, leading to altered mRNA processing and an unstable mRNA[55]. Heterozygous animals are normal, but develop mild siderosis late in life. This model should be useful to explore further the pathophysiology of the corresponding human disorder in addition to other aspects of iron metabolism.

Transferrin–immune complex disease

A single case report demonstrates the occurrence of an autoimmune basis for apparent inactivation of transferrin's function to transport iron to erythroid cells[56]. As in hereditary atransferrinemia, iron was diverted to other tissues and the patient had pigment cirrhosis, bronze skin, diabetes mellitus, and anemia with absence of hemosiderin in bone marrow macrophages and of sideroblasts. Immunosuppressive therapy produced a partial remission.

Familial hypochromic, microcytic anemia, hyperferremia, and parenchymal siderosis

In two siblings, impaired uptake of iron by erythroid precursors is responsible for severe hypochromic, microcytic anemia, increased plasma iron concentrations, marked siderosis and fibrosis of the liver, and scant reticuloendothelial iron deposits in bone marrow and liver[57]. A defect in the transferrin–transferrin receptor pathway at the level of the erythroid cell has been postulated to explain this disorder[58]. Two similar cases in sisters were described by Stavem et al.[59]. In these patients, marrow ferrochelatase activity was ~20% of normal, but there was no evidence of protoporphyria. The pathogenesis of these unique conditions remains to be elucidated.

Hereditary aceruloplasminemia

Aceruloplasminemia is an autosomal recessive disorder with marked accumulation of iron in the nervous system (basal ganglia and retina), pancreas, and liver[60–62]. To date, 26 families with aceruloplasminemia have been detected (JD Gitlin, personal communication). The clinical features become evident after middle age and include neurological symptoms and signs of choreo-athetoid involuntary movements, cerebellar ataxia, dementia, retinal degeneration, diabetes mellitus, and iron overload. Mild hypochromic, microcytic anemia is present in most patients. In addition to very low serum copper concentrations and absence of serum ceruloplasmin, biochemical analyses reveal subnormal serum iron, normal transferrin, and increased ferritin concentrations. Heterozygotes have half-normal serum copper and ceruloplasmin values and are asymptomatic.

Heterogeneous mutations in the ceruloplasmin gene have been identified in seven families[63–68, 91] (Table 43.4), and lead to an unstable protein because amino acids that constitute its essential copper ligands are eliminated. The pathogenesis of the tissue iron accumulation is consistent with impaired oxidation of iron for its normal release from tissues to transferrin that requires the ferroxidase function of ceruloplasmin[69]. To what extent intestinal absorption of iron is increased has not been determined. The neurologic involvement as the unique and most prominent feature of this iron overload disorder can be attributed to the recently demonstrated role of glia-specific expression of ceruloplasmin in basal ganglia and the retina[70, 71]. The mild

Table 43.4. Mutations in the ceruloplasmin gene in aceruloplasminemia

Mutation (Exon)	Reference
Insertion or deletion	
607insA (3)	63
128insTACAC (7)	64
2389delG (13)	65
Nonsense	
Trp858ter (15)	66, 67
Splice site	
1209-2A-G (7)	91
3019-1G-A (18)	68

anemia is probably related to impaired recycling of iron consequent to its reduced release from macrophages[72].

The clinical course resembles that of hemochromatosis and is dependent on the gradual tissue accumulation of toxic levels of iron. The increased plasma and cerebrospinal fluid lipid peroxidation in patients with aceruloplasminemia may be an added factor of free radical-mediated tissue injury[73, 92]. Early diagnosis of the disorder by detecting absence of serum ceruloplasmin and administration of ceruloplasmin or deferoxamine can permit prevention of iron accumulation and clinical manifestations[61, 74.] Treatment with deferoxamine reduced clinical symptoms, iron deposition in the brain, and plasma lipid peroxidation in one case[74]. The anemia must be distinguished from that of iron deficiency.

Hereditary hemolytic anemias

Non-transfusional iron overload is occasionally encountered in patients with hereditary hemolytic anemia and is consistent with co-inheritance of hemochromatosis alleles. The two disorders are disassociable by pedigree analysis.

Hereditary spherocytosis

At least 17 patients with hereditary spherocytosis and concomitant hemochromatosis have been reported[75, 76]. In hereditary spherocytosis, intestinal absorption of iron is normal[3] and serum ferritin concentrations are usually in the normal range[77]. Although the hemolytic process may contribute to the magnitude of the iron loading, the latter is due primarily to independent inheritance of hemochromatosis alleles. Thus iron accumulation continues after splenectomy-induced remission of the hemolysis[78]. In three family studies, the independent inheritance of the two disorders was demonstrated[76, 77, 79]. Prolonged consumption of iron supplements accelerates the iron loading[80]. Late diagnosis of the hemochromatosis has resulted in fatal hepatocellular failure and hepatoma[75, 81, 82]. The combination of hemochromatosis heterozygosity and hereditary spherocytosis may also lead to significant iron overload[75, 83].

Pyruvate kinase deficiency

Approximately 15 patients have been reported who had pyruvate kinase deficiency anemia, the most common hereditary non-spherocytic hemolytic anemia, and non-transfusional iron overload[84–86]. In several patients, hepatic cirrhosis had developed. For this combination of heritable diseases, family studies have not been performed to demonstrate the likely independent inheritance of the two disorders. This would now be facilitated by DNA analysis for the *HFE* gene mutations.

Unstable hemoglobin

A family with hemoglobin Köln hemolytic anemia and co-inherited hemochromatos due to the Cys 282 Tyr mutation has been described[94]. In this instance, iron overload occurred only in homozygotes for the mutation.

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44

Hemochromatosis, iron overload, and porphyria cutanea tarda

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Introduction

The term *porphyria,* derived from the Greek word *porphyra,* meaning purple, is apt because a hallmark of most of the porphyrias is the overexcretion of porphyrins in urine or feces. The porphyrins have a dark-red color that borders on purple. In most porphyrias, there are enzyme defects in the pathway of normal heme biosynthesis that cause the accumulation of porphyrins and porphyrin precursors. A summary of the pathway of heme biosynthesis showing the primary enzymatic defects in the porphyrias is displayed in Fig. 44.1. A classification of the porphyrias, emphasizing that either the liver or the bone marrow is the major site of overproduction of porphyrins and porphyrin precursors in these diseases, is shown in Table 44.1.

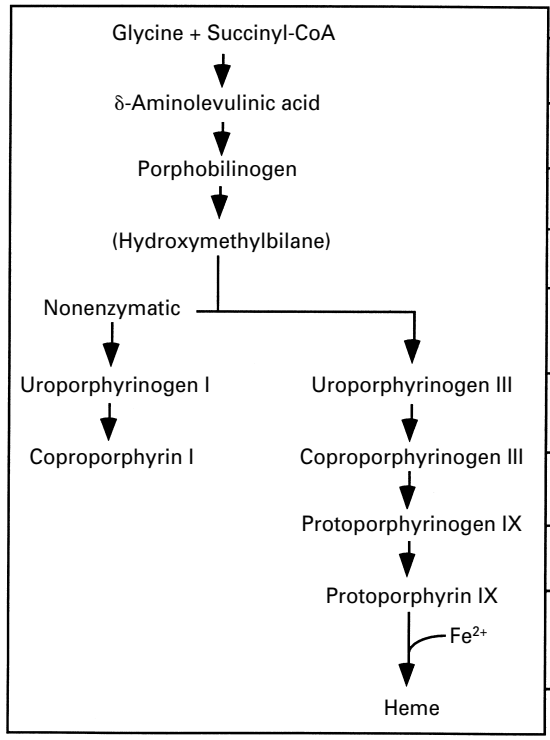
The major clinical features of the porphyrias are dermatologic and/or neurologic abnormalities. The types of porphyria in which dermatologic features predominate include porphyria cutanea tarda (PCT), the most common form of porphyria encountered world-wide. Hepato-erythropoietic porphyria is due to a rare, hereditary, severe deficiency in activity of the enzyme uroporphyrinogen decarboxylase (Uro-D), the enzyme that is also decreased in activity in PCT (Fig. 44.1). The severe deficiency in hepato-erythropoietic porphyria is due to homozygosity or compound heterozygosity for deficiency of Uro-D activity. Congenital erythropoietic porphyria (Günther's disease), also rare, is a form of usually severe cutaneous porphyria due to homozygous or compound heterozygous deficiency of the enzyme in the heme biosynthetic pathway immediately preceding Uro-D, namely, uroporphyrinogen III synthase (cosynthase).

The diseases hereditary coproporphyria, variegate porphyria, and protoporphyria may also cause dermatologic abnormalities. In addition, patients with hereditary coproporphyria and variegate porphyria may present mainly with neurological features typical of acute attacks of porphyria. The most severe form of porphyria causing such neurologic disease is acute intermittent porphyria, due to a partial deficiency in activity of the third enzyme of the heme biosynthetic pathway, porphobilinogen (PBG) deaminase (Fig. 44.1). The final type of porphyria with which patients present with mainly neurologic features is porphyria due to severe deficiency of δ-aminolevulinic acid (ALA) dehydratase, the second enzyme of the heme biosynthetic pathway. In this disorder, homozygous or compound heterozygous deficiency of ALA dehydratase leads to severe overproduction of the substrate for the enzyme, ALA, and may cause severe heme deficiency in the liver and other organs, and to numerous neurological abnormalities. A more complete description of the clinical and biochemical features of porphyrias other than porphyria cutanea tarda is beyond the scope of this chapter, but these have been reviewed recently[1, 2].

Major features of porphyria cutanea tarda (PCT)

History, genetics, and incidence

It is difficult to trace the history of our understanding of PCT, because many cases of hereditary coproporphyria or variegate porphyria were almost certainly included in early descriptions of the cutaneous porphyrias. The term *porphyria cutanea tarda* was coined by the eminent Swedish clinician Jan Waldenström who made many seminal clinical observations in study of the porphyrias. From early studies and more recent observations, particularly those related to the association of chronic hepatitis C infection and PCT (see below), it seems clear that PCT is sometimes an acquired disorder that complicates exposure to toxic substances such as hexachlorobenzene or other



ENZYMES	DISEASES
ALA synthase	
ALA dehydratase	ALA dehydratase deficiency
Porphobilinogen deaminase	Acute intermittent porphyria
Uroporphyrinogen III synthase (cosynthase)	Congentital erythropoietic porphyria
Uroporphyrinogen decarboxylase	Porphyria cutanea tarda; Hepatoerythropoietic porphyria
Coproporphyrinogen oxidase	Hereditary coproporphyria
Protoporphyrinogen oxidase	Variegate porphyria
Ferrochelatase	Protoporphyria

Fig. 44.1. The heme biosynthetic pathway, showing sites of primary enzymatic defects in the porphyrias.

halogenated aromatic hydrocarbons, alcohol, iron overload, chronic viral hepatitis, or other factors that may cause hepatic disease. This acquired or sporadic form of PCT has been called Type I PCT. Approximately 25% of patients with PCT have an inherited partial defect in activity of Uro-D[3, 4]. Most of these patients have heterozygous deficiency of Uro-D that is detectable in lysates of erythrocytes, liver, and probably all other cells and tissues. This common inherited form of PCT has been called Type II PCT. There is also evidence for a much rarer form of PCT, in which apparently hereditary decreased activity of Uro-D is limited to the liver, called Type III PCT. Because only one human gene that encodes for Uro-D has been found, it is unclear how or why this latter form of PCT (Type III) occurs. Perhaps the heritable defect actually lies in other gene(s), the product(s) of which somehow cause a secondary decrease in activity of hepatic Uro-D. A classification of the forms of PCT is given in Table 44.2.

The incidence of recognized, clinically manifest PCT varies markedly in different parts of the world. In general, the incidence and prevalence of PCT are higher in countries near the equator, where there is more exposure to natural light of high intensity. It is also higher in regions where chronic hepatitis C, iron overload, and/or heavy alcohol use are common. Exposure to environmental toxins, such as the halogenated aromatic hydrocarbons, also plays a lesser role in determining the incidence and prevalence of PCT. Formerly, the disease was very frequent among the Bantus of South Africa, in whom it was associated with excessive ingestion of native or Kaffir beer that was traditionally brewed in iron pots and contained high concentrations of iron. High prevalence rates (approximately 1 in 300) have also been described among populations of Czechoslovakia and China[5]. Patients with PCT should be questioned carefully regarding risk factors for development of the disease (Table 44.3).

Clinical features

Cutaneous findings

PCT presents clinically with blisters, vesicles, and/or milia on the dorsal aspects of the hands (Fig. 44.2). Unlike the lesions of protoporphyria, the skin lesions of PCT do not occur due to acute and immediate photosensitivity reactions. Rather, lesions develop on sun-exposed skin that is also prone to mild trauma or other insults. Other skin manifestations include fragility, hypertrichosis (especially involving the lateral aspects of the face), chloracne and

Table 44.1. Classification of the porphyrias

According to major clinical features	According to major site of overproduction of precursors of heme
Neuro-visceral features	*Hepatic porphyrias*
Acute or inducible porphyrias	Acute or inducible porphyrias
ALA dehydratase deficiency porphyria	ALA dehydratase deficiency porphyria
Acute intermittent porphyria	Acute intermittent porphyria
Hereditary coproporphyria	Hereditary coproporphyria
Variegate porphyria	Variegate porphyria
Protoporphyria	Chronic forms
(Rare, severely affected patients, especially just after liver transplantation.)	Hepatoerythropoietic porphyria
	Porphyria cutanea tarda
Cutaneous features	*Erythropoietic porphyrias*
Chronic vesiculo-bullous features	Congenital erythropoietic porphyria
Congenital erythropoietic porphyria	Protoporphyria
Hepatoerythropoietic porphyria	
Hereditary coproporphyria	
Porphyria cutanea tarda	
Variegate porphyria	
Acute light-induced burning, edema, itching, urticaria	
Protoporphyria	

Table 44.2. Classification of porphyria cutanea tarda

Type	Common designation	Percent of PCT patients	Inheritance	Site(s) of defective activity of Uro-D
I	Acquired or sporadic	72–84	None[a]	Liver
II	Inherited – common form	16–28	Autosomal partial deficiency of Uro-D	All cells
III	Inherited – uncommon form	<5	Autosomal defect (gene unknown) leading to deficiency of hepatic Uro-D	Liver

Note:

[a] Many patients with acquired (Type 1) PCT have defect(s) outside the heme biosynthetic pathway that predispose them to develop PCT. The best-defined of these is heterozygosity or homozygosity for HLA-linked hereditary hemochromatosis (mutations in the *HFE* gene).

chronic hyper- and/or hypo-pigmentation, sclerodermoid changes, dystrophic calcification with ulceration, scarring alopecia, and onycholysis.

The vesicles or bullae, the most common primary lesions, vary from 2–30 millimeters in diameter. If large bullae are aspirated or punctured, a pink fluid is obtained that contains high concentrations of uro- and heptacarboxyl porphyrins. After their rupture, the bullae heal slowly, often requiring six weeks to resolve. The healing phase may be characterized by development of dry crusted lesions overlying shallow ulcerations that eventually form atrophic scars. Not infrequently, the bullae or erosions become secondarily infected, leading to further delay in healing and greater likelihood of scarring. Milia are small (1–2 mm in diameter), firm, yellow-white papules that represent asymptomatic structures in the superficial dermis. They occur commonly in patients with active PCT. Not uncommonly patients with active PCT exhibit cutaneous lesions in all stages of evolution.

Facial hypertrichosis is frequent in PCT and more noticeable in women. The excess hair growth usually becomes evident first on the upper lateral aspects of the cheeks and the periorbital areas, at the temples. Other areas of the body, including the trunk, arms, and legs

Table 44.3. Major risk factors for development of porphyria cutanea tarda

Chemical or toxic exposure
Hexachlorobenzene
2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD)
Other halogenated aromatic hydrocarbons
(Chemicals that deplete GSH or ascorbate or otherwise increase oxidative stress)
Iron overload
Heterozygosity for HLA-linked hemochromatosis
Homozygosity for HLA-linked hemochromatosis
Inheritance of other genetic abnormalities that lead to increased hepatic iron (e.g., African, non HLA-linked iron overload, iron-loading heritable anemias)
Heavy intake of alcohol
Chronic liver diseases
Alcoholic liver disease
Chronic hepatitis C
Drug exposure
Estrogens
(Barbiturates)
(Other inducers of hepatic cytochrome(s) P-450, especially CYP I A2)

Note:
Factors listed within parentheses have been shown to trigger or exacerbate experimental uroporphyria that resembles PCT biochemically. Thus, they are considered theoretical risks in humans.

may be involved. Cutaneous hyperpigmentation is less common and usually not generalized. It appears mainly on the face in periorbital malar distributions, mimicking melasma. Rarely melanosis is so widespread that it simulates the hyperpigmentation of Addison's disease or hemochromatosis. Scarring, alopecia, and sclerodermatous changes occur in 15–20% of patients with PCT. These firm, hypopigmented, pale-yellow plaques are not limited to areas of sun exposure, but may occur on areas usually covered with clothing, such as the back or chest. When severe, the condition can resemble the skin changes of progressive systemic sclerosis (scleroderma). Unlike other cutaneous changes of PCT, these chronic fibrosing changes rarely respond to treatment. They may also be complicated by development of dystrophic calcification, and by the formation of non-healing ulcers in areas of the sclerodermatous plaques. Such calcium deposits usually occur in the pre-auricular regions of the face, but may also occur on the scalp and neck.

Other physical findings

Other than the changes of the skin, the major physical findings in PCT are limited to the abdomen. Approximately one-third to one-half of patients, especially those that drink alcohol heavily, have hepatomegaly. This may be due to steatosis, to cell swelling, to inflammatory cells, to fibrosis, or to a combination of these changes. Patients exposed to toxins other than alcohol may show similar changes, particularly hepatocytic swelling and fat accumulation. A minority of patients (~10%) have advanced hepatic fibrosis or cirrhosis. Some of these patients develop portal hypertension and its complications, including splenomegaly, a prominent venous pattern on the abdomen (perhaps with *caput medusae*), gastroesophageal varices, or portal hypertensive gastropathy. Alcohol, chronic hepatitis C, heavy iron overload and other conditions may cause cirrhosis in addition to PCT. Cirrhosis, with or without PCT, is the major risk factor for development of hepatocellular carcinoma. Thus, a small minority of patients with PCT may present with findings typical of hepatocellular carcinoma, including progressive hepatomegaly, abdominal pain (particularly in the upper abdomen), a vascular thrill over the liver, and development of hepatic and/or portal vein thrombosis with possible complications of Budd–Chiari syndrome or progressive splenomegaly and portal hypertension. Patients with advanced liver disease may develop all of the usual stigmata of such disease, including palmar erythema, spider angiomas, gynecomastia, ascites, edema, and encephalopathy.

Laboratory features of PCT

Most patients with PCT have normal blood counts and urinalyses, except that the urine may appear pink to red in color due to the marked increase in porphyrins in the urine. Despite the red color, tests for heme, hemoglobin or myoglobin in the urine are all negative, because the porphyrins that are excreted do not contain iron in the center of the porphyrin macrocycle. Serum concentrations of hepatic enzymes are usually mildly abnormal; increased concentrations of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transpeptidase are most common. Depending upon the underlying etiology of hepatic disease (e.g., alcoholic or chronic hepatitis C), the levels of serum AST or ALT may be only mildly increased with AST greater than ALT (alcoholic liver disease) or vice versa (chronic hepatitis C). It is very uncommon for patients with PCT to have evidence of hepatic decompensation when they first present with skin

lesions and the initial diagnosis is made. Thus, most have no elevation in serum concentrations of bilirubin, or prothrombin time, or partial thromboplastin time values; levels of serum albumin, total protein, and platelets are within normal limits. Evidence of iron overload is usually found, with increased values of serum transferrin saturation (45–65%) and serum ferritin concentrations (300–3000 ng/ml). The degree of elevation of the serum ferritin concentration is quite variable, depending upon the etiology of the underlying liver disease and the presence and degree of iron overload. Most patients have a modest increase in serum ferritin concentration (less than three times the upper limit of normal), but values of ten times normal are occasionally observed. The hepatic iron concentration of most patients with PCT is mildly to moderately increased, and is in the range often observed in patients who are heterozygous for HLA-linked hemochromatosis. Thus, their hepatic iron concentrations are 2–5 times normal and their hepatic iron indices are usually 1.0–2.0.

It is relatively common for patients with active PCT to have non-insulin-dependent diabetes mellitus with hyperglycemia. This is probably due to acquired, rather than inherited, defects in glucose regulation. For example, chronic hepatitis C, now recognized to be a major trigger for PCT (see below), also causes type II diabetes mellitus, and hepatic cirrhosis of any cause produces insulin resistance and hyperglycemia. Many patients with PCT, especially those exposed to alcohol or other hepatotoxins, have increased serum cholesterol or triglyceride concentrations and excess deposition of triglyceride in the liver (fatty liver). The functions of endocrine glands other than the pancreatic islet cells and renal function are usually within normal limits in patients with PCT. However, patients with severe chronic renal insufficiency have increased risk to develop PCT. This is likely due to several factors, including a high prevalence of chronic hepatitis C infection in patients on chronic hemodialysis, some degree of iron overload, and the lack of any avenue for excretion of excess uro- or hepta carboxyl-porphyrins, due to the failure of renal excretion.

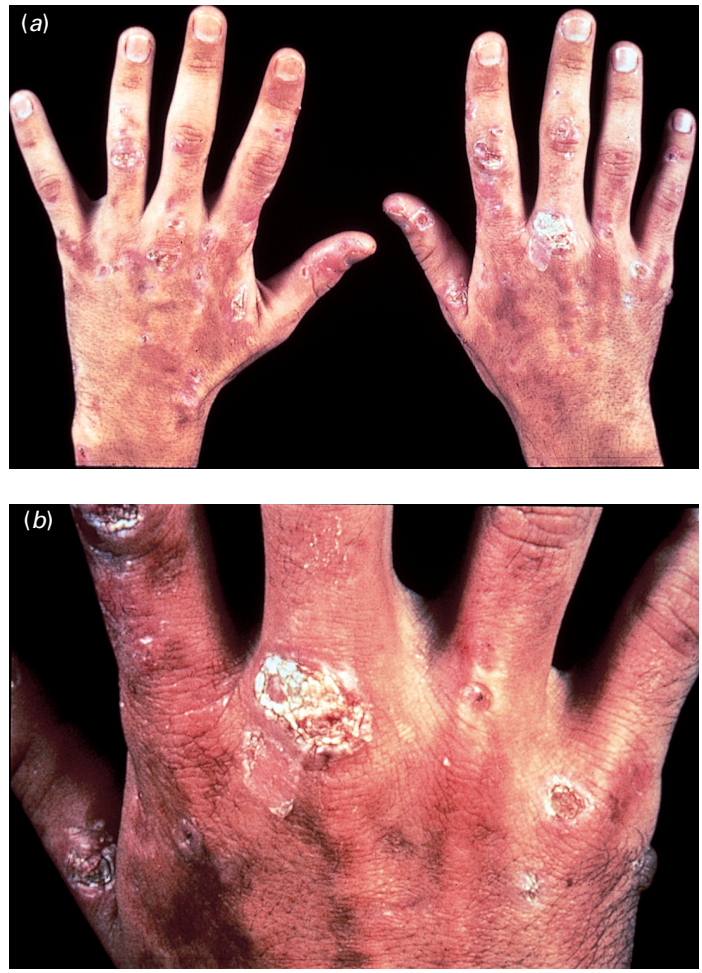


Fig. 44.2. Typical cutaneous lesions of active porphyria cutanea tarda. (*a*) Overview. (*b*) Close-up view, showing vesiculo-bullous lesions at all stages of development.

Special studies

The biochemical hallmark of PCT is the marked hepatic overproduction of uro- and heptacarboxyl-porphyrins. After binding and storage sites within hepatocytes have been saturated, these porphyrins leak into the plasma by which they are carried to other tissues. They are deposited in the skin, bones, teeth, and other sites, and are also filtered by renal glomeruli and excreted in the urine. Due to the high degree of water solubility of these porphyrins with numerous carboxyl group side chains, very little of them is secreted into the bile or excreted into the stool. The urine of patients with active PCT often has mildly increased ALA, but the levels of PBG (porphobilinogen) are always normal.

Standard porphyrin analyses of the stool of PCT patients may reveal a moderate increase in 'coproporphyrin'. More than 30% of this is an isomer of coproporphyrin, and not coproporphyrin III itself, the usual form in which coproporphyrin appears in normal stool or in the stool of patients with other types of porphyria. The isocoproporphyrin that accumulates in PCT is thought to arise due to the action of coproporphyrinogen oxidase, the next enzyme in the heme biosynthetic pathway after uroporphyrinogen decarboxylase (Fig. 44.1) that acts on a pentacarboxyl porphyrinogen intermediate that has accumulated due to the deficient activity of uroporphyrinogen

Table 44.4. Summary of major clinical features of the porphyrias

	Clinical manifestations		
Porphyria	Neuro-visceral	Cutaneous	Liver damage
ALA dehydratase deficiency porphyria	Yes	No	No
Acute intermittent porphyria	Yes	No	No (or mild)
Hereditary coproporphyria	Yes	Yes (bullae, fragility)	No
Variegate porphyria	Yes	Yes (bullae, fragility)	No
Porphyria cutanea tarda	No	Yes (bullae, fragility)	Yes
Hepatoerythropoietic porphyria	+/−	Yes (bullae, fragility)	Yes
Congenital erythropoietic porphyria	No	Yes (bullae, fragility)	Occasional
Erythropoietic protoporphyria	Rarely[a]	Yes (urticaria, erythema)	Yes (10%)

Notes:
The porphyrias are listed with respect to their dominant symptomatology with neuro-visceral or cutaneous manifestations and the presence of potential hepatic damage.
[a] (E)PP with end-stage hepatic disease, especially just after liver transplantation, is rarely associated with neuro-visceral manifestations.

decarboxylase. Thus, measurement of the ratio of fecal iso-coproporphyrin to coproporphyrin helps to distinguish PCT from hereditary coproporphyria, or variegate porphyria.

In addition, all patients with biochemically manifest and clinically active cutaneous porphyria, including PCT, have distinctly increased concentrations of porphyrins in the plasma. Those with the erythropoietic forms of disease (hereditary erythropoietic porphyria or protoporphyria; see Table 44.1) also have increased porphyrin concentrations in erythrocytes. The fluorescence spectra of porphyrins in plasma are valuable for differential diagnosis. In PCT or other conditions in which uroporphyrin or coproporphyrin is increased, the wavelength for maximal excitation of porphyrins is 398 nm and for emission is 619 nm. In contrast, in protoporphyria the wavelength maxima are 409 nm and 634 nm, respectively, and in variegate porphyria they are 405 nm and 626 nm, respectively. Thus, measurement of plasma porphyrin concentration and, if increased, determination of fluorescence spectral patterns, are useful in the differential diagnosis of photosensitivity disorders with clinical features suggestive of porphyria[4, 6]. Typical biochemical findings in the porphyrias are summarized in Table 44.4.

Histopathologic features of PCT

Cutaneous histopathology

Specimens obtained from vesicular or bullous lesions show that the fluid is subepidermal, and that dermal papillae rise irregularly into the cavities of the vesicles. This is often termed 'festooning' of the dermal papillae. Evidence of solar-induced degeneration (homogeneous thickening of superficial dermal vessels and mild inflammatory cell infiltrate) are also observed in the dermis. Periodic acid-Schiff-positive, diastase-resistant glycoproteins are present in and around vessels in the upper dermis and at the dermal–epidermal junction. Alcian blue staining is positive in areas of superficial dermal vessels, indicating the presence of acidic glycosaminoglycans. With immunofluorescent staining, deposition of immunoglobulin G, and sometimes immunoglobulin M or complement, can be detected around the upper dermal vessels and at the dermal–epidermal junction. Electron photomicrographs reveal changes consistent with repeated injury to endothelial and basal cells, including reduplication of the basal lamina at the dermal–epidermal junctions. The histopathologic changes of the sclerodermatous lesions that sometimes complicate PCT are indistinguishable from those of progressive systemic sclerosis or morphea. None of the above changes is pathognomonic or diagnostic of PCT[7].

Hepatic histopathology

Excess porphyrins

In fresh specimens of liver from patients with active PCT, red autofluorescence is striking (Fig. 44.3). This is not specific for PCT, because a similar finding may occur in hereditary coproporphyria, variegate porphyria, and

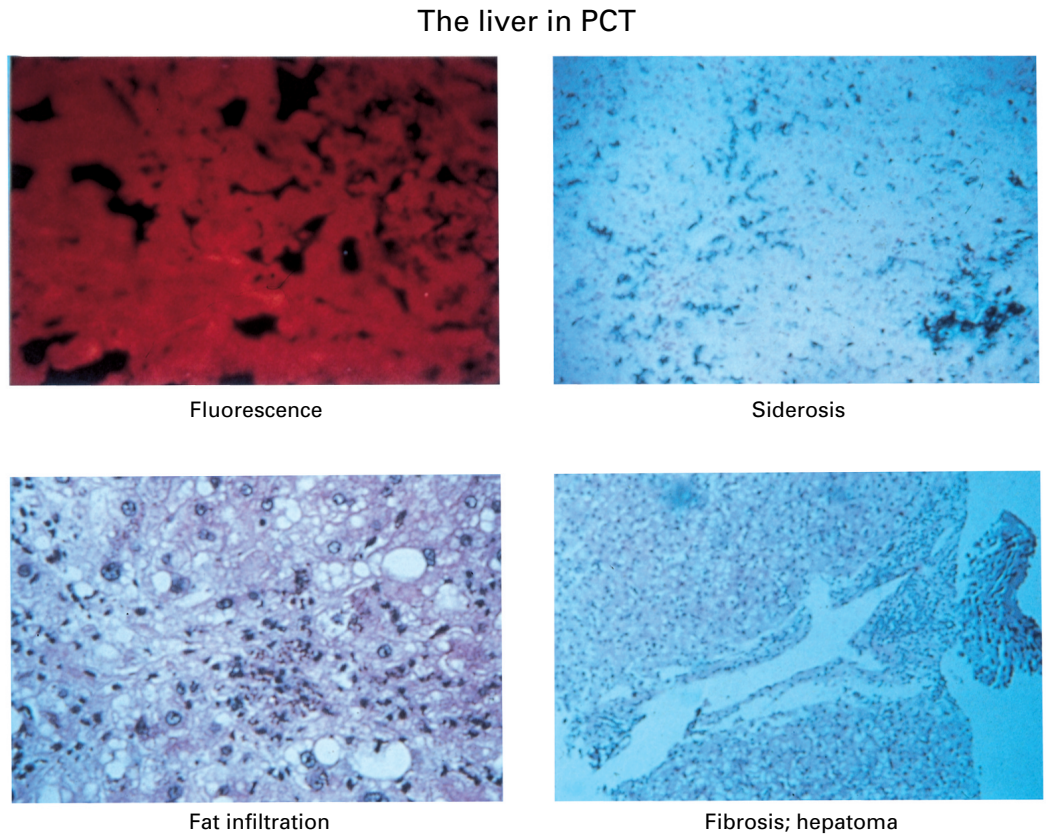


Fig. 44.3. Major hepatic histopathologic features in porphyria cutanea tarda. Photomicrographs (~100×) of typical sections from livers of patients with active PCT. *Upper left panel*: red fluorescence of a frozen, unstained section excited with light of 405nm (the Soret band); *upper right panel*: a section stained with Prussian blue, containing moderately heavy (3+) iron deposition; *lower left panel*: section stained with hematoxylin and eosin showing lobular unrest, chronic inflammation, and moderate fatty change; *lower right panel*: section stained with trichrome stain showing fibrosis and hepatocellular carcinoma at bottom of the picture. (Figure kindly provided by JR Bloomer.)

protoporphyria. In frozen unstained cryostat sections or unstained paraffin sections, needle-shaped inclusions can be found in the cytoplasm of hepatocytes of most hepatic biopsy specimens obtained from patients with PCT[8]. It seems likely that these needle-shaped crystals are composed mainly or totally of uro- and/or heptacarboxyl-porphyrin. They can be seen in sections stained with hematoxylin and eosin, provided that rinsing with water is rapid. Because the crystals are water-soluble (as are the porphyrins), they disappear when washing of sections is prolonged beyond 1–2 minutes. They are birefringent but are sometimes very small and difficult to detect. They are preserved in semi-thin toluidine blue stain sections and in sections for electron microscopy. They have been found in hepatic biopsy specimens of patients with increased urinary porphyrins even before the development of cutaneous abnormalities. These crystals have been co-localized to areas of the cytoplasm that contain ferritin granules (Fig. 44.4). It is likely that high concentrations of iron play a role in oxidation of porphyrinogens (Fig. 44.5) and precipitation of porphyrins in these subcellular locations.

Excess iron

The majority of patients with PCT have evidence of abnormal iron metabolism. Concentrations of serum ferritin and values of serum transferrin saturation are often increased. In ≥80% of patients, hepatic biopsy specimens of untreated patients show evidence of siderosis, especially involving periportal hepatocytes. The typical findings are like those seen in heterozygotes for HLA-linked hereditary hemochromatosis. Granules that appear to be ferritin are present throughout the cytoplasm of such hepatocytes, with a clustering in association with crystals of porphyrin. Concentrations of non-heme iron and total body iron stores are increased in ≥60% of patients. Fewer than 20%

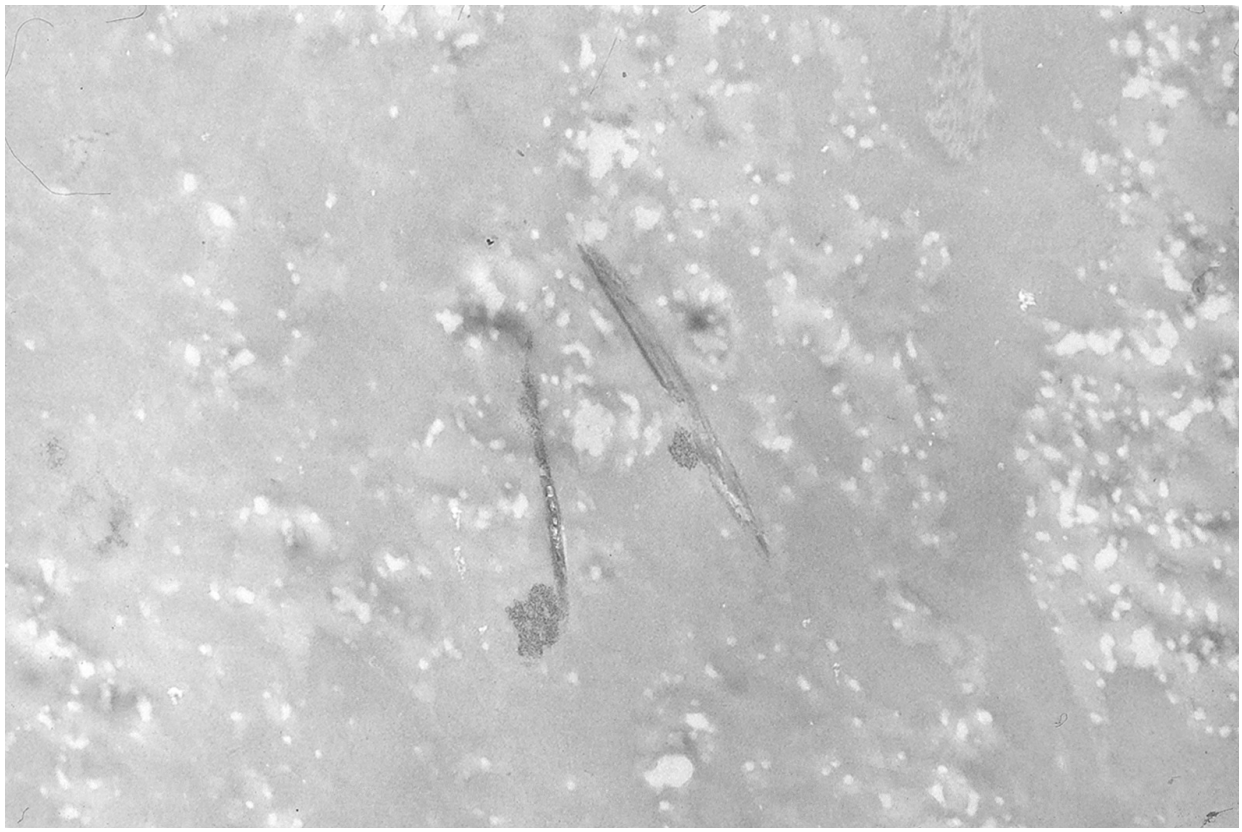


Fig. 44.4. Electron photomicrograph of hepatocytic cytoplasm from a patient with active porphyria cutanea tarda. Two needle-like crystals of porphyrin with abutting clusters of ferritin granules are shown. Unstained section, ~6000×.
(Figure kindly provided by PD Siersema.)

have severe iron overload suggestive of possible homozygosity for hemochromatosis, as judged by the presence of grade 3–4 iron staining of hepatic biopsy specimens, iron stores determined by therapeutic phlebotomy in excess of 4 grams, or serum transferrin saturation at baseline >62%. The association of PCT with clinically overt homozygous hemochromatosis is uncommon[9]. However, with the cloning of the hemochromatosis gene and development of DNA-based detection tests, the hemochromatosis mutation has recently been detected in 44% of British and Dutch patients with Type I PCT, of whom 15–20% are homozygous for the hemochromatosis mutation C282Y[10, 11]. Thus, hemochromatosis appears to be a major contributing cause to development of PCT and is a major reason for the iron overload that occurs with regularity in the livers of patients with PCT.

Other histopathologic features

Other frequent abnormalities are mild steatosis, focal necrosis of individual hepatocytes associated with groups of pigment-laden tissue macrophages (Kupffer cells), focal lipofuscin deposition, and mild portal fibrosis with variable lympho-histiocytic inflammation. Hepatic cirrhosis is observed in a minority of patients with PCT, usually less than 15%. When cirrhosis is present, it is associated with a high risk of development of hepatocellular carcinoma that may be greater than the risk of development of this tumor in other types of cirrhosis. In a minority of cases, hepatocellular carcinoma occurs in the absence of cirrhosis. Due to the importance of heavy alcohol ingestion and chronic hepatitis C infection in the pathogenesis of PCT, it is often impossible to attribute the histopathologic changes in livers of PCT patients to porphyria alone. However, mild abnormalities have been observed in young women whose disease appeared to be precipitated solely by estrogen therapy. Because most of these observations were made prior to availability of serologic or PCR-based testing for hepatitis C infection, virtually all of the literature on the hepatic histopathology of PCT prior to 1990 must be interpreted with caution.

Table 44.5. Differential diagnosis of PCT-like cutaneous features (vesiculo-bullous eruptions)

Porphyrias
Congenital erythropoietic porphyria (late onset)
Hepatoerythropoietic porphyria
Hereditary coproporphyria
Porphyria cutanea tarda
Variegate porphyria
Hepatic tumors that overproduce porphyrins (porphyrins with 8, 7, 6, 5, or 4-COOH groups)
Drug reactions (pseudo-porphyrias)
Vesiculo-bullous skin changes in patients on long-term hemodialysis

Differential diagnosis

Vesiculo-bullous and other disorders of the skin typical of PCT may occur in several other forms of porphyria, including hepatoerythropoietic porphyria, congenital erythropoietic porphyria (that may occasionally resemble PCT due to late onset), hereditary coproporphyria, and variegate porphyria (Table 44.5). Rarely, benign or malignant hepatic tumors may overproduce porphyrins and lead to a PCT-like syndrome. A variety of cutaneous reactions to drugs resemble closely the lesions seen in PCT. Some patients with chronic renal failure who require long-term hemodialysis also develop vesiculo-bullous skin changes that resemble PCT.

Occasional patients with variegate porphyria exhibit urinary porphyrin and porphyrin precursor excretion patterns similar to those of PCT. Therefore, analysis of urine alone cannot provide an unequivocal diagnosis of PCT. This must be supplemented by plasma or fecal porphyrin determinations[6]. Fluorescence emission scanning of plasma is simpler and less expensive than fecal analysis. In the drug-induced pseudo-porphyrias, porphyrin excretions are normal. In patients undergoing chronic hemodialysis, fecal porphyrin analysis is the most specific test, because plasma porphyrin concentrations, although generally higher in overt PCT, are increased in both conditions.

Pathogenesis of PCT

The central issues for understanding the pathogenesis of PCT, especially the association of this disease with hemochromatosis and iron overload, involve explanations of how elevated iron levels lead to increased porphyrin levels, and why only some individuals with increased hepatic iron stores develop clinically overt PCT.

Mechanism of uroporphyrin overproduction

Heme is synthesized from glycine and succinyl-CoA, and normally this occurs with little accumulation of uroporphyrin (Fig. 44.1). The hepatic production of uroporphyrin may be increased if: (i) the rate of decarboxylation of uroporphyrinogen to coproporphyrinogen (catalyzed by Uro-D) is decreased; (ii) the rate of oxidation of uroporphyrinogen to uroporphyrin is increased; (iii) the amount of ALA synthesized by, or imported into, hepatocytes is increased; or (iv) a combination of these events occur.

Decreased Uro-D activity can be the primary cause of overproduction of uroporphyrin and heptacarboxylporphyrin[12, 13], because increased substrate concentrations can result from the need to maintain the rate of hepatic heme synthesis. The most straightforward examples of this are provided by Type II PCT and hepato-erythropoietic porphyria[14, 15], in which mutations in the Uro-D gene have been determined at the molecular level, and the effects of these mutations on enzyme activity are known. Even in these cases, little accumulation of uroporphyrin occurs unless Uro-D activity levels are decreased to $<25\%$ (or in some cases, $<10\%$) of the normal values[16], indicating that, under normal circumstances, Uro-D is present in considerable excess.

The oxidation of uroporphyrinogen to uroporphyrin (and of heptacarboxylporphyrinogen to heptacarboxylporphyrin) is also an important factor in porphyrin accumulation. The rate of this oxidation can be influenced by many factors, including the activity of cytochrome P-450 1A2. When rodents, rodent hepatocytes, or primary cultures of chick embryo hepatocytes are exposed to polyhalogenated aromatic hydrocarbons, such as hexachlorobenzene, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, or some congeners of polychlorinated biphenyls (all of which are inducers of cytochrome P-450 1A2), they can develop profoundly increased uroporphyrin and heptacarboxylporphyrin levels[17, 18]. This has been attributed, in part, to the cytochrome P-450 1A2-mediated increase in the oxidation rate of porphyrinogens to porphyrins, and to the formation of non-porphyrin products of porphyrinogen oxidation that inhibit Uro-D activity[19–21]. However, these Uro-D inhibitors have not been characterized, and the overproduction of uroporphyrin in cultured hepatocytes can be prevented by inhibitors of cytochrome P-450[17, 22]. One hypothesis that has been proposed to account for the occurrence of Types I and III PCT is that patients with these

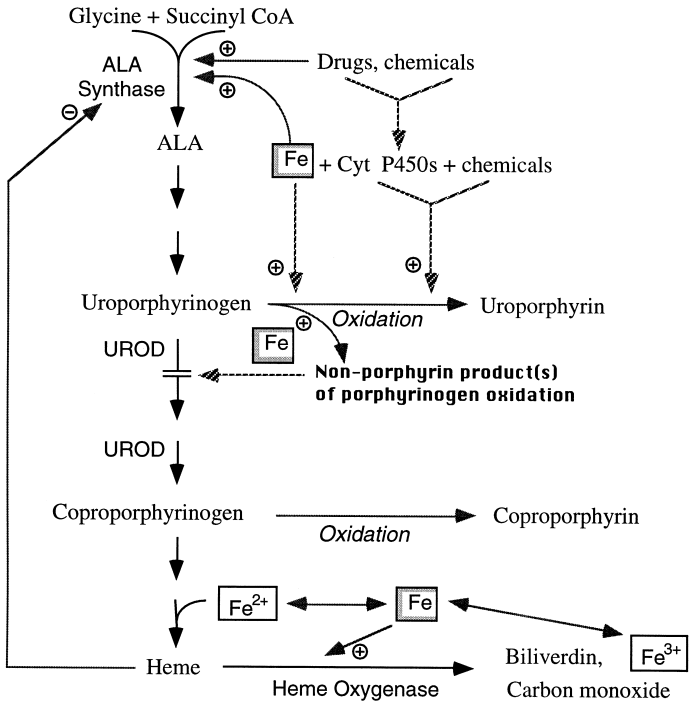


Fig. 44.5. The role of iron in the pathogenesis of porphyria cutanea tarda. The rate-controlling enzymes for hepatic heme biosynthesis (ALA synthase) and breakdown (heme oxygenase) are shown, as is uroporphyrinogen decarboxylase (UROD), the enzyme that carries out the stepwise decarboxylation of uroporphyrinogen to coproporphyrinogen. UROD activity is decreased in PCT. Iron, especially in concert with cytochromes P450, increases oxidation of porphyrinogens to porphyrins. It also appears to enhance production of a nonporphyrin inhibitor of UROD that is probably derived from uroporphyrinogen. Iron also induces heme oxygenase, causing depletion of a regulatory heme pool and thus derepression of ALA synthase which further increases the production of uroporphyrinogen and uroporphyrin.[57]

forms of PCT have inherited or acquired increases in hepatic levels of cytochrome P-450 1A2. However, no such increases have as yet been demonstrated. An earlier claim that levels of total cytochrome P-450 are markedly increased in patients with PCT[23] was later shown to be an artifact caused by the high level of uroporphyrin and the method of assay employed[24]. Further evidence to support the idea that oxidation of porphyrinogens is important in the process of uroporphyrin accumulation comes from observations that the antioxidant ascorbate can partially prevent uroporphyrin accumulation in ascorbate-deficient rats treated with hexachlorobenzene[25], and that some PCT patients have low plasma levels of ascorbate[26].

The amount of ALA that is synthesized by, or imported into, hepatocytes, can affect the accumulation of porphyrins significantly. Because ALA synthase is normally the rate-controlling enzyme for hepatic heme biosynthesis, its level of activity may play a role in uroporphyrin overproduction in experimental models of porphyria and in porphyria patients. In experimental models of porphyria, phenobarbital and other drugs that induce ALA synthase (and thus increase the production of ALA) markedly increase porphyrin accumulation[27]. The administration of ALA to SWR mice caused a decrease in Uro-D activity and an increase in levels of uroporphyrin, but these changes were not observed in other strains of mice tested[28]. When hepatic ALA synthase activity has been measured in patients with PCT, the levels are not always increased[29].

The role of iron in uroporphyrin overproduction

A large number of clinical observations and experimental studies have shown two important facts about the role of iron in uroporphyrin overproduction. First, the presence of iron overload, whether from hemochromatosis or other causes, is rarely sufficient to cause uroporphyria in the absence of other predisposing factors. Second, when predisposing factors are present, increased iron levels can greatly increase the severity, or decrease the time to onset, of porphyrin overproduction. Thus, iron overload acts to exacerbate uroporphyrin overproduction, but it is not the primary cause.

Iron can enhance uroporphyrin overproduction in a variety of ways, including: (i) increasing the rate at which uroporphyrinogen and heptacarboxylporphyrinogen are oxidized to their corresponding porphyrins; (ii) decreasing the activity of uroporphyrinogen decarboxylase; or (iii) increasing the amount of ALA present in the cell (Fig. 44.5). Iron can catalyze the formation of reactive oxygen species, which can, in turn, catalyze the oxidation of porphyrinogens to porphyrins[30, 31]. Similarly, certain forms of cytochrome P-450 (CYP 1A2 in rodents and CYP 2H in chickens) that contain iron in the form of heme in their active sites catalyze the NADPH-dependent oxidation of uroporphyrinogen[32, 33]. Crystals of uroporphyrin sometimes found in proximity to ferritin molecules in the livers of patients with PCT (Fig. 44.4) and in highly uroporphyric rodents suggest that ferritin may function as a catalytic site for the oxidation of uroporphyrinogen[8].

Iron can also increase uroporphyrin overproduction by decreasing the activity of Uro-D. This does not appear to be a direct effect of the iron on the enzyme, because purified Uro-D is not inhibited by either Fe^{2+} or Fe^{3+} under physiological conditions[34], and because neither acute nor chronic iron overload typically cause uroporphyrin accumulation.

A more plausible role for iron in decreasing Uro-D activity is its ability to enhance the formation of non-porphyrin products of porphyrinogen oxidation that are the direct inhibitors of Uro-D (Fig. 44.5). This effect of iron is probably responsible for the uroporphyria produced in some strains of mice by iron overload alone[35].

Iron can also increase uroporphyrin overproduction by increasing the amount of ALA present in the cell (Fig. 44.5). Through induction of ALA synthase[36]. This is mediated primarily by the iron-mediated induction of heme oxygenase that depletes the regulatory heme pool in hepatocytes, and thus increases the activity of ALA synthase[27, 37, 38]. These actions of iron are summarized in Fig. 44.5.

Alcohol, estrogens, and viral hepatitis in pathogenesis of PCT

Other important factors that contribute to development of PCT include heavy use of alcohol, use of estrogens, and, in some parts of the world, chronic hepatitis C infection. A major cause of chronic hepatitis C is intravenous use of illicit drugs, and many users of such drugs also drink alcohol heavily. Thus, these risk factors often occur in the same patients[39].

All of the known risk factors for PCT (alcohol, estrogens, iron overload, and chronic hepatitis C), probably act mainly by increasing oxidative stress and porphyrinogen oxidation within hepatocytes.

Acute intake of ethanol induces hepatic ALA synthase to a modest degree[40, 41]. Exposure of hepatocytes to longer chain alcohols (e.g., propanol, butanol, pentanol) has a greater inducing effect; such alcohols are found particularly in red wines and 'dark' spirits, such as scotch and bourbon. Chronic exposure to these alcohols also induces hepatic cytochromes P-450, particularly those of the 2E family, and this may increase hepatic demand for heme synthesis. However, they have not been shown to affect the activity of hepatic Uro-D. Thus, how or why uro- and heptacarboxylporphyrin accumulate in the liver after alcohol ingestion is not readily explained by the known effects of alcohol on hepatic heme metabolism. The most plausible explanation for the effect of alcohol on PCT is that it increases oxidative stress in hepatocytes. This effect has been shown in a variety of experimental systems. Recent studies, in which this effect was synergized by modest degrees of hepatic iron overload (2–3 times above normal; similar to those typically encountered in heterozygous hemochromatosis and PCT), emphasize the importance of inciting factors acting together[42]. Oxidative stress in hepatocytes probably leads to increased oxidation of porphyrinogens to porphyrins and to other consequences already described.

Like alcohol, estrogens induce hepatic ALA synthase and porphyrin accumulation in cultured hepatocytes[43], but have no known effect on Uro-D. Estradiol is an excellent substrate for cytochromes P-450 1A2 and 3A4 that catalyze hydroxylation in the 2-position, forming a catechol derivative[44]. Catechols are involved in redox cycling reactions, and it is possible that estradiol increases oxidative stress and porphyrinogen oxidation in hepatocytes. Possible synergistic effects of estrogens with alcohol or iron are not well studied.

Chronic liver disease of many causes may also increase oxidative stress in hepatocytes. With respect to PCT, chronic hepatitis C is particularly important because in some parts of the world (e.g., France, Italy, and Spain), 70–90% of patients with PCT have chronic hepatitis C[45–47]. In contrast, where chronic hepatitis C is less prevalent (e.g., Northern Europe, Australia, and New Zealand) but hemochromatosis is more prevalent, 0–20% of PCT patients have chronic hepatitis C[4, 39]. In the USA, the prevalence of chronic hepatitis C in patients with PCT is intermediate (56%)[48]. Chronic hepatitis C increases hepatic oxidative stress[49]. A mild decrease (~20%) in hepatic Uro-D activity was described in one study[50], but whether this decrease is unique to chronic hepatitis C, or occurs also in other liver diseases, was not tested. In addition, whether such a mild decrease in an enzyme present in considerable excess would cause porphyrin accumulation remains unclear. PCT complicating chronic hepatitis C has been associated exclusively with genotype lb hepatitis C virus in Northern Italy[51]. In the USA, all cases thus far described have belonged to genotype 1, and both subtypes a and b were detected[48]. This is not surprising, because genotypes la and lb account for approximately 75% of all chronic hepatitis C in the USA, and the liver disease caused by these viral strains is severe and not usually responsive to therapy with alpha-interferons. Patients with chronic hepatitis C often have increased serum ferritin concentrations, transferrin saturation values, and hepatic iron concentrations. Patients with these changes do not respond to alpha-interferon therapy as well as those with normal values of iron studies. Iron reduction alone usually decreases concentrations of serum aminotransferases in patients with chronic hepatitis C, but has little, if any, effect on serum viral RNA levels. Iron reduction may also improve the end-of-treatment and sustained response rates to alpha-interferon therapy[52].

An apparent association between infection with the human immunodeficiency virus (HIV) and PCT has also been described[53]. Others have argued that it is actually co-infection with HCV, a common occurrence in HIV-infected patients, that increases the risk of PCT[54].

Table 44.6. Management of porphyria cutanea tarda

Avoid precipitating or exacerbating agents
Stop intake of alcohol, estrogens, drugs that induce cytochrome(s) P-450
Stop intake of medicinal iron supplements
Restrict intake of foods rich in heme iron
Protect skin from light or other injury
Limit sun exposure
Wide-brimmed hat, long sleeves, etc.
Special clothing impervious to sunlight
Opaque sunscreen (titanium- or zinc-oxide)
(Regular sunblocks are not effective)
Remove iron
Therapeutic phlebotomies at least weekly (as for HHC)
Chronic, slow infusions of deferoxamine (as for secondary HC due to anemias)
Erythropoietin for those with chronic renal failure (to increase iron mobilization and incorporation into erythrocytes)
Antimalarials (chloroquine, hydroxyquine)
Start with low dose (125mg twice or thrice per week)
Antiviral therapy – treat chronic hepatitis C, HIV infections
(Alkalinization of urine – increase excretion of filtered porphyrins)
(Supplemental antioxidants/sulfhydryl donors)
(Vitamins A, C, E)
(*N*-acetyl cysteine)
(S-adenosylmethionine)
(Silymarin)

Note:
Factors listed within parentheses are perhaps of theoretical benefit but have not been shown to be beneficial in clinical trials.

Management of PCT

Clinical remission of PCT may occur with only cessation of alcohol ingestion or estrogen therapy (Table 44.6). However, if numerous active skin lesions are present or urinary porphyrin excretion is markedly increased (>2500 μg/d), more active treatment is advisable. The treatment of first choice is removal of iron. In most patients this can be carried out by a regimen of therapeutic phlebotomies performed at least weekly, as for therapy of hemochromatosis. In a small minority of patients with PCT and secondary forms of iron overload due to anemias, chronic infusions of deferoxamine may be needed. Patients with severe chronic renal failure who have a defect in iron mobilization and erythrocyte production may require injections of erythropoietin to increase iron mobilization and blood hemoglobin concentrations sufficiently high to permit therapeutic phlebotomies[1, 2, 55].

Alternatively, or in addition to iron removal, low-dose antimalarial drugs may be used. These agents are taken up into the acidic endosomal compartment of hepatocytes where they increase the pH and form water-soluble complexes with porphyrins. These complexes are released from the liver, probably due to stimulation of exocytosis. In addition, the antimalarials may inhibit synthesis of porphyrins. The starting dose of antimalarials should be 125 mg orally given 2–3 times weekly. Higher doses may lead to acute symptomatic hepatotoxicity, and even the low doses may be associated with mild transient increases in serum aminotransferase concentrations at the start of treatment. It is recommended that antimalarial treatment be continued until the urinary excretion of porphyrins is <500–600 μg/24 h. This usually requires 4–6 months of therapy.

In those PCT patients with evidence of chronic infection with hepatitis C virus or human immunodeficiency virus, specific antiviral therapy should be considered. Because the removal of iron from the liver may itself reduce the severity of chronic viral hepatitis and increase responsiveness to antiviral therapy, we recommend iron depletion prior to initiation of antiviral therapy for chronic hepatitis C.

Increasing the urine pH will lead to greater excretion of porphyrins that have been filtered at the glomerulus by trapping a larger proportion of them in the renal tubules and inhibiting tubular reabsorption. Thus, in patients who are particularly severely affected or who are anxious to ameliorate their disease as rapidly as possible, alkalinization of the urine with administration of sodium bicarbonate or Sholl's solution may be considered. Finally, there is theoretical rationale for the administration of supplemental anti-oxidants and, perhaps, compounds to replete or supplement hepatic glutathione levels in PCT. The use of vitamin C, however, can increase enteral absorption of iron and may also increase mobilization of iron from non-hepatic iron stores.

Middle-aged and older patients with PCT, particularly those with a history of heavy alcohol intake or evidence of chronic hepatitis C infection, should undergo hepatic biopsy. Those with extensive (bridging) fibrosis or cirrhosis should be considered for life-long screening for hepatocellular carcinoma with periodic measurement of serum alpha-fetoprotein concentrations and hepatic imaging. Patients with PCT and cirrhosis are at high risk for development of hepatocellular carcinoma, perhaps even higher than those with cirrhosis due to other causes[56]. However, early detection of hepatocellular carcinoma that is possible with regular screening of patients with cirrhosis has not been shown to increase the life expectancy in prospective, randomized trials.

Because the mutation for hemochromatosis is very frequent among Caucasians of Northern European origin with PCT[10, 11], it seems sensible to test all such patients for the C282Y mutation of the *HFE* gene. First-degree relatives of those who have this mutation should also undergo screening for hemochromatosis. Due to the rarity of familial (Type II) PCT, routine screening of first-degree relatives of probands for the presence of PCT is probably not indicated or cost-effective.

Conclusions

Porphyria cutanea tarda is the most common form of porphyria. It presents as a skin disease, although virtually all patients also have evidence of hepatic injury. Approximately 80% of patients have a sporadic and apparently acquired form (Type I) in which decreased activity of uroporphyrinogen decarboxylase is restricted to the liver. Most of the others have a familial form (Type II) in which mutations in the Uro-D gene are inherited in an autosomal dominant pattern, but have low clinical penetrance. Less than 5% of PCT patients have an apparently familial form of disease, in which deficiency of enzymatic activity is limited to the liver (Type III). In Type III PCT, it appears that an inherited defect in some other enzyme or protein in the liver leads to a secondary decrease in Uro-D activity.

Most patients with PCT have mild to moderate iron overload. Among those of Northern European extraction, 40–60% are heterozygous or homozygous for the C282Y mutation that is associated with hemochromatosis. Other factors that are important in pathogenesis are alcohol abuse, chronic hepatitis C infection, HIV infection, and estrogen intake. The pathogenesis of PCT depends upon an inherited and/or acquired decrease in activity of hepatic Uro-D by a process that involves iron and a non-porphyrin precursor of heme, the formation of which may be accelerated by induction of hepatic cytochrome(s) P-450 and increased oxidative stress in the liver.

The management of PCT involves avoidance of alcohol, estrogens, iron, and other chemicals, drugs, or toxins that precipitate or exacerbate the overproduction of porphyrins. The skin should also be protected from light and other injury. Specific treatment involves removal of iron from the liver and/or administration of antimalarial drugs such as chloroquine or hydroxyquine that increase excretion of porphyrins.

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Interactions of alcohol, iron and hemochromatosis

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Introduction

There has been much debate about the role of excessive alcohol ingestion in the development of iron overload and hemochromatosis. Some have suggested that hemochromatosis is the end-point of alcoholism[1, 2]; others suggest that excessive alcohol ingestion and hemochromatosis are unrelated. Recent observations helped resolve these controversies. First, the hepatic iron index is a reliable diagnostic tool for differentiating hemochromatosis from alcoholic liver disease[3, 4]. Second, alcohol ingestion can cause perturbations of several non-invasive iron status measurements used to screen for hemochromatosis[5]. Third, and most importantly, the genetic basis of many cases of hemochromatosis has been established. This was initially accomplished by means of elegant and pioneering linkage studies that revealed that the hemochromatosis allele resides on chromosome 6 in proximity to the HLA-A locus[6, 7]. The recent description of the putative hemochromatosis gene[8] and the production of animal gene knockout models[9, 10] should lend greater specificity to the molecular and biochemical characterization of the disorder. Further, African iron overload is a condition in which alcohol excess has been incriminated as an etiologic agent in the development of iron overload[11], although there is a possible genetic component of this disorder also[12]. This chapter delineates the effects of alcohol on iron measurements performed as screening tests for hemochromatosis, reviews the interactions between the use of excess alcohol and the pathogenesis of hemochromatosis, and explores the possible role of alcohol in the development of African iron overload.

Alcohol and the assessment of iron status

Traditionally, screening for iron overload has been accomplished by means of indirect, non-invasive measurements including the serum ferritin concentration and the percentage saturation of transferrin (a composite of the serum iron concentration and the unsaturated serum iron-binding capacity or the total serum iron-binding capacity). The serum ferritin concentration has a good positive correlation with the amount of iron present in body tissues, because there is an equilibrium established between the amount of ferritin synthesized intracellularly and the amount of ferritin secreted by the same cells[13]. Ferritin is synthesized intracellularly as a polymer of H (acidic) subunits and L (basic) subunits. Synthesis of the subunits and assembly of the polymer is regulated in direct proportion to intracellular iron content. Synthesis of ferritin provides the mechanism whereby iron is stored intracellularly to limit its toxic intracellular potential. A small amount of ferritin polymer undergoes glycosylation and secretion by the cell. Thus, serum ferritin concentrations are directly proportional to the cellular iron content and intracellular ferritin synthesis. This relationship is a precise one: 10 μg of cellular iron is equivalent to 1 μg ferritin/l, when serum ferritin is in the range of 12–300 μg/l[13, 14]. Therefore, serum ferritin concentration provides the simplest, non-invasive measurement of iron storage status and has been widely employed in screening for hemochromatosis.

At higher values of serum ferritin concentration, the relationship to cellular iron becomes less precise for several reasons[15]. Some of the commercial assays display a 'hook effect,' meaning that, for a given increment in the serum ferritin concentration, there is a disproportionately small increase in the measured absorbance. To overcome this problem, the assay needs to be optimized over the

appropriate concentration range. Another problem is that most serum ferritin assays are optimized over the 12–300 μg/l range. When a high ferritin value is obtained, some laboratories dilute the specimen, and errors of dilution and dilutional perturbations of the assay can lead to variable serum ferritin concentration measurements. Once the body iron burden has increased significantly, cellular injury related to the biochemical effects of increased iron develops, and there may be leakage of non-glycosylated tissue ferritin from cells that is detectable in the serum ferritin assay[16, 17]. This can lead to overestimation of the serum ferritin concentration. This phenomenon can be corrected by repeating the assay after passage of the specimen over an affinity column to remove glycosylated ferritin. Then, the true serum ferritin concentration can be calculated as the difference in the two assay results[18]. However, this is too labor-intensive for routine application in many laboratories.

The percentage saturation of transferrin has high sensitivity in screening for hemochromatosis[19]. The increased values of transferrin saturation observed in persons with hemochromatosis generally have two components. First, the serum iron concentration is markedly increased in hemochromatosis, and is directly related to the metabolic basis of the disorder (absorption of iron in excess of body iron needs). Increased body and hepatocellular iron deposition resulting from the disorder down-regulates transferrin synthesis such that the serum transferrin concentration or total iron-binding capacity is reduced. The quotient of serum iron concentration and total iron-binding capacity, referred to as the percent saturation of transferrin, is therefore elevated (usually >62%) in a majority of persons with hemochromatosis[19]. This increased saturation causing supersaturation of the normal carrier mechanism for iron in plasma results in the generation of reactive, non-transferrin bound iron. This form of highly available iron is believed to lead to increased iron uptake by and subsequent injury to parenchymal organs characteristic of the clinical hemochromatosis phenotype. The combination of percentage saturation of transferrin and serum ferritin concentration have been used to screen for and non-invasively diagnose hemochromatosis, and the information provided by these two measurements is complementary. The finding of supersaturated transferrin identifies a subject who is absorbing excessive iron and is at risk for progressive iron loading with consequent parenchymal organ damage. Serum ferritin concentration identifies subjects in whom progressive iron loading has already occurred.

Excessive alcohol ingestion can have a profound impact on these measurements, confounding the diagnosis of the clinical hemochromatosis phenotype by elevating, sometimes strikingly, the serum ferritin concentration. In a carefully conducted longitudinal study evaluating this phenomenon, markedly elevated serum ferritin concentrations in alcoholics undergoing detoxification declined precipitously when alcohol was withdrawn[20]. Further, the concentration of ferritin closely paralleled another hepatic indicator of alcohol ingestion, the serum concentration of g-glutamyl transpeptidase (GGT). In patients admitted to an alcohol withdrawal unit and in whom hemochromatosis was excluded, 58% had elevated serum ferritin concentrations; 11% had values >1000 μg/ml. These values showed a 400% reduction over the first 2–6 weeks of abstinence[21]. Similarly, 16% of patients admitted to an alcohol withdrawal unit had elevated serum ferritin concentrations, and the serum ferritin values declined with abstinence and were correlated with serum aspartate aminotransferase concentrations[22].

The mechanisms whereby alcohol ingestion leads to increased serum ferritin concentrations are incompletely understood. An obvious mechanism involves alcohol-induced hepatic damage and leakage of non-glycosylated tissue ferritin into the circulation. Alcohol may induce ferritin synthesis. A study of glycosylated and non-glycosylated serum ferritin in alcoholic patients being treated in a withdrawal center revealed that there were proportionate increases in both free and concanavalin A-bound ferritin; both forms of ferritin decreased during alcohol withdrawal[23]. Alcohol exposure at high levels for long periods increased H and L subunit synthesis and corresponding subunit mRNA levels in hepatoblastoma cells[24]. In rat liver cell cultures, low levels of alcohol increased the synthesis of L subunits. These data are consistent with the hypothesis that alcohol increases serum ferritin concentration as a consequence of hepatocyte injury and its stimulatory effect on ferritin synthesis.

These effects of alcohol on serum ferritin concentration can influence the results of any screening program for hemochromatosis profoundly[25, 26]. The strong association between alcohol ingestion and serum ferritin concentration was confirmed in a large Danish population survey[27]. If alcohol is suspected of causing an elevated serum ferritin concentration, surrogate markers of excess alcohol ingestion can be evaluated. These include blood urea nitrogen (often markedly reduced in persons who abuse alcohol), serum GGT (often elevated in persons who consume much alcohol), and erythrocyte mean corpuscular volume (MCV) (frequently mildly increased in heavy drinkers). Excess alcohol consumption is also associated with the presence of desialated transferrin (carbohydrate-deficient = CD) transferrin. Commercial assays for CD

transferrin are commercially available and may provide further clarification of the cause of an elevated serum ferritin concentration. Overall, it appears that CD transferrin is more sensitive than GGT or MCV. Elevation of the CD transferrin concentration is 60–70% sensitive and 80% specific for ingestion of >60 g of alcohol daily[28, 29]. There are reports of superiority of CD transferrin over the other markers[29, 30], although this finding is not uniform[31]. Other unrelated hepatic disease and hemochromatosis can confound the interpretation of the measurement[32]. Because CD transferrin and GGT measurements are not directly correlated, the most accurate testing for excess alcohol ingestion may be a combination of measurements[33].

Alcohol can also affect the measurement of the percentage saturation of transferrin. Hepatocellular injury is frequently associated with release of cellular iron into the circulation, resulting in an elevated serum iron concentration. Hepatocellular injury may also cause reduced transferrin synthesis, such that the total iron-binding capacity becomes reduced. Alcohol excess may also suppress erythropoiesis and reduce iron utilization and plasma iron turnover, thus increasing the serum iron concentration. In alcohol-induced sideroblastic anemia, alcohol may almost completely inhibit iron utilization in erythroid precursors. Alcohol excess has also been associated with reduced erythrocyte survival, and this may be associated with an increase in the serum iron concentration in alcoholics who develop folic acid deficiency, hypersplenism, or the hemolytic state known as Zieve's syndrome. In one series of patients admitted to an alcohol withdrawal unit, 16% had either a transferrin saturation >62% or a serum ferritin concentration >1000 ng/ml[22]. In another series, 15.2% of all persons admitted to an alcohol withdrawal unit had a percentage saturation of >62%[21]. These elevated saturation values in alcoholic patients return to normal rapidly after alcohol withdrawal and re-establishment of normal food intake.

Although the percentage saturation of transferrin and serum ferritin concentrations are useful screening measurements for the hemochromatosis phenotype, alcohol ingestion can confound the screening. Previously, the only reliable way to resolve certain of these situations was to perform a hepatic biopsy to obtain tissue for measurement of the total or non-heme iron concentration. This concentration, corrected for the patient's age and expressed as the hepatic iron index, has been reliable in distinguishing hemochromatosis from alcohol excess[3, 4]. Some degree of iron overload occurs in the livers of patients thought to have alcoholic disease of the liver[34–40], although this has not been a uniform finding[41, 42]. If present, the degree of hepatic iron loading determined either qualitatively (Prussian blue staining) or quantitatively (biochemical measurement of non-heme iron; total iron measured using atomic absorption spectrophotometry) is modest. The tissue distribution of the iron, different from that in hemochromatosis, is predominantly in macrophages in alcoholics and predominantly in hepatocytes in persons with hemochromatosis[5], even though most of these subjects frequently have increased percentage saturation of transferrin and serum ferritin concentrations. Other factors that can contribute to increased hepatic iron content in persons who consume excess alcohol include the iron content of alcoholic beverages, and possible effects of alcohol in promoting non-heme iron absorption by the gastrointestinal tract and enhancing hepatic iron uptake. High iron intake from alcoholic beverages in the developed world is unusual, because the iron content of alcoholic beverages manufactured in western countries is usually low. However, alcoholic brews with high iron contents and highly bioavailable iron are common in sub-Saharan Africa, and may contribute to the development of African iron overload[11].

Alcohol ingestion may stimulate mucosal iron uptake[43], possibly via an alcohol-induced non-carrier-mediated paracellular route[44], although this effect appears to be minimal[45, 46]. Polyphenols in many alcoholic beverages complex iron, thereby limiting its mucosal absorption[47]. Excess alcohol ingestion may cause shortened erythrocyte survival due to hemolysis or hypersplenism, or may cause ineffective erythropoiesis, largely due to folic acid deficiency. Hemolytic anemia and ineffective erythropoiesis may cause enhanced mucosal iron absorption[48]. Alcoholic cirrhosis is often associated with the development of intrapulmonary arteriovenous shunting of blood via fistulae that leads to systemic hypoxia, a factor that enhances iron absorption[39].

The hepatic uptake of radiolabeled iron from tagged serum transferrin is five-fold greater in persons with alcoholic cirrhosis than in normal subjects[49]. Although the cause for this is incompletely understood, one potential explanation is related to observations that desialated transferrin is present in increased concentrations in the sera of alcoholics. Hepatocytes acquire iron by mechanisms other than the transferrin receptor expressed on the surfaces of most cells. One mechanism involves the asialoglycoprotein receptor on the surface of hepatocytes that participates in the clearance of desialated proteins from the circulation[50]. However, this proposed mechanism is not fully consistent with the qualitative hepatic iron data in alcoholic subjects indicating that much of the increased iron is deposited in hepatic macrophages (Kupffer cells), not hepatocytes.

Although many factors may contribute to increased hepatic iron content in persons who consume much alcohol, the hepatic iron index discriminates between alcoholism and hemochromatosis. Alcohol-associated iron overload is always associated with a hepatic iron index of <2.0, whereas hemochromatosis is usually associated with a hepatic index of >2.0. Caucasian subjects with hepatic iron indices >2.0 are believed to have hemochromatosis, even though they may be heavy drinkers[39, 40]. Furthermore, persons who are heterozygous for the hemochromatosis gene do not develop clinically evident iron overload unless they exhibit excessive alcohol intake[39, 40, 51].

These observations demonstrate that excess alcohol intake confounds the screening for and diagnosis of hemochromatosis when biochemical measurements of phenotypic characteristics are employed, particularly serum ferritin concentration and percentage saturation of transferrin. Although invasive testing by quantification of iron in hepatic biopsy specimens may be ambiguous in the presence of alcohol excess, calculation of the hepatic iron index differentiates hemochromatosis from alcoholism. The genetic basis of most hemochromatosis that occurs in Caucasians has been identified[8]. This includes the identification of two mutations of the MHC class I-like *HFE* gene. Once heterogeneity of mutations within the *HFE* and other hemochromatosis-associated genes are characterized, the diagnosis of hemochromatosis can be made in many affected persons by genotyping rather than by measurement of phenotypic criteria. This will largely eliminate alcohol excess as a compounding variable in the diagnosis of hemochromatosis.

Alcohol and the pathophysiology of hemochromatosis

A consistent relationship between heavy alcohol consumption and hemochromatosis has long been recognized. In one report, one-third of persons with hemochromatosis consumed >50 g of alcohol daily[52]. In a recent evaluation of 105 phenotypically and genotypically (by linkage) defined hemochromatosis patients, 15% consumed >80 g of alcohol daily. Despite prior reports[40], histologic features of heavy alcohol consumption are usually not present in hepatic biopsy specimens of such hemochromatosis patients[53]. Persons with hemochromatosis who ingested much alcohol had a higher prevalence of hepatic cirrhosis, and their survival is reduced significantly. Accordingly, there is little doubt that alcohol can contribute to the organ damage associated with hemochromatosis, or accelerate the rate of development of symptomatic or pathologic disease otherwise associated with iron overload.

The pathogenesis of tissue damage in hemochromatosis is clearly related to increased cellular iron content, but the specific mechanisms are incompletely defined. The evidence linking iron to the pathogenesis of hepatic cirrhosis is based upon three fundamental observations. These are that (i) hepatic cirrhosis is rarely evident before a critical level of iron loading has been reached (usually a ten-fold increase in hepatic iron content); (ii) iron depletion therapy by phlebotomy can prevent the development of cirrhosis in homozygotes who are diagnosed before symptoms appear; and (iii) there is a positive correlation between regional intrahepatic iron load and the degree of hepatic fibrosis. There are two major theories regarding the mechanism of iron-related hepatic injury and cirrhosis. The first is that iron induces lipid peroxidation of organelle membranes leading to cellular injury and death[54]. The mechanism whereby this process excites a fibrogenic response is unknown, but it is possibly related to peroxidation products that stimulate stellate cells to produce collagen, or Kupffer cells to produce profibrogenic cytokines[55]. The second theory is that iron directly stimulates collagen synthesis in the liver, a hypothesis supported by observations that iron is associated with increased levels of procollagen I mRNA[56–59]. Fibrogenesis appears to be mediated by hepatic stellate cells that, when activated, change their expression from type III to type I collagen genes. Whether these theories are mutually exclusive or complementary is unknown.

Much evidence supports the hypothesis that there is synergy between alcohol and iron in causing hepatocellular injury and hepatic fibrosis. However, experimental models rely largely on rodents fed non-physiologic forms of iron and alcohol. The development of genetic knockout mice that develop a hemochromatosis-like phenotype[9, 10] should greatly facilitate these investigations. There are many reports of a potentiating effect of alcohol on hepatic injury in murine models of iron overload[60–63]. Although evidence of synergy of iron and alcohol in causing hepatocellular toxicity is abundant, the evidence for synergy in causing fibrogenesis has been more difficult to establish. In one study, there was no evidence of synergy[60], although the same investigators used a modified model and demonstrated a composite effect of iron and alcohol in causing fibrogenesis[64].

Little work has been conducted to investigate possible interactions of alcohol consumption and iron with respect to other target organs of iron overload in hemochromatosis, particularly the heart and the pancreas. A genetic knockout mouse model that develops a hemochromatosis-

like phenotype could provide an excellent model for conducting these evaluations. With the identification of the *HFE* gene and its mutations that are associated with hemochromatosis, it will be important to evaluate interactions of iron and alcohol at the genomic level.

Nutritional iron overload

There appears to be one circumstance in which heavy alcohol consumption is related to the development of parenchymal organ loading of iron with consequent organ dysfunction: iron overload in sub-Saharan Africa[11, 12]. In this setting, locally brewed beers contain large amounts of iron. The acidic environment produced by fermentation leaches iron out of the iron brewing containers. The organic acids in the brew, particularly lactic acid, have a marked effect in promoting gastrointestinal absorption of iron[65], and these circumstances appear to override the normal control mechanisms governing iron absorption and homeostasis. However, this interpretation has been questioned in a recent study that suggests that African iron overload may be related to a non-HLA-linked genetic factor[12, 66]. One confounding factor is the relatively high frequency of hemoglobinopathies in the region that could increase iron absorption in affected persons[67].

Conclusions

Excessive alcohol consumption does confound the interpretation of abnormal serum iron parameters and diagnosis of hemochromatosis when phenotypic criteria are used, a problem that may be alleviated by development of genotypic diagnostics. Alcohol appears to be a significant co-factor in the development of parenchymal organ dysfunction in persons with iron overload. Alcohol ingestion rarely causes significant iron overload, with the possible exception of African iron overload.

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Iron overload in African Americans

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Introduction

Primary iron overload is a problem among African Americans[1–4], but the condition is not often recognized by clinicians. The prevalence is not known, and whether a genetic defect is responsible is uncertain. In contrast, hereditary iron overload in the form of HLA-linked hemochromatosis is a well-recognized condition among whites in the United States[5], and a mutation in the newly described *HFE* gene on chromosome 6 is responsible for most cases[6]. Several explanations for primary iron overload in African Americans are possible: (i) an admixture of the genetic defect for HLA-linked hemochromatosis from the Caucasian population of northern European extraction into the African–American population via interracial mating; (ii) a distinct but unidentified inherited defect that may be implicated in iron overload in Africa; and (iii) a sporadic occurrence of iron overload due to unidentified mechanisms. The *HFE* defect is rare in the African–American population and has not been identified in most African Americans with iron overload investigated. These observations suggest the possibility that primary iron overload in African Americans may be related to the iron-loading disorder found in the people of sub-Saharan Africa. Iron overload is a common condition in Africa where, in many areas, the prevalence of excessive body iron of a severity to cause damage to the liver is 10% or more[7]. The condition is related to a diet high in iron, but recent studies of pedigrees suggest that a genetic defect may also be implicated in iron overload in Africans and that the putative defect is different from the *HFE* mutation in Caucasians[8–10]. One half or more of the ancestors of African Americans came from regions of Africa where iron overload is common. A recent increase in the number of reports of African Americans with primary iron overload[3, 4] may represent early indications that this form of iron overload is more common than previously believed. In this chapter, iron overload in Africa is reviewed and the possibility that a related condition is present in the African–American population considered.

Iron overload in Africa

Distinction from alcoholic siderosis and hemochromatosis in Caucasians

Iron overload has been recognized in sub-Saharan Africa for over 60 years[11]. The condition is known as 'dietary iron overload,' because the consumption of a traditional fermented beverage rich in iron is strongly associated with the development of iron-loading[12]. Two observations are important in characterizing iron overload in Africa: (i) the condition is distinct from alcoholic hepatic disease, although the excess iron is often derived from a traditional beer that contains alcohol. Hepatic iron concentrations do not exceed 90–180 μmol/g dry weight in alcoholic hepatic disease[13, 14]. In iron overload in Africans, liver iron concentrations often exceed these levels[15–18] and the histological changes of alcohol effect are almost always absent[18, 19]. (ii) Although the clinical manifestations of African iron overload and HLA-linked hemochromatosis are similar, the pattern of iron accumulation in iron overload in Africans differs from that of Caucasian HLA-linked hemochromatosis. Iron deposition is prominent both in macrophages and in hepatic parenchymal cells in African iron overload[20, 21]. By contrast, HLA-linked hemochromatosis is marked by relatively reduced amounts of iron in macrophages[21, 22]. This raises the possibility that two distinct metabolic defects are responsible for the two conditions.

General description

Iron overload in southern African blacks was first described by A.S. Strachan[11] in the context of a necropsy study of more than 500 adults from Botswana, Lesotho, Malawi, Mozambique, South Africa, Swaziland, and Zaire who died in South Africa from 1925 to 1928. The investigator concluded that: 'Haemochromatosis is a not uncommon disease in the South African native; the chief factor in its production appears to be the diet. The development of the complete picture of bronzed diabetes depends on the degree of deposition of the pigment and the rate of its deposition. In mild cases pigmentation alone may occur (this was seen in the younger natives); in slightly more severe cases, some cirrhosis may be added and so on until the complete picture is produced.' While Strachan's findings were treated with skepticism by JH Sheldon in his classic monograph on hemochromatosis in 1935, the high prevalence of the condition was later confirmed in a number of studies[15-18, 23–29].

Iron overload in southern African blacks was initially thought to be the result of some metabolic defect induced by chronic malnutrition[23], but the dietary intake of iron was subsequently shown to be very high, with most of the iron being derived from the iron drums and cans used for the brewing of traditional alcoholic beverages[12, 26, 27, 30]. The histological findings were distinctive, with the bulk of the iron being deposited in the hepatocytes and in the cells of the mononuclear-macrophage system in most subjects[25]. Several direct and indirect sequelae were documented. Direct sequelae included micronodular cirrhosis[15, 16, 20] and diabetes mellitus[31] and indirect sequelae included ascorbic acid deficiency and osteoporosis[32–34]. African iron overload may also be associated with the development of esophageal carcinoma[17].

Recent studies in African iron overload

Over the past 15 years a series of studies indicate that iron overload is a common, contemporary problem in much of central and southern Africa, and that an iron-loading locus which is distinct from the HLA-linked hemochromatosis gene may be involved in pathogenesis.

Iron overload has been reported in at least 15 countries of southern, central, east and west Africa. In certain necropsy studies from each of these geographical areas, the prevalence of hepatic iron loading of a degree to cause cirrhosis has been greater than 10%[7]. Although a study in the 1970s indicated that the prevalence and severity of iron overload in urban black South Africans was declining substantially due to decreased consumption of traditional beer prepared at home[17], four studies in the 1980s and 1990s showed that iron overload remains a common condition in rural areas of Africa where 80% of the population resides.

Five hundred and five adult members of a rural community in western Zimbabwe were surveyed in 1985, including 393 apparently healthy individuals and 112 inpatients or out-patients at a small hospital. Among traditional beer drinkers, 49 of 297 men (16.5%) had a high serum ferritin concentration and a transferrin saturation of greater than 70%, a combination that indicates a risk of hepatic disease and other pathological effects from excess body iron. One of 99 apparently healthy men who were not beer drinkers (1.0%) also had the combination of elevated serum ferritin concentration and transferrin saturation. No women had the combination of elevated iron tests, but the sample size was small and few women were traditional beer drinkers[29]. In a separate study conducted in a rural community in central Zimbabwe in 1994, four of 28 traditional beer-drinking men (14.3%) had the combination of elevated serum ferritin concentration and transferrin saturation[9]. These studies indicate that iron overload continues to be an important public health issue in rural Africa. The finding that only a minority of traditional beer drinkers had serologic evidence of toxic iron overload led to the hypothesis that, in addition to increased amounts of dietary iron, an inborn error of metabolism may also be responsible for iron overload in Africans.

To corroborate the finding of a high prevalence of iron overload in rural Zimbabwe, a similar study was conducted in a rural community in South Africa in 1988. Three hundred and seven men were surveyed, 127 of whom were apparently healthy and 180 of whom were hospitalized. Seventy-six percent of the men gave a history of traditional beer consumption. The combination of a transferrin saturation greater than 70% and elevated serum ferritin concentration was found in 18% of all beer drinkers and in 16% of apparently healthy beer drinkers from the community[18]. This study indicates that iron overload is currently a common condition in rural South Africa, with a prevalence similar to that in Zimbabwe.

To determine if iron overload is at present an important disease in a third African country and to examine the clinical consequences of the serological evidence of iron overload in the community surveys described above, 29 consecutive diagnostic hepatic biopsy specimens were studied in 1988 at a hospital in Swaziland that caters to a largely rural population. Twenty-one (72%) of the subjects undergoing hepatic biopsy were regular traditional beer drinkers. Nine (43%) of the beer-drinking subjects had hepatic non-heme iron concentrations of >360 μmol Fe/g dry weight (normal

<30 μmol/g dry weight), extremely high iron levels that carry a substantial risk for hepatic damage and cirrhosis. Two of these subjects were females. Seven (33%) of the subjects had the combination of excessive hepatic iron and cirrhosis, and one of these subjects was a woman[18]. This study indicates that iron overload is a contemporary problem in a third African country, that the condition may be an important cause of hepatic disease in Africa, and that iron overload is a problem in females and males.

Two studies suggest that the spectrum of pathology related to African iron overload is broader than previously suspected. Specifically, this form of iron overload may be a risk factor for hepatocellular carcinoma and for death from tuberculosis. Hepatocellular carcinoma is a complication of HLA-linked haemochromatosis in people of European ancestry, but the highly prevalent dietary iron overload of Africa has not been regarded as a risk factor for this malignancy. Both conditions of iron overload cause cirrhosis.

The first study of iron overload in Africans, conducted between 1925 and 1928, was re-examined to determine whether this common condition is associated with death from hepatocellular carcinoma and/or tuberculosis[35]. In the original study, necropsies were performed on 714 adult blacks from southern Africa. Hepatic and splenic iron levels were measured semi-quantitatively in 604 subjects and one of five iron grades was assigned. Deaths from hepatocellular carcinoma or from tuberculosis were examined in the context of age, sex, the presence of cirrhosis or other diagnoses that might be influenced by iron status, and tissue iron grades. Nineteen percent of men and 16% of women had the highest grade of hepatic iron. After adjustment for the presence of cirrhosis, hepatic iron grade was the variable most significantly associated with death from hepatocellular carcinoma ($p=0.021$). The odds of death from hepatocellular carcinoma in subjects with the highest grade of hepatic iron was 23.5 (95% confidence interval 2.1–225) times the odds in subjects with the three lowest grades. Splenic iron was the variable most significantly associated with death from tuberculosis ($p<0.001$). The odds of death from tuberculosis with the highest grade of splenic iron were 16.9 (4.8 to 59.9) times the odds with the two lowest grades. These findings suggest that iron overload in black Africans may be a risk factor for death from hepatocellular carcinoma and for death from tuberculosis.

More than 300 patients who experienced diagnostic hepatic biopsies processed at the University of Zimbabwe from 1992 to 1994 were reviewed and 215 patients whose hepatic biopsy specimens were suitable for the histological assessment of hepatocellular iron were assessed in detail[36]. The subjects were stratified according to hepatocellular iron grades of 0 to 2+ (normal levels to mild siderosis; $n=157$) and grades of 3+ or 4+ (distinctly elevated levels consistent with iron overload; $n=32$). Thirty-six subjects had hepatocellular carcinoma. Logistic regression modeling revealed a significant association between iron overload and hepatocellular carcinoma after adjustment for age, sex, and the presence of cirrhosis ($p=0.040$) The odds of hepatocellular carcinoma in subjects with iron overload were 3.2 (95% confidence interval 1.1–9.9) times that of subjects without iron overload. This finding is consistent with the idea that iron overload contributes to the pathogenesis of cirrhosis in Africa and suggests a possible etiologic association of African iron overload with hepatocellular carcinoma.

In contrast to hereditary hemochromatosis which in white populations is inherited through a gene linked to the HLA locus, iron overload in sub-Saharan Africa until recently was believed to result solely from increased dietary iron derived from traditional home-brewed beer. Two studies conducted in southern and central Africa have now tested the hypothesis that iron overload in Africans also involves a genetic factor.

A segregation analysis of 236 members of 36 African families was conducted. The families were chosen because they contained index cases with iron overload as determined by hepatic biopsies or clinical and laboratory evidence[8]. Likelihood analysis was used to test for an interaction between a gene (hypothesized iron-loading locus) and an environmental factor (increased dietary iron) that determines transferrin saturation and unsaturated iron-binding capacity. In index subjects, increased hepatic iron was present in both hepatocytes and macrophages. Among family members with increased dietary iron due to traditional beer consumption, transferrin saturation was distributed bimodally with 56 values normal (less than 60% saturation) and 44 elevated. The pedigree analysis provided evidence of both a genetic effect ($p<0.005$) and an effect of increased dietary iron ($p<0.005$) on transferrin saturation as well as on unsaturated iron-binding capacity. In the most likely model, increased dietary iron raised the mean transferrin saturation from 30% to 81% and decreased the mean unsaturated iron-binding capacity from 38 μmol/l to 13 μmol/l in heterozygotes for the iron-loading locus. The condition appeared to follow an autosomal dominant pattern of inheritance in the presence of increased dietary iron and a recessive pattern without increased dietary iron. The hypothesis of tight linkage to HLA was rejected. These findings suggest that iron overload in Africa may be caused by an interaction between the amount of dietary iron and a gene distinct from any HLA-linked gene.

To determine if serum ferritin concentrations follow a genetic pattern and to confirm the previous observation, a second pedigree study of 351 Zimbabweans and South Africans from 45 families was conducted. The families ranged in size from 2 to 54 members[10]. Iron status was characterized with repeated morning measurements of transferrin saturation, unsaturated iron-binding capacity, and serum ferritin concentration after supplementation with vitamin C. For each measure of iron status, segregation analysis was consistent with an interaction between an iron-loading gene and dietary iron content ($p<0.01$). In the most likely model, the transferrin saturation would be 75% and the serum ferritin concentration would be 985 μg/l in a 40-year-old male heterozygote with an estimated lifetime traditional beer consumption of 10000 liters, whereas the saturation would be 36% and the ferritin concentration would be 233 μg/l in an unaffected individual with identical age, sex, and beer consumption. This segregation analysis provides further evidence consistent with a genetic influence on the development of iron overload in Africans.

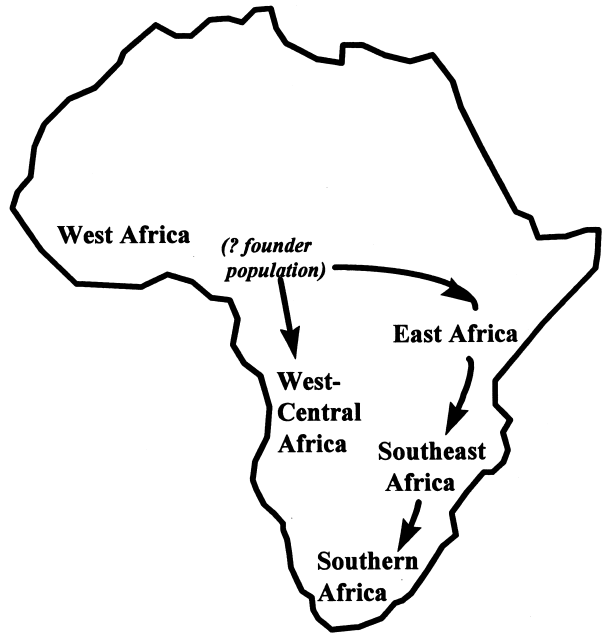

Fig. 46.1. A schematic map of Africa depicting (i) geographic regions referred to in the text, and (ii) location of postulated founder population of Bantu-speaking peoples with western and eastern migration patterns beginning approximately 4000 years ago.

Population migrations within Africa and the occurrence of iron overload

Studies that enlisted the prevalence of African iron overload have described this condition in predominantly four regions of the continent; (i) East Africa including Uganda[37], Kenya[38], and Tanzania[39]; (ii) Southeast Africa including Mozambique, Malawi[40], Zambia[41], and Zimbabwe[42]; (iii) Southern Africa including Lesotho, Botswana[28], South Africa[15], and Swaziland[18]; and (iv) West-Central Africa, that includes Angola[40] and Zaire[11]. These areas of Africa are all populated by descendants of Bantu-speaking people. Given that Caucasian hemochromatosis may have arisen from a single gene mutation in a solitary founder population that ultimately spread throughout Europe[6, 43], the possibility exists of a comparable etiology for *African* hemochromatosis, i.e., a single gene mutation in a founder population that subsequently spread throughout Africa. Although the presence of a gene for iron overload in Africa has not been proven, substantial evidence supports a hereditary patter to the condition[8, 10]. The possible genetic defect of African iron overload might be expected to be different than that of hemochromatosis because certain histological features of these two disease states are distinct[7].

Today, interrelated Bantu languages are spoken by more than 200 million people spread over an area of close to nine million square kilometers of sub-Saharan Africa[44]. It is among these Bantu-speaking people of East, Southeast, Southern, and West–Central Africa that most studies of African iron overload have been conducted. Archeologic and linguistic evidence demonstrates that this wide distribution of language exists as a result of a relatively recent (within the last 3000–4000 years) migration of people from an area that is now Cameroon and eastern Nigeria[45] (Fig. 46.1). This migration is thought to have occurred in two broad movements of peoples (Fig. 46.1). Linguists divide modern Bantu languages into two major groups, spoken respectively in the western and eastern parts of subequatorial (East, Southeast, Southern and West–Central) Africa[46–48]. The western group was the earliest expansion of Bantu-speakers from eastern Nigeria/Cameroon and advanced southward to Gabon, Zaire, and Angola[48]. The eastern group of Bantu-speaking people apparently migrated through or north of the equatorial forest in an easterly route from Nigeria/Cameroon to Lake Albert on the border of Uganda. Ultimately, the eastern Bantu expanded south from the lakes of East Africa to the Eastern Cape of South Africa early in the first millennium[44, 46, 49].

Archeologic evidence begins with the appearance of a characteristic type of pottery associated with metallurgy and mixed farming around the beginning of the first mil-

lennium AD throughout much of subequatorial Africa, signifying the beginning of the early Iron Age. Virtually all areas belonging to this cultural complex were inhabited by Bantu-speaking people. The pottery, metallurgy, and farming of the early Iron Age culture represent the introduction of major technical innovations to those geographic areas, suggesting a rapid and coherent physical movement of substantial numbers of people who brought with them a well-developed culture independent of the stone tool-using hunter gatherers whose lands they were entering. It is believed that these people were speakers of Bantu languages[44, 50]. Thus, both archaeologic and linguistic evidence supports the theory that Bantu-speaking people originated from the same founder population in Cameroon and eastern Nigeria, and that they subsequently migrated south to Angola and also to the east, reaching as far as Kenya and then the Eastern Cape of South Africa. This common ancestry is consistent with the theoretic possibility of a single gene mutation in a founder population giving rise to a hereditary form of iron overload in East, Southeast, Southern and West–Central African families.

The widespread distribution of iron overload in Africa also raises the question of whether a possible African iron-loading locus may influence iron metabolism in the African–American population. Interestingly, a significant proportion of the African slaves sold in North America were captured from areas populated by Bantu-speaking people where iron overload has been found. From British records of the African slave trade to North America, an estimated 24.5% of imported slaves were captured from West–Central Africa (Angola) and 1.6% from Southeast Africa (Madagascar and Mozambique), yielding a total of 26.1% of African slaves from Bantu-speaking origins during the peak of the slave trade from 1700–1809[51]. Later, based on the analysis of archives from British, Portuguese, French, Dutch, North American, and Danish vessels, it was estimated that 37.8% of the Atlantic slave trade was acquired from West–Central Africa during 1700–1809[52]. Even more complete British, Portuguese, French, Dutch, North American, Danish, and Swedish accounts from 1700–1867 have been examined to provide an estimate that 47.3% of the African slaves imported into North America were of Bantu-speaking origins, with 43.0% coming from West–Central Africa and 4.3% from Southeast Africa[53]. Because 25–50% of all slaves imported into North America were from areas populated with Bantu-speaking populations where iron overload has been documented to occur, a possible gene for African iron overload might be expected to occur in the African–American population.

Although no linguistic or archeologic evidence currently exists, proving that West African populations have a common ancestry with the Bantu-speaking peoples, iron overload has been described in this geographic region[54–56]. If iron overload in West Africa is familial, then even the 50–75% of the ancestors of African Americans who originated from this region may have been at risk for carrying the mutation.

Iron overload in African Americans

A seldom-reported condition

Very few articles regarding African Americans with 'hemochromatosis' have appeared in the literature[57–60], and, in general, primary iron overload has not been considered as a potential problem in African Americans. Nonetheless, the observations described in the previous sections raise the possibility that iron overload might be expected to occur in African Americans with some frequency. In brief, a non-HLA-linked iron-loading locus may be fairly common among Africans and homozygotes for this locus may develop iron overload in the absence of high dietary iron. Linguistic and archeologic studies indicate that most African populations affected by African iron overload derived from a single founder population 3000–4000 years ago. Thus, one mutation in the founder population could have given rise to a disorder such as a tendency to enhanced iron absorption. At least 25–50% of the ancestors of African Americans came from populations that manifest iron overload. Two recent reports emphasize that primary iron overload does occur in African Americans[3, 4], and some preliminary evidence suggests that the condition may not be rare[4]. It is therefore conceivable that some African–American subjects with iron overload might be homozygotes for a possible African iron-loading locus who manifest iron overload in the absence of increased dietary iron.

As a population, African Americans have an admixture of Caucasian genes[61]. In theory, the HLA-linked hemochromatosis gene may contribute to primary iron overload in some African–American subjects. Information presented by several investigators at the Centers for Disease Control and Prevention's Iron Overload, Public Health and Genetics Conference in March, 1997, and the International Symposium on Iron in Biology and Medicine in Saint Malo, France in June, 1997, indicates that the *HFE* mutation responsible for iron overload in Caucasians[6] is not present in the African Americans with primary iron overload studied so far. The presence of this mutation is virtually absent in African populations and very low in the African–American population. In this section, a series of

investigations are described that show that primary iron overload occurs in African Americans and that suggest the condition is more common than perceived at present.

Case reports of iron overload in African Americans

Individual case reports of possible primary iron overload in African Americans published between 1950 and 1996 are presented in Table 46.1. In these patients, secondary causes of iron overload such as blood transfusions or ineffective erythropoiesis were excluded, and the degree and pattern of iron-loading could not be explained by inflammation or malignancy. Most subjects gave a history of alcohol use, but the degree of iron-loading in the individual cases could not be accounted for based on the effect of alcohol alone.

Two recent publications have provided the most extensive documentation of the clinical presentation in African–American patients with primary iron overload. The first article reported seven patients with iron overload from a single internal medicine practice in Alabama[3]. Two patients from this article are excluded from Table 46.1 because they had normal hepatic iron indices. The second paper reported eight cases: four patients diagnosed during life with iron overload and four diagnosed in a review of autopsy cases[4]. All of the cases in whom hepatic iron concentration could be determined had hepatic iron indices ≥ 1.9[4]. The hepatic iron index is the hepatic iron concentration in μmol/g dry weight divided by the age in years. This index is useful for distinguishing primary iron overload from the moderate siderosis that may accompany alcoholic liver disease. The normal index is <1.0. An index ≥ 1.7 cannot be explained by alcohol effects and an index ≥ 1.9 indicates the magnitude of iron-loading found in Caucasian homozygous HLA-linked hemochromatosis[14, 62, 63]. The four patients with iron overload diagnosed during life were males, 27–50 years of age, with one or more of the following: hepatomegaly, cirrhosis, cardiomyopathy, diabetes mellitus, and impotence. All of the subjects had hepatic dysfunction and half had cardiac failure. Four (1.2%) of 326 African–American autopsy subjects had evidence of primary iron overload[4]. These two recent studies provide contemporary evidence that primary iron overload occurs and might be fairly common in African Americans.

A summary of clinical characteristics of the 19 published case reports is shown in Table 46.2. The pattern of iron-loading in the majority of subjects is characterized by the deposition of parenchymal and macrophage iron, a pattern that resembles the histologic findings of African iron overload. Serum and tissue iron measurements are consistent with the degrees of iron-loading found in HLA-linked hemochromatosis and African iron overload. Clinical findings associated with iron overload are common, including hepatic fibrosis or cirrhosis (71%), cardiomyopathy (12.5%), diabetes mellitus (31.3%), arthritis (31.3%), and impotence or decreased libido (25%). Strikingly, 43.8% of the case reports in which appropriate clinical information was available were diagnosed with a malignancy. The degree of iron overload observed in the majority of these patients could not be due to inflammatory changes secondary to malignancy alone. This raises the possiblity that primary iron overload in African Americans may be a risk factor for malignancy. An increased risk of extrahepatic malignancies has been described in African iron overload and HLA-linked hemochromatosis [17, 64, 65].

Taken together, these studies provide compelling evidence that primary iron overload occurs in African Americans. The majority of these cases closely resemble the form of iron overload that occurs in African people of Bantu origins. The findings are consistent with the possibility that an inherited genetic defect that originated in Africa may be present in African Americans.

Analysis of NHANES II data for African Americans

To determine if transferrin saturation values in African Americans reflect the presence of a gene that influences iron metabolism, the distribution of transferrin saturation values was analyzed in 808 African Americans studied in the second National Health and Nutrition Examination Survey. Transferrin saturation values were adjusted for the time of day of collection, for sex, and for the potential presence of the HLA-linked hemochromatosis gene. The findings were examined to determine whether they were consistent with population genetics for a major locus effect in which the proportion of normal homozygotes is q^2, of heterozygotes is 2 pq, of affected homozygotes is p^2, and in which $q + p = 1$. Three subpopulations based on transferrin saturation were present ($p<0.001$) and the fit to a mixture of three normal distributions was good. An estimated proportion of 0.867 of the African Americans studied were included in a subpopulation with a lower mean (± 1 S.D.) saturation of $26 \pm 6\%$; 0.125 comprised a subpopulation with an intermediate mean saturation of $40\% \pm 6\%$; and 0.008 formed a subpopulation with a higher mean saturation of $67 \pm 6\%$. These proportions are consistent with population genetics for a single major locus because the sum of the square roots of the lower proportion ($q = 0.9314$) and of the higher proportion ($p = 0.0854$) is approximately 1 (1.017). The results are compatible with the possibility that

Table 46.1. Published reports of African Americans with possible primary iron overload

No.	Age	Sex	Transferrin saturation (%)	Serum ferritin (μg/l)	Hepatic iron concentration (μmol/g)	Hepatic iron index	Estimated iron mobilized (g)	Liver parenchymal iron grade	Kupffer cell/ macrophage iron	Hepatic histology	Other findings	Reference
1	42	M	—	—	—	—	—	Large amount	Present	Cirrhosis	Diabetes mellitus	[57]
2	—	—	—	—	—	—	—	Large amount	Present	Cirrhosis	Iron in pancreas and other organs	[58]
3	64	M	37	242	331	5.2	12	3+	Minimal	No cirrhosis or fibrosis	Arthritis 2nd and 3rd MP joints	[59]
4	60	F	86	823	—	—	—	—	—	—	—	[1]
5	28	M	>75	285	—	—	—	—	—	—	—	[1]
6	50	M	96	2450	—	—	—	Large amount	Minimal	Cirrhosis	Arthritis, sickle cell disease, HLA-A3, B7, colon adenocarcinoma	[2]
7	48	M	29	495	—	—	4.6	—	—	—	Impotence, arthritis	[3]
8	33	M	62	382	143	4.3	6.7	4+	Present	No cirrhosis	Impotence, arthritis	[3]
9	67	F	53	3175	111	1.7		3+	Present	No cirrhosis	Arthritis, plasma cell myeloma, adenocarcinoma of the colon	[3]
10	63	M	37	1403	—	—	6.8	2+	Present	No cirrhosis	Decreased libido, weakness	[3]
11	42	M	92	2633	—	—	6.0	2+	Present	Cirrhosis	Weakness, diabetes mellitus, hyperpigmentation, hepatitis C	[3]
12	27	M	100	>600	545	20.2	>10	—	Present	—	Cardiomyopathy	[4]
13	44	M	95	1740	104	2.3	—	3+	Present	Cirrhosis, chronic hepatitis	Diabetes mellitus, hepatitis C	[4]
14	47	M	97	>1,000	541	11.5	22.7	—	—	—	Lung adenocarcinoma	[4]
15	50	M	99	3535	—	—	>10	4+	Present	Cirrhosis	Cardiomyopathy, diabetes mellitus, impotence, testicular atrophy	[4]
16	50	F	—	—	143	2.1	—	2+	Present	Fibrosis, steatosis	Diabetes mellitus, chronic lymphocytic leukemia	[4]
17	55	M	—	—	105	1.9	—	2+	Present	Fibrosis, steatosis, mallory bodies, metastatic carcinoma	Lung adenocarcinoma	[4]
18	57	F	—	—	552	5.6	—	4+	Present	Fibrosis, metastatic carcinoma	Pancreatic carcinoma	[4]
19	63	M	—	—	148	2.2	—	3+	Present	Fibrosis, steatosis	Carcinoma of esophagus and prostate, intestinal carcinoid	[4]

Table 46.2. Clinical characteristics of 19 published cases of African Americans with possible primary iron overload[a]

Characteristic	*n*, observation available	Result	Normal range
Age, years	18	50 (27–67)	
Males:Females	18	14:4	1:1
Transferrin saturation, %	13	89 (29–100)	20–45
Serum ferritin, μg/l	13	1403 (242–3535)	20–200
Hepatic iron concentration, μmol/g dry weight	10	146 (104–552)	<30
Hepatic iron index	10	3.3 (1.9–20.2)	<1.0
Hepatocellular iron grade	11	3+ (2+-4+)	0–1+
Macrophage iron present; *n*, %	15	13 (86.7%)	—
Iron mobilized by phlebotomy, g	8	6.8 (4.6–22.7)	<1.5
Hepatic fibrosis or cirrhosis present; *n*, %	14	10 (71.4)	Absent
Cardiomyopathy; *n*, %	16	2 (12.5)	Absent
Arthritis; *n*, %	16	5 (31.3)	Absent
Diabetes mellitus; *n*, %	16	5 (31.3)	Absent
Impotence or decreased libido; *n*, %	16	4 (25.0)	Absent
Malignancy; *n*, %	16	7 (43.8)	Absent

Note:

[a] Results expressed as median (range) unless otherwise indicated.

an altered gene that influences iron metabolism may be present among African Americans.

Conclusions

Iron overload is a common condition in Africa. The disorder has been studied most thoroughly in southern and central Africa, where both high dietary iron and an iron-loading locus appear to be involved in the pathogenesis. Iron overload also occurs in West–Central and West Africa, the ancestral origins of most African Americans. In general, observations from Africa are consistent with a hypothesis that an iron-loading mutation occurred in a founder population in Cameroon/Nigeria several thousand years ago and spread through population migrations to most of Africa south of the Sahara. Iron overload occurs in African Americans, and the condition does not appear to be related to the *HFE* gene mutation found in Caucasians. One autopsy study suggests that primary iron overload in African Americans may be more common than previously believed. As with HLA-linked hemochromatosis in Caucasians, the insidious onset of clinical manifestations of iron overload in African Americans and the common occurrence of associated conditions in the general population may make this disorder difficult to recognize without a high index of suspicion. Physicians need to consider the diagnosis of primary iron overload in African–American patients with medical disorders which may be a manifestation of excessive deposition of tissue iron including hepatic disease, diabetes mellitus, congestive heart failure, arthritis, impotence, decreased libido, weakness, and malignancy. HLA-linked hemochromatosis, once believed to be exceedingly rare, is now known on the basis of population studies to be one of the most common inherited disorders in Caucasians. Similarly, prospective studies need to be done to define the prevalence, clinical consequences, and molecular biology of primary iron overload in African Americans. Whether an iron-loading gene unique to Africa is implicated in the pathogenesis will require further investigation.

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Part X

Animal models of hemochromatosis and iron overload

47

β_2-microglobulin-deficient mice as a model for hemochromatosis

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Introduction

The roles of specific components of iron metabolism associated with gross iron maldistribution have been evaluated by identifying mutations in genes related to human diseases and by generating and phenotyping genetically altered mice. A list of new genes involved in iron metabolism that have been identified, cloned, and characterized recently is presented in Table 47.1[1–41].

Absorption of low molecular weight iron

Although iron is an abundant element, its availability is reduced because the oxidized form of the metal, iron (III), is extremely insoluble at neutral pH. Thus, complex mechanisms for its acquisition, utilization, and preservation have evolved in even the most primitive organisms. The process of intestinal iron absorption in mammals occurs in phases[42] (Fig. 47.1). In the initial uptake phase, iron is transported into the intestinal epithelial cell. Ferric iron in the lumen of the gut is reduced to ferrous iron by a ferric reductase. Next, the ferrous iron product is transported into the cell by a ferrous transporter, recently identified as DCT1/*Nramp2*[1], which is mutated in microcytic anemia *(mk)* mice[2]. Mucosal uptake is influenced by many intraluminal factors, including the chemical state of the iron in the test dose (ferric or ferrous, heme or non-heme), the amount of iron, the composition of the test dose, gastric and intestinal secretions, and the state of the brush border of the mucosal cells.

After uptake, the iron becomes part of a cellular iron pool and can be stored as ferritin or transported across the basolateral membrane of the cell (mucosal transfer) to complete absorption[42]. A novel gene encoding a multicopper ferroxidase called hephaestin (Heph)[15], which is defective in *sla* mice[16,17], has been identified as necessary for iron egress from enterocytes. The protein(s) involved in the transport across the basolateral membrane remains unknown. Mucosal transfer depends less on intraluminal factors than mucosal uptake, and therefore reflects body iron status more accurately. The absence of a functional *HFE*-β_2m complex, like that which occurs in β_2m-deficient mice, can decrease the iron that gains access to the mucosal cell via transferrin receptor[9]. This leads to low ferritin expression and increased expression of ferrous transporters at the microvillus and basolateral membranes. The overall expression of this sequence of events could lead to the observed paradox, i.e., that intestinal absorptive cells can behave as if they were iron-deficient, despite elevated iron body stores. To allow discrimination between mucosal uptake and mucosal transfer, a nonabsorbable indicator ($^{51}CrCI_3$) is used in iron absorption measurements[43], and this method has been adapted for use in small animal models[44–46].

The $\beta_2m^{-/-}$ mouse as a model for hemochromatosis

The availability of appropriate animal models can augment our understanding of the mechanism(s) of iron absorption. Interest in the β_2-microglobulin knockout mouse as a model for human hemochromatosis had two roots: one derived from the clinic[10], and another from an interest in duodenum-associated molecules possibly involved in the regulation of iron absorption[11]. The existence of hepatic iron overload in β_2-microglobulin (β_2m)-deficient mice was revealed similar to that found in hemochromatosis: pathologic iron deposits occur predominantly in hepatic parenchymal cells[10]. In these mice, cell surface expression of major histocompatibility complex (MHC) class I molecules is also severely decreased, and consequently they lack $CD8^+$ lymphocytes.

Table 47.1. Homologous genes that affect iron metabolism in humans and animal models

Gene	Function	Phenotype of animal disruption	Phenotype of human disease
DCT1/Nramp2[1]	Transports iron across microvillus membrane; endosomal iron transport	*mk mice*[2]; microcytic anemia; low intestinal uptake of iron; *b rat*[3] failure of iron transport out of endosomes	? Autosomal recessive iron-deficiency anemia, unresponsive to iron therapy[4,5]
HFE/MS2[6,7]	Associates with transferrin receptor[8,9]; regulates intracellular iron levels?	*β2m*$^{-/-}$ *mice*[10–12]; increased iron absorption; parenchymal iron overload *HFE*$^{-/-}$ *mice*[13]; iron overload	Hereditary hemochromatosis[14]; increased iron absorption; parenchymal iron overload
sla[15]	Transport of iron across intestinal basolateral membrane	*sla mice*[16,17]; *sex-linked anemia* microcytic anemia; decreased testinal iron transfer	?
Ceruloplasmin	Ferroxidase activity	*Fet3p – yeast homologue*; deficient growth on low Fe[18,19]	Aceruloplasminemia[20,21] deficient iron mobilization; low serum iron; tissue iron overload
hemox1 hemox2	Catabolysis of cellular heme to bilirubin, carbon monoxide and free iron; iron recycling	*hemox1*$^{-/-}$ *mice*[22]; anemia; tissue iron overload; chronic inflammation *hemox2*$^{-/-}$ *mice*[23]; lung iron accumulation	? ? idiopathic pulmonary siderosis[24]
Transferrin[25]	Iron transport in plasma and into cells	*hp mice*[26]; hypotransferrinaemic; microcytic anemia; parenchymal iron overload; increased iron absorption[27,28]	Hypotransferinemia[29,30]; anemia; parenchymal iron overload
Frataxin[31]	Mitochondrial iron transport	*Yfh1p – yeast homologue*; deficient growth on non-fermentable carbon sources due to mitochondrial iron overload[32,33]	Friedreich's ataxia[34–36]; neurodegeneration and cardiac myopathy
L-ferritin[37]	Iron storage	?	Hereditary hyperferritinemia-cataract syndrome[38–41]

These observations were later confirmed by Rothenberg and Voland[11]. They extended these findings, documenting that β_2-microglobulin-deficient mice develop progressive hepatic iron deposition that increases with supplemental dietary iron and that the mice have an increased incidence of hepatocellular carcinoma. Subsequently, it was demonstrated that β_2m-deficient mice have a four-fold increase in plasma iron concentrations, increased transferrin saturation (>80%), and increased hepatic iron when compared with normal control mice[12]. Furthermore, mucosal uptake of ferric (but not ferrous) iron and its subsequent mucosal transfer into the plasma is inappropriately increased in β_2-microglobulin knockout mice[12, 46]. Importantly, iron absorption was similar in mice in which the CD8 molecule has been disrupted (CD8$^{-/-}$ mice), in mice that lack the endoplasmic reticulum transporter for class I-associating peptides (TAP1$^{-/-}$ mice), and in normal control mice[12]. Taken together, these observations suggest that a MHC class I-like, β_2m-dependent gene product is involved in iron absorption. The finding of a novel gene of the MHC class I family (*HFE*) that is mutated in the majority of hemochromatosis patients provides further independent support for the proposed causative role of a β_2m-dependent gene in hemochromatosis and its involvement in iron absorption[6]. The recently characterized *HFE*$^{-/-}$ mice[13] confirm that mutations that disrupt the function of the *HFE* gene product result in development of the hemochromatosis phenotype.

Adaptive response of iron absorption In β_2m$^{-/-}$ mice

Although body iron content is the principal factor that regulates iron absorption, there are other physiological variables that also influence iron absorption, e.g., rate of erythropoiesis[47], hypoxia[48], and inflammation[49]. If the rate of erythropoiesis is stimulated by blood loss, dyserythro-

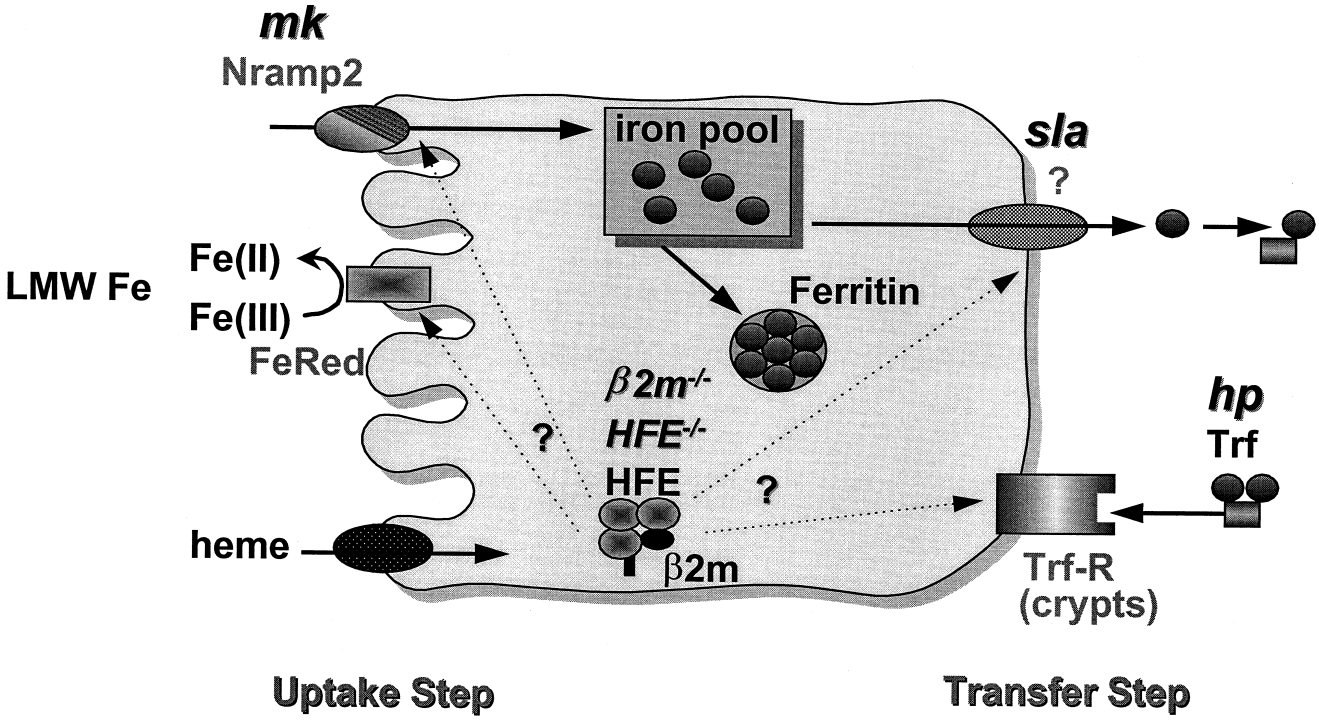

Fig. 47.1. Intestinal iron absorption. Gene products involved in iron absorption are represented schematically and relevant animal models in which a particular gene is mutated are represented in italics. Mouse models: *mk*, microcytic anemia; *sla*, sex-linked anaemia; *hp*, hypotransferrinemia; $\beta_2 m^{-/-}$, β_2-microglobulin knockout; $HFE^{-/-}$, *HFE* knockout; LMW Fe, low molecular weight iron; FeRed, ferric reductase; Trf, transferrin; Trf-R, transferrin receptor.

poiesis, or acute hemolysis, the efficiency of iron absorption is increased in normal persons. Conversely, if erythropoiesis is inhibited by hypertransfusion, starvation, or descent from high altitude to sea level, iron absorption is decreased. In $\beta_2 m^{-/-}$ mice, the adaptive response of iron absorption to an increase in the rate of erythropoiesis, stimulated either by blood loss or acute hemolysis, is indistinguishable from that of control mice[46]. This suggests that the regulatory mechanism(s) of iron absorption operating in response to these stimuli are independent of the expression of *HFE*.

In contrast, when iron stores are altered through dietary manipulations or parenteral iron injections, mucosal uptake and mucosal transfer are affected in $\beta_2 m^{-/-}$ mice. Indeed, $\beta_2 m^{-/-}$ mice respond by increasing or decreasing iron absorption appropriately. However, their ultimate retention of iron is invariably higher than that of control wild-type mice (Fig. 47.2). Therefore, the expression of the defect in iron absorption in the $\beta_2 m^{-/-}$ mice is quantitative and may involve the initial step of mucosal uptake of ferric iron and the subsequent step of mucosal transfer of iron to the plasma[12, 46]. The mechanism by which *HFE* interferes with iron absorption remains to be elucidated. However,

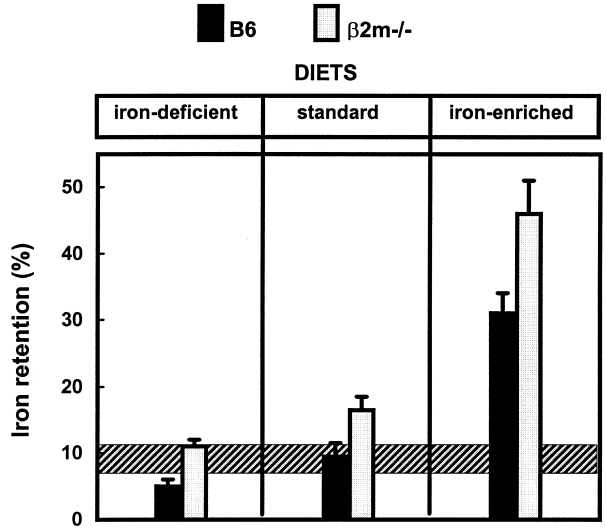

Fig. 47.2. Adaptive response of iron absorption to dietary manipulations in B6 and $\beta_2 m^{-/-}$ mice (modified from ref.[46]). Shadowed rectangle represents range for iron absorption of wild-type (B6) mice fed a standard diet. The data are presented as mean ± S.E.M.

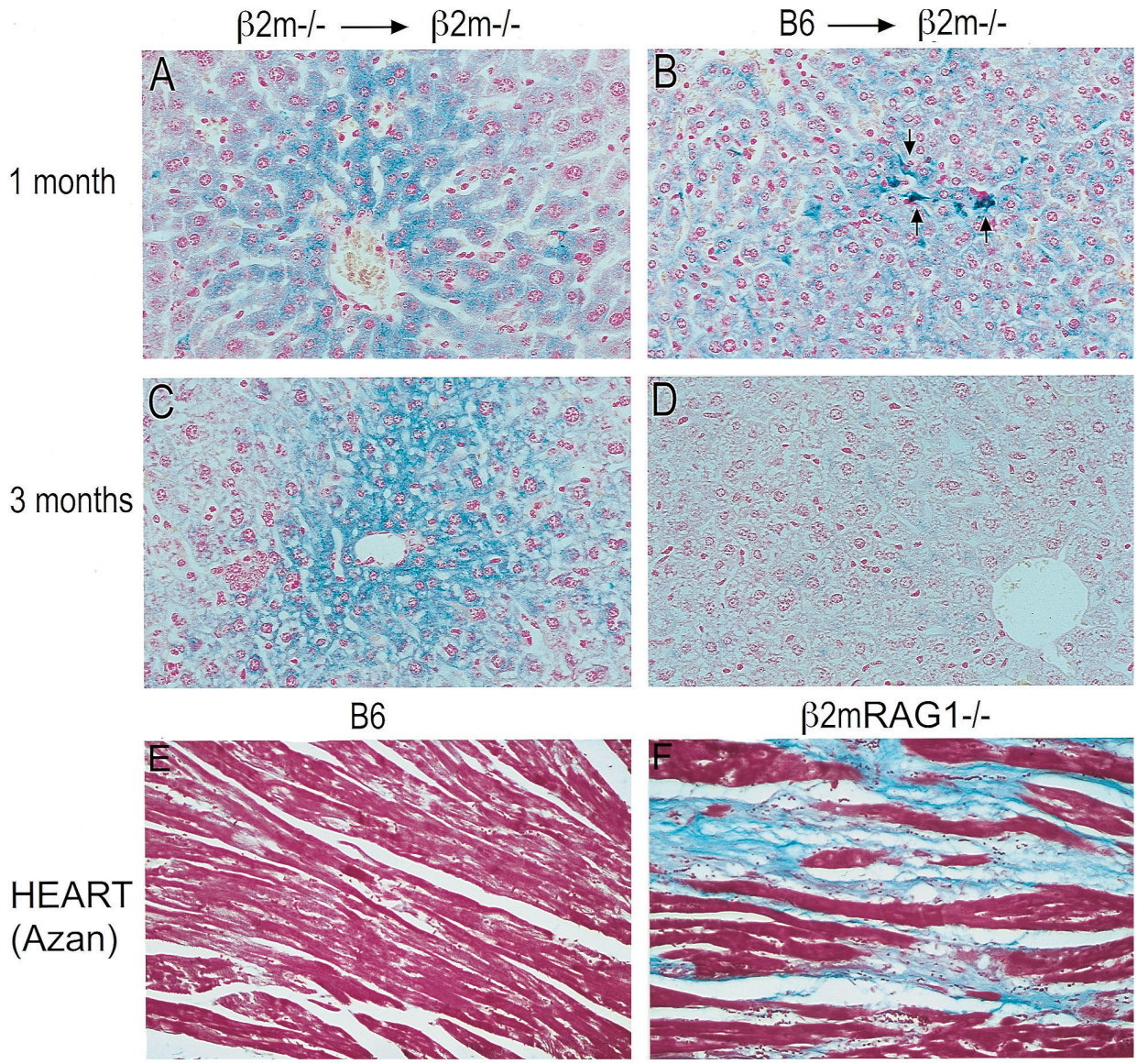

Fig. 47.3. Liver iron in radiation chimeras (Perls' Prussian blue staining). All animals were two months old at the beginning of this study. A. and C. Liver sections from $\beta_2m^{-/-}$ mice one month A. and three months C. after reconstitution with fetal liver cells from $\beta_2m^{-/-}$ donor mice. B. and D. Liver sections from $\beta_2m^{-/-}$ mice one month A. and 3 months C. after reconstitution with fetal liver cells from B6 donor mice. Heavily iron-loaded Kupffer cells (arrows) are visible one month after reconstitution. After three months, no iron deposits are present. Original magnification ×300. (Reprinted with permission from ref.[12].) Heart fibrosis in double mutants β_2mRAG1$^{-/-}$ mice six months of age (Azan staining). E. Heart sections from B6 wild-type mice with normal histology and F. from β_2mRAG1$^{-/-}$ mice showing extensive collagen deposition. Original magnificaton ×300.

the inappropriate iron absorption in $\beta_2m^{-/-}$ mice suggests that heterodimers, rather than α-heavy chain or β_2m subunits, exert this influence.

Parenchymal iron overload in $\beta_2m^{-/-}$ mice

Reconstitution of lethally irradiated β_2m-microglobulin-deficient mice with normal hematopoietic donor cells results in a redistribution of iron from parenchymal cells to Kupffer cells in the liver[12] (Fig. 47.3, A–D), but does not correct the inappropriately great iron absorption. This suggests that the lack of appropriately conformed *HFE*-β_2m complexes in cells from the mononuclear phagocytic system in $\beta_2m^{-/-}$ mice lead to defective iron storage in these cells, similarly to the defect seen in intestinal cells. Greater amounts of iron absorbed by the intestine are deposited in the liver in $\beta_2m^{-/-}$ mice than in wild-type

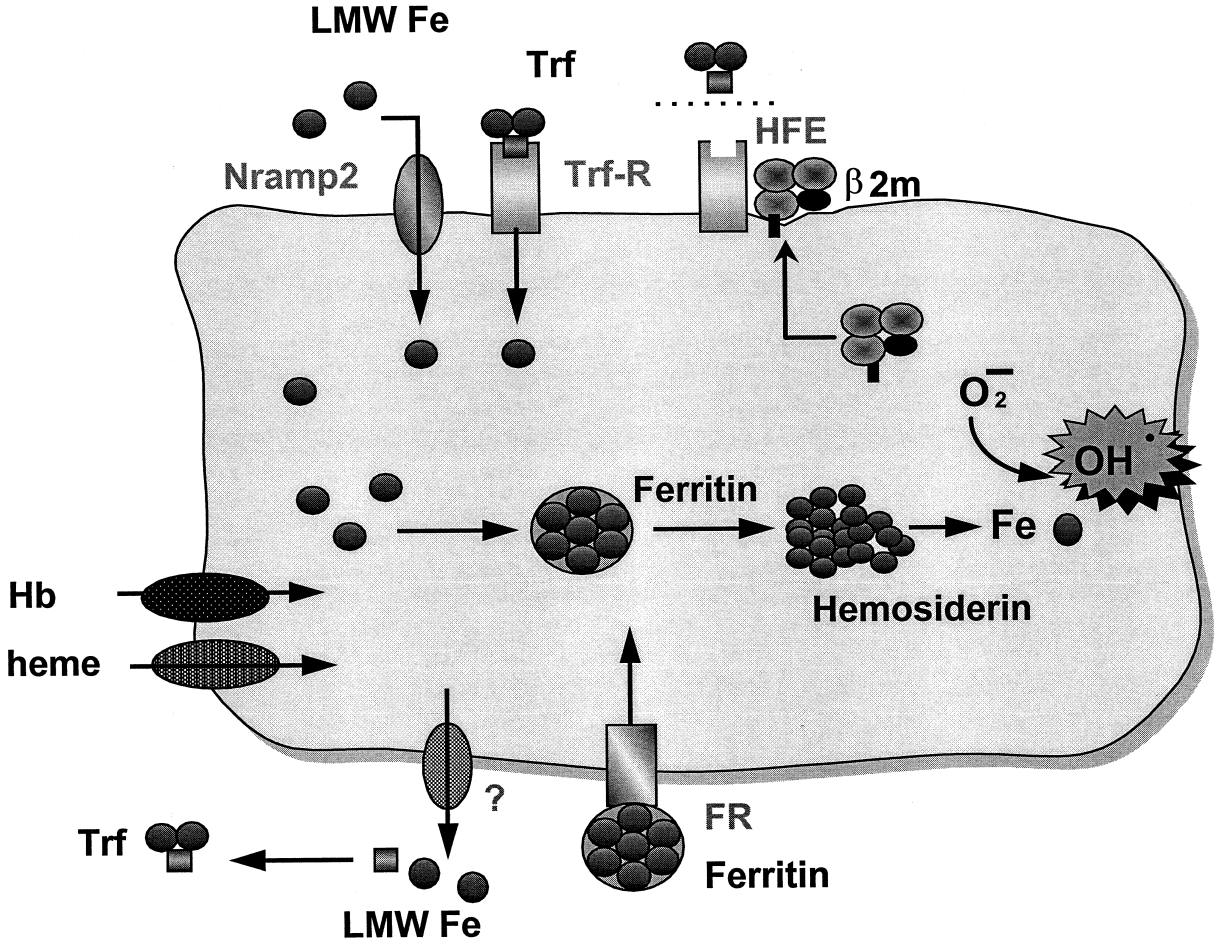

Fig. 47.4. Diagram of normal hepatocyte iron metabolism and effects of hemochromatosis. LMW Fe, low molecular weight iron; Trf, transferrin; Trf-R, transferrin receptor; Hb, hemoglobin; FR, ferritin receptor.

mice. Taking into account that $\beta_2m^{-/-}$ mice have greater transferrin saturation and reduced concentrations of plasma apotransferrin[12], this indicates that absorbed iron is released from mucosal cells independent of the availability of transferrin in plasma and is deposited in the liver. This is in agreement with previous findings in hypotransferrinemic mice, which are characterized by a heritable reduction in circulating transferrin that leads to anemia and increased hepatic iron storage[27]. In hypotransferrinemic mice, the absorbed iron not bound to transferrin is deposited in the liver, suggesting that there is an uptake system for non-transferrin-bound iron[28] (Fig. 47.4).

It appears that the mutations in the recently discovered *HFE* gene in hemochromatosis patients cannot explain the heterogeneity of the disease per se. Therefore, other genetic and environmental factors must certainly affect the severity of iron overload. In this context, the role of lymphocyte subpopulations has been suggested from previous observations of lymphocyte abnormalities in hemochromatosis patients[50, 51]. Moreover, it has been suggested that there is a linkage of cell-mediated immunity to iron metabolism involving cytokines and nitric oxide in the pathogenesis of anemia of chronic disease[52–54].

To test the role of lymphocytes in cellular iron handling directly, we generated a double knockout mouse by interbreeding $RAG1^{-/-}$ and $\beta_2m^{-/-}$ mice. RAG1-single-deficient mice lack mature lymphocytes[55], but have a normal capacity to regulate iron absorption. When these mice are given excess dietary iron, hepatic iron deposition occurs in a similar pattern to that seen in $\beta_2m^{-/-}$ mice. The doubly mutant progeny from a cross between $\beta_2m^{-/-}$ and $RAG1^{-/-}$ mice accumulate excess dietary iron in parenchymal cells of the liver. Heavy iron deposition also occurs in other tissues, such as the pancreas and the heart of double knockout mice, but not in normal or single knockout mice. Older mice develop fibrosis of heart (Fig. 47.3, E and F) and pancreas, even when they consume a standard diet.

At present, it is not clear whether *HFE* has a role in the immune system[56,57] and whether it can be recognized by T-lymphocytes. No studies have been done to determine how knocking out *HFE* may affect MHC class I expression and lymphocyte numbers. However, other non-classical MHC class I molecules, such as MICA and MICB, are recognized by T-lymphocytes[58]. MHC class I-related molecules are expressed in response to several forms of stress, and represent a mechanism that is effective in eliminating infected and damaged cells. The expression and recognition of such molecules may serve the broad physiologic function of removing damaged cells.

Conclusions

The β_2m-deficient mouse model faithfully reflects many abnormalities observed in hemochromatosis, i.e., elevated plasma iron concentration, transferrin saturation, inappropriately increased iron absorption, hepatic iron content, and parenchymal distribution of hepatic iron with relative paucity of iron storage in mononuclear phagocytic cells. In addition, the development of iron overload pathology in $\beta_2m^{-/-}$ mice is enhanced when a RAG1 mutation that causes a lack of mature lymphocytes is introduced. The possibility that lymphocytes may influence tissue iron accumulation may be useful in understanding the basis of the clinical heterogeneity of the expression of hemochromatosis in man.

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Animal models of iron overload based on excess exogenous iron

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Introduction

The purpose of an animal model of iron overload is to mimic hemochromatosis in humans. In hemochromatosis, excess iron accumulation in parenchymal cells of the liver results in toxicity, hepatic fibrosis and ultimately cirrhosis[1]. Characteristically, the excess iron is initially deposited in periportal hepatocytes in hemochromatosis; with progression, hepatocytes across the entire acinus become heavily iron loaded[2]. Although there is some Kupffer cell iron overload late in the course of iron loading, hemochromatosis is an iron overload disorder in which parenchymal cells are predominantly affected.

There are many models of iron overload, and they can be categorized into two main groups according to the route of iron administration. The first are enteral or dietary iron overload models in which carbonyl iron, ferrocene, and ferric ammonium citrate are administered to experimental animals. The second are parenteral iron overload models that involve administration of iron chelates such as iron–dextran, iron–sorbitol, or ferric nitrilotriacetate. Some have been proposed to represent the clinical and physiological manifestations of hemochromatosis. Some mimic the pattern of iron accumulation of hemochromatosis. A few induce hepatic fibrosis. Most do not faithfully reproduce all of the pathophysiologic characteristics of hemochromatosis. However, they provide experimental systems in which to examine the pathways of iron metabolism and to study the toxic effects of excess iron on normal physiology. This chapter discusses the pathophysiologic consequences of excess hepatic iron and outlines the different animal model systems used to study the pathophysiology of iron overload and its relationship to hemochromatosis.

Iron toxicity

Elaborate transport and storage mechanisms for iron exist in most organisms because free iron is toxic. The precise mechanisms of iron toxicity are not fully understood. However, it is believed that free radicals generated through iron-catalyzed reactions contribute to the pathogenesis of iron overload disease in patients with hemochromatosis[3, 4]. Low molecular weight forms of iron can initiate free radical production by catalyzing the degradation of preformed lipid peroxides, and the Haber–Weiss reaction that lead to the production of hydrogen peroxide and the highly reactive hydroxyl radical, •OH. The hydroxyl radical can attack many cellular components, including polyunsaturated fatty acids of membrane phospholipids, thus precipitating the process of lipid peroxidation[3–5].

Evidence of hepatic mitochondrial and microsomal lipid peroxidation in iron overload in vivo suggests that membrane phospholipids of subcellular organelles are damaged directly by iron-induced peroxidation of polyunsaturated fatty acids[6–13]. This mechanistic hypothesis may underscore the numerous theories that relate increased hepatic iron to cellular damage in iron overload conditions[14]. Although hepatic lipid peroxidation and associated organelle dysfunction have been demonstrated in experimental iron overload, these alterations do not result in significant necrosis or inflammation[2, 15–17]. The formation of iron-catalyzed free radicals is not the only means by which iron may be toxic to cells. Hepatic fibrosis is also associated with increased activity of prolyl hydroxylase, an enzyme involved in the post-translational biosynthesis of collagen. Weintraub and colleagues[18] suggest that this effect on fibrosis may be a direct consequence of iron overload.

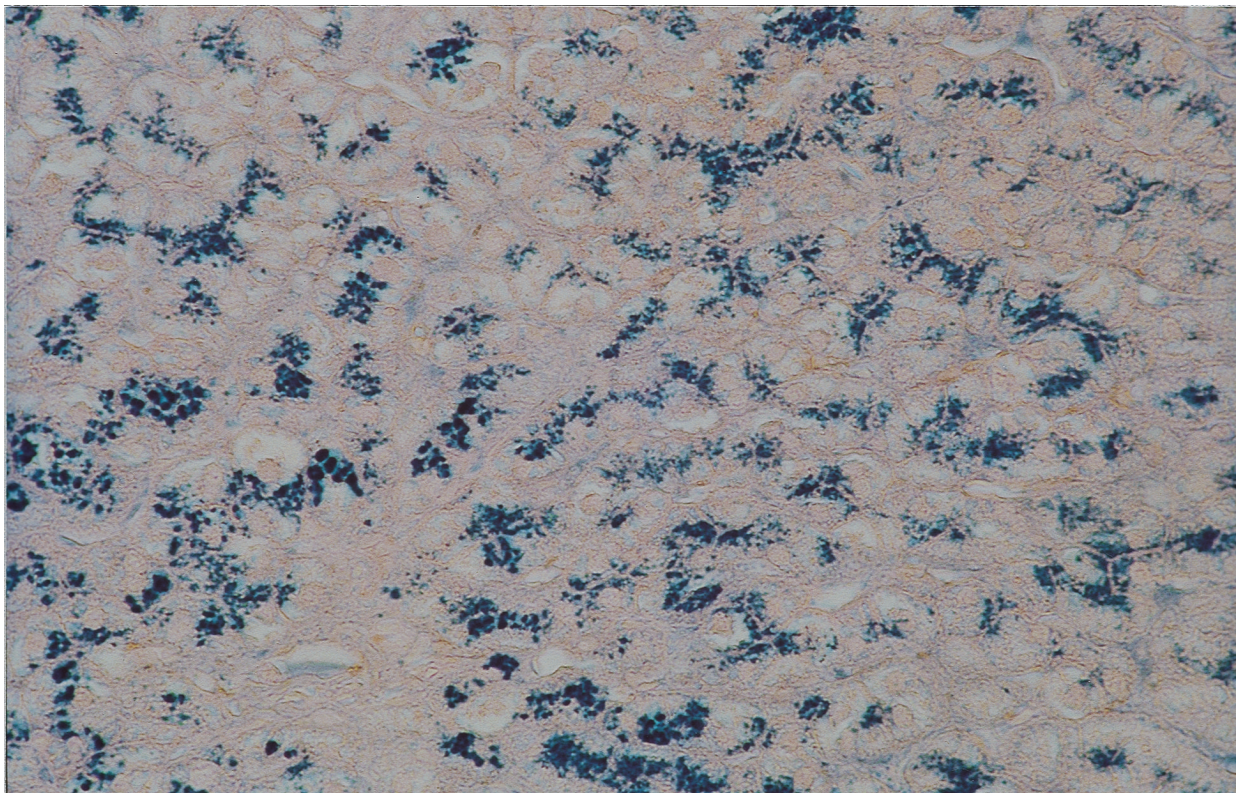

Fig. 48.1. Hepatic iron overload in a section of liver from a carbonyl iron-loaded rat. Iron is observed in a periportal distribution and is located predominantly within hepatocytes (Perls' Prussian Blue; 400×).

Pathophysiologic consequences of iron overload

The development of hepatic cirrhosis is the most important adverse prognostic factor in hemochromatosis; the five-year survival of untreated patients with cirrhosis is as low as 50%[19]. Hepatic fibrosis usually occurs in patients with an hepatic iron concentration of 250–400 μmol/g dry weight[20, 21]. The other major complication of iron overload in hemochromatosis is the development of hepatocellular carcinoma. There is a 200-fold increase in relative risk of developing primary hepatic cancer in patients with hemochromatosis and cirrhosis[22]. These observations were confirmed in separate studies by Niederau and colleagues[19, 23], who suggest that the prognosis of hemochromatosis and its complications, including hepatocellular carcinoma, are dependent on the hepatic iron concentration, the duration of iron loading, and the presence of cirrhosis[23]. Iron-induced DNA damage may be necessary to initiate the development of neoplasia and the progression to hepatocellular carcinoma in iron overload[3, 4].

Advances have been made toward understanding the pathophysiologic mechanisms involved in iron-induced hepatic fibrosis in hemochromatosis[24], but the precise pathways whereby excess iron initiates fibrogenesis have not been elucidated. Hepatocyte iron loading appears to be a prerequisite to the development of fibrosis, whereas Kupffer cell iron-loading does not generally result in fibrosis at similar hepatic iron concentrations[25]. The use of an appropriate animal model of iron-induced fibrogenesis would assist in evaluating these mechanisms. Unfortunately, the lack of a model that reproduces all of the pathophysiologic manifestations of hemochromatosis has been a great obstacle to the advance of knowledge of hepatic fibrogenesis in iron overload. Although many iron overload models generate markedly increased concentrations of hepatic iron, the resulting fibrosis is usually very mild. However, these models have allowed many of the pathways of iron metabolism in normal and iron-loaded conditions to be elucidated (see below).

Dietary iron overload

The development of experimental models of iron overload in laboratory animals has been necessary to elucidate the mechanisms involved in hepatic iron metabolism in

hemochromatosis. Because hemochromatosis is a consequence of increased intestinal iron absorption, many experimental models rely on increased dietary iron.

Carbonyl iron

In 1983, Bacon and colleagues evaluated the role of chronic iron overload in promoting hepatic lipid peroxidation in vivo by examining subcellular fractions of liver from chronically iron-loaded rats for the presence of lipid-conjugated dienes, intermediates in the peroxidative degradation of polyunsaturated lipid membranes[7]. They used two different methods of experimental chronic iron loading in vivo. The first involved the parenteral administration of ferric nitrilotriacetate (FeNTA). The second method involved the supplementation of the diet with 2.5% (wt/wt) carbonyl iron, a highly purified form of elemental iron. This feeding regimen predominantly produces iron-loading of hepatocytes in a periportal distribution (Fig. 48.1), similar to that of hemochromatosis. Significant quantities of iron are deposited after as early as two weeks in rats fed the carbonyl-iron diet[26]. Iron continues to accumulate in hepatocytes for three months when stores of iron in hepatocytes from carbonyl iron-fed rats were 40–90 times those of control animals and signs of mild hepatic necrosis had developed. At this time, iron deposits also become more prominent in Kupffer cells and macrophages. After 8 months, iron stores in both hepatocytes and Kupffer cells are massive and there is evidence of hepatic fibrosis in some animals that becomes more pronounced after 12 months on the diet. Therefore, dietary supplementation with carbonyl iron appears to provide an experimental model of iron overload that reproduces the pathophysiology of hemochromatosis.

Many groups have since used the carbonyl iron model of iron overload to study in vivo pathways of iron metabolism, the hepatotoxicity of iron overload, and the mechanisms of iron-induced hepatic fibrosis. Other studies have used this model to demonstrate the hepatotoxic effects of excess iron on peroxidation of organelle membrane lipids[7–14]. Elevated levels of thiobarbituric acid (TBA)-reactive products such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) are indicators of the extent of lipid peroxidation, and have been detected in the livers of carbonyl iron-loaded rats[27, 28]. Similar findings also occur in isolated hepatocytes[29] and lysosomes[30]. The carbonyl iron model has also been used to demonstrate the toxicity of excess hepatic iron on organelle function, including induction of lysosomal fragility[30, 31], impairment of mitochondrial oxidative metabolism[9], abnormalities in calcium transport[32, 33], reduction in the levels of cytochromes a, b and c, and cytochrome C oxidase activity[34], and disruption of metabolic functions of other subcellular organelles[3, 4].

Although the pathways of iron uptake and metabolism have largely been investigated through in vitro studies or using non-iron loaded animals, the mechanisms involved in the excretion of iron from the liver requires in vivo experiments. Biliary excretion is the major pathway by which iron is removed from the liver in carbonyl iron-loaded rats[31, 35]. Using carbonyl iron, the hepatic iron concentration can be as much as 45-fold greater than in normal rats[31], but biliary iron excretion is increased only two- to four- fold above the excretory rates in control animals[31, 35]. In situations of increased iron absorption, the inability of biliary excretion to compensate fully for increased hepatic iron uptake leads to progressive accumulation of iron within the liver. Presumably, hepatotoxicity occurs when cellular storage capacity is overwhelmed.

Hemochromatosis is manifested by increased intestinal iron absorption that may ultimately lead to hepatic fibrosis. Adams and colleagues transplanted the liver of carbonyl iron-loaded rats into normal rats and observed a marked decrease in intestinal iron absorption[36]. Because the transplantation of iron dextran-loaded livers into normal rats had no effect on iron absorption, they proposed that hepatocyte iron stores are involved in regulating iron absorption. Other advances have come through cell biology and the identification of specific hepatic cellular functions. Smith and Yeoh[37] demonstrated increased proliferation of periportal oval cells in carbonyl iron-loaded rat liver. The activated phenotype of hepatic stellate cells (also known as lipocytes, Ito cells, or fat-storing cells)[38] have been induced in carbonyl iron-loaded rat livers[39, 40] and in livers from hemochromatosis patients[41]. These cells are responsible for the production of increased collagen in iron overload[39, 40]. Some investigators have used the carbonyl iron-loaded animal model to induce hepatic fibrosis[26, 42], although others have been unable to demonstrate histologic evidence of fibrosis reproducibly[43–45] (see pp. 501–2).

Ferrocene

One of the earliest uses of diets enriched with 3, 5, 5-trimethylhexanoyl ferrocene or TMH-ferrocene as a model of iron overload were studies that examined new chelation therapy[46, 47]. Longueville and Crichton[46] demonstrated that TMH-ferrocene feeding leads to a more than two-fold increase in ferritin protein and a four- to eight-fold increase in ferritin iron. In TMH-ferrocene-loaded rats,

Ward and colleagues[48] demonstrated increased hepatic lysosomal membrane fragility and a significant negative correlation between increasing hepatic iron concentration and a decrease in lysosomal α-tocopherol levels, implying free radical-mediated damage. Others have also reported evidence of lipid peroxidation in this model, including decreased hepatic α-tocopherol and ubiquinol 9/10, and markedly increased ethane exhalation[49].

A number of ferrocene derivatives have been examined for their comparative proficiency in iron absorption, delivery to tissues, cellular and tissue iron-loading, and fibrogenic capacity. Ferrocene, TMH-ferrocene, and 1,1′-bis TMH-ferrocene were compared with ferrous sulfate[50]. Bioavailability of iron from TMH-ferrocene is two-fold greater than for ferrocene and six-fold greater than for ferrous sulphate or 1,1′-bis TMH-ferrocene. In addition, the intestinal absorption of TMH-ferrocene is not regulated by total body iron stores and the bioavailability of iron was excellent, because almost all the iron is released from the parent molecule within the liver. These results differ somewhat from those obtained using carbonyl iron, the intestinal absorption of which depends on the dose used and the iron status of the animal[51]. In other studies of TMH-ferrocene-loaded rats, hepatic iron stores do not decrease at the end of iron-loading, unlike iron dextran-loaded rats that show a rapid depletion of iron stores two weeks after loading[47]. Thus, this model produces rapid and progressive iron overload in rats, with an acinar and cellular distribution of iron similar to that seen in hemochromatosis[47, 51]. The demonstration of mild perisinusoidal and portal fibrosis in long-term TMH-ferrocene fed rats[52] has led some investigators to propose that this model is the most encouraging animal model of experimental hemochromatosis[51].

Ferric ammonium citrate

Ferric ammonium citrate has been used to examine lipid peroxidation[53–55], lysosomal fragility[56], membrane potential and contractility[57], and the effect of chelators[53, 58] on cultured iron-loaded myocardial cells, but there is scant evidence for its use to create a model of iron overload in hemochromatosis. Richter described rats treated with a cyclic feeding regimen of ferric ammonium citrate and starvation over six months[59]. These rats develop increased hepatic iron levels in hepatocytes similar to persons with hemochromatosis, although the rats develop no evidence of hepatic fibrosis. Follow-up studies examined the storage of ferritin and its transition to hemosiderin under iron overload conditions[60], but the potential of this model to mimic hemochromatosis has not been investigated.

Parenteral iron administration using iron chelates

Iron–dextran

The iron–dextran complex is a solution of elemental iron that has pH stability and low toxicity, and is easily absorbed into the circulation from intramuscular injections[61]. Parenteral injection of iron–dextran results in the initial deposition of iron primarily in cells of the reticuloendothelial system (Fig. 48.2), with redistribution of iron to the parenchymal cells of the liver[62–64], the kidney, and interstitial cells of the testis[62]. The concentrations of iron deposited in the liver may be as much as 50 times those of controls, depending on the dose and duration of treatment[62]. As with the carbonyl iron model, iron dextran-loaded animals have been used extensively to investigate the mechanisms of iron-induced lipid peroxidation[29, 65–67]. In addition, this model has been used to examine the protective role of α-tocopherol as an antioxidant in iron-induced oxidant stress and lipid peroxidation[4]. Iron dextran-loaded rats have also been employed to elucidate the metabolic pathways of iron and ferritin. Ramm and colleagues have demonstrated that tissue ferritin is cleared from the circulation of normal and iron-loaded rats by a microtubule-dependent process, probably through receptor-mediated endocytosis[68, 69] that requires chloroquine-sensitive vesicles[70]. These studies also suggest that the release of ferritin into serum and bile of iron dextran-loaded rats requires intact microtubules, microfilaments, and chloroquine-sensitive vesicles[68–70]. Increasing the hepatic iron stores does not alter the clearance of ferritin via the hepatic ferritin receptor[68–71]. Iron dextran-loaded animals have developed histologic evidence of hepatic fibrosis in some studies[72–74] but not in others[62, 75] (see pp. 501–2).

One of the most interesting models of iron dextran-induced histologic fibrosis involves the Mongolian gerbil. Carthew and colleagues[72] described iron-induced hepatic fibrosis in male Mongolian gerbils, in which micronodular cirrhosis developed three months after a single large injection of iron dextran (1 mg Fe/g body weight). Within six weeks, hepatic siderosis and fibrosis developed, and the cellular source of increased collagen type I and III appeared to be hepatic stellate cells. Large intracellular deposits of iron and ferritin were observed within hepatic stellate cells, an uncommon observation in iron overload. The authors postulated that this is a model of ferritin-induced fibrosis, although no direct supportive evidence was provided.

Iron–dextran-loaded mice and rats in this study did not accumulate ferritin within hepatic stellate cells, nor did

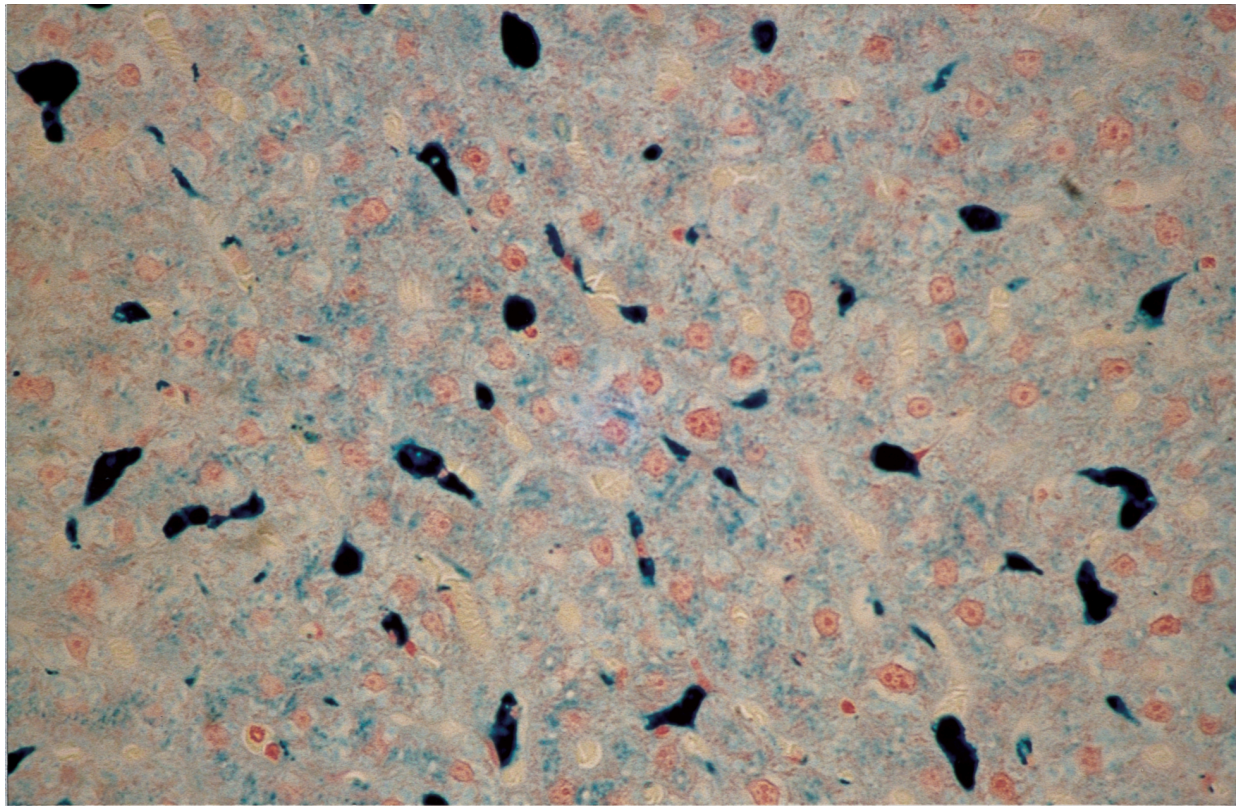

Fig. 48.2. Hepatic iron overload in a section of liver from an iron dextran-loaded rat. Iron is observed across the acinus and is located predominantly within Kupffer cells (Perls' Prussian Blue; 400×).

they develop fibrosis or cirrhosis. There are two potential explanations for these observations: the physiology of the gerbil is different from that of other rodents (which seems unlikely), or another potentiating 'co-factor' or hepatotoxin possibly exists in gerbils. This latter possibility was substantiated by the results of another study. Iron accumulation, hepatic fibrosis, and micronodular cirrhosis occurred in gerbils injected with endotoxin lipopolysaccharide (LPS), derived from *Escherichia coli*, in the absence of excess exogenous iron[76]. The histopathology in these gerbils was almost identical to that of gerbils in the first study injected with iron–dextran[72]. It is likely that the endotoxin-induced hemorrhagic necrosis in the livers of gerbils injected with LPS[76] accounts for the micro-inflammatory foci seen in Carthew's initial report[72], and this may have promoted the development of fibrosis and cirrhosis. The presence of *Bacillus piliformis*, the causative agent of Tyzzer's disease, in various gerbil tissues causes Tyzzer's disease that may contribute to the hemorrhagic focal necrosis seen in gerbil livers[77]. It is possible that the presence of these bacteria in gerbil liver or gut may lead to the release of endotoxin into the liver and cause hepatic hemorrhage, focal iron accumulation, and fibrosis. In pathogen-free gerbils injected with iron-dextran according to the method of Carthew and colleagues[72], increased collagen mRNA and mild focal fibrosis surrounding iron-laden macrophages occurred, but bridging hepatic fibrosis or cirrhosis did not occur after four months[75]. Thus the iron-loaded gerbil is not an ideal model of the hepatic injury resulting from hemochromatosis because the gerbils preferentially develop Kupffer cell iron-loading and they are predisposed to have pre-existing micro-inflammation due to infection or endotoxemia. However, the use of Mongolian gerbils may be useful in investigations of the mechanisms involved in iron-potentiated fibrogenesis during hepatic inflammation. Pietrangelo and colleagues[73] demonstrated that dietary supplementation with α-tocopherol in gerbils is associated with a marked decrease in hepatic MDA concentrations, inhibits iron dextran-induced hepatic fibrosis, and prevents the development of cirrhosis. They proposed that the mechanisms involved in the inhibition of fibrogenesis were mediated through the antioxidant effect of α-tocopherol on the iron-induced proliferation of non-parenchymal cells.

Ferric nitrilotriacetate

The administration of FeNTA to experimental animals has been used often as a model of hemochromatosis[7, 78–84]. FeNTA induces diabetes mellitus in rats and is associated with extensive iron deposition in exocrine pancreatic cells, toxicity, and tissue damage[78, 79]. FeNTA also causes renal injury and oxidative damage[85–90] and induces the development of tumors[91–94]. The administration of FeNTA to experimental animals results in the homogeneous deposition of iron throughout the hepatic lobule in both Kupffer cells and hepatocytes[7], unlike the specific lobular distribution of excess iron seen in hemochromatosis. Iron-loading experimental animals using FeNTA is laborious, involves frequent intramuscular or intraperitoneal injections, and produces only modest degrees of iron loading. The greatest problem with this model is the associated toxicity[4]. The FeNTA iron-loaded rat has been used to study iron-induced lipid peroxidation of intracellular organelle membranes in the liver[7, 80, 95]. In baboons iron-loaded by FeNTA administration, Brissot and colleagues[81] demonstrated that hepatic toxicity is manifest by increased serum transaminase concentrations and markedly decreased hepatic levels of 4-prolyl hydroxylase activity. However, significant hepatic fibrosis did not occur although occasional foci of perisinusoidal fibrosis were observed. This model has been employed to examine hepatic iron uptake via transferrin[96] and transferrin receptor expression and distribution in the liver[83, 84]. Lu and colleagues used immunohistochemistry to study transferrin receptor expression in normal, iron-deficient, and iron-loaded rats. They demonstrated that the expression of transferrin receptors is negatively regulated by tissue iron stores[83]. Transferrin receptor expression is increased on the sinusoidal membranes of acinar zone 1 hepatocytes and decreased across the acinus, resulting in much less stainable iron in acinar zone 3 hepatocytes[84]. This may be due in part to the zonal heterogeneity of hepatocytes[97, 98], and their relative metabolic functions under differing conditions of oxygen tension.

Iron–sorbitol

There are few reports using the chelate iron–sorbitol to iron-load experimental animals and none were designed as a model of hemochromatosis. In one study, an iron-sorbitol/citric acid complex (Jectofer®) was injected into rats. By electron microscopy, ferritin-like granules were observed in hepatocytes and Kupffer cells, and were localized to 'lysosomal-like' structures and phagocytic vacuoles[99]. Hultcrantz and colleagues[100] demonstrated evidence of increased lipid peroxidation in the liver and kidney of rats injected with Jectofer® (0.5 g Fe/kg). Two other studies examined the effects of iron sorbitol-loading on mitochondrial lipid peroxidation[101], and, using a lower dosage (0.25 g Fe/kg), on biliary excretion and metabolism[102]. In these studies, iron did not enhance mitochondrial lipid peroxidation[101], but the ethinyl estradiol-induced suppression of bile flow was reversed[102].

Models of iron-potentiated hepatic injury

Hemochromatosis was previously believed to be a type of alcoholic injury of the liver, wherein increased hepatic iron deposition was derived from intestinal absorption of iron in alcoholic beverages[103]. Although a series of investigations clearly demonstrated the genetic basis of hemochromatosis[104–106], clinical data suggest that patients with alcoholic hepatic injury have histologic evidence of increased hepatic iron[107], and consumption of ethanol may expedite the development of hepatic fibrosis in patients with hemochromatosis[20]. Therefore, it has been postulated that the association of increased hepatic iron and alcoholic hepatic injury, or of increased ingestion of alcohol in persons who have iron overload conditions, synergistically exacerbate the injurious effect of either hepatotoxin and thus initiate hepatic fibrosis or cirrhosis prematurely. Indeed, the outcome of many animal models of iron overload discussed in this chapter indicate that iron per se is a relatively poor initiator of hepatic fibrosis. However, the presence of excess iron may exacerbate fibrogenesis stimulated by other hepatotoxins, possibly via oxidative stress pathways. This view is expounded by Pietrangelo[24], who suggests that the pro-oxidant action of iron may act as a co-initiator or a propagator and amplify the effect of hepatic toxicity in certain instances. Therefore, there is much interest in the development of animal models designed to evaluate the potential synergistic effect of iron and other hepatotoxic agents.

Iron and ethanol

In 1993, Stål and Hultcrantz[108] developed a model to examine the possible synergistic interaction between iron overload and chronic ethanol administration in the induction of hepatocellular damage. This incorporated feeding rats a diet supplemented with 3% carbonyl iron (wt/wt) for eight weeks, followed by four weeks of a Lieber–DeCarli liquid ethanol-containing diet[109]. They demonstrated a five-fold increase in serum alanine aminotransferase concentrations in rats fed iron + ethanol compared to control groups. Hydroxyproline concentrations were elevated in

the livers of the rats treated with iron + ethanol, and there was ultrastructural evidence of phagocytosed necrotic hepatocytes within Kupffer cells. Although this model demonstrates biochemical and morphological evidence of hepatocellular damage, there is no histologic evidence of hepatic fibrosis. Others have also used iron + ethanol in a Lieber-DeCarli liquid diet[110–115]. After a similar treatment regimen, animals developed increased hepatocellular damage[110, 112, 113, 115] and augmented increases in hepatic hydroxyproline concentrations[112, 114] in the iron + ethanol group compared to either the iron group or the ethanol group. However, potentiation of the effect on hepatic lipid peroxidation was not evident[113–115], and none of these studies observed potentiation of fibrosis in the iron + ethanol groups[110, 112–114].

The duration of the iron + ethanol treatment might be too brief to allow the development of fibrosis in some studies. Accordingly, Olynyk and colleagues[110] examined the interaction of iron and ethanol over a 26-week period in rats receiving carbonyl iron (6 g/1000 ml liquid diet). Some rats developed mild hepatic fibrosis associated with periportal macrophages, but no potentiating effect of ethanol on fibrosis was observed. Different results were obtained by Tsukamoto and colleagues[112]. Carbonyl iron (0.25% wt/vol) was infused with a high-fat liquid diet (HFD) containing increasing concentrations of ethanol into the stomachs of rats for 16 weeks. Iron significantly potentiated hepatocellular damage, and increased concentrations of MDA, 4-HNE and hydroxyproline. There was also a significant correlation between hepatic 4-HNE concentrations, hydroxyproline concentrations, and collagen accumulation. Thus, Tsukamoto and colleagues proposed that these results, together with those of another study showing 4-HNE stimulation of procollagen α_1 (I) mRNA in cultured hepatic stellate cells[116], provide strong evidence for an integral role of lipid peroxidation in hepatic fibrogenesis. In addition, from a total of 21 rats prepared with HFD/ethanol + iron, 12 developed perivenular fibrosis, seven developed bridging hepatic fibrosis, and two had micronodular cirrhosis. Only 50% of rats in the HFD/ethanol group had perivenular fibrosis, none had cirrhosis, and there were no HFD + iron rats that developed any form of histologic fibrosis or cirrhosis. Although this may be an excellent model for studying hepatic fibrogenesis, it is not an ideal model for evaluating iron-induced injury in hemochromatosis.

Iron + carbon tetrachloride

In 1989, Younes and colleagues[117] reported the results of a study designed to examine the possible toxic effects of iron used in combination with a number of additional hepatotoxins, including ethanol, acetaminophen, bromobenzene, and carbon tetrachloride (CCl_4). Rats were treated with either parenteral iron-dextran to mimic secondary hemochromatosis, or dietary iron as iron-fumarate (3.5% wt/wt) as a model of hemochromatosis. Lipid peroxidation was enhanced by the presence of each of the hepatotoxins in iron fumarate-loaded rats, whereas hepatotoxicity was magnified only by CCl_4 treatment, consistent with the toxicity and fibrogenicity of CCl_4 administration[118]. Others have also used CCl_4 as a hepatotoxin in combination with iron overload. Kent and colleagues[119] described the augmentation of fibrogenesis and cirrhosis in iron dextran-loaded rats subjected to CCl_4 administration compared to that of CCl_4 treatment alone. They observed increased numbers of iron-laden macrophages and fibroblasts within the septae of the cirrhotic bands and the presence of extracellular iron as ferritin in the connective tissue matrix. The authors proposed that the accumulation of excess iron in the septae appeared to exert an effect on the connective tissue and ultimately resulted in the augmentation of fibrosis. These are important observations due to the role of activated hepatic stellate cells, or 'myofibroblast-like' cells of the liver in the development of hepatic fibrosis and cirrhosis (see pp. 501–2). Iron within fibroblasts or hepatic stellate cells is uncommon in iron overload per se, and thus this regimen may be useful in investigating the mechanisms involved in the iron-induced activation of hepatic stellate cells.

Iron + ethanol + carbon tetrachloride

Mackinnon and colleagues[120] combined three hepatotoxins (iron, ethanol and a subtoxic dose of CCl_4) to create a model of hepatic fibrosis with potential relevance to hemochromatosis. The authors used Porton rats that were fed the Lieber–DeCarli liquid diet that included 3% carbonyl iron and an ethanol consumption of 4–5 g/kg/d, and were exposed to subtoxic doses of CCl_4 vapour (iron + ethanol + CCl_4) daily. Some hepatic fibrosis was demonstrated after ten weeks of CCl_4 exposure in all groups receiving ethanol or iron, but not in the control group. Hepatic fibrosis was evident as early as four weeks after commencing CCl_4 exposure in the iron + ethanol + CCl_4 group, and after ten weeks all animals had either developing or established cirrhosis. This represents an excellent model by which to examine the mechanisms involved in hepatic fibrogenesis and the synergism between iron and other hepatotoxins.

Iron + ethionine

Ethionine-induced hepatic cirrhosis in rats is potentiated by iron loading[121, 122], although other reports have disputed these observations[123, 124]. In 1968, Orfei and colleagues[122] administered intraperitoneal injections of iron–dextran to rats (100 mg Fe/kg body weight given every three days for 1 month), and gave them 0.5% ethionine in their drinking water. Ethionine-induced hepatic injury thus induced could be divided into an initial hepatitis phase followed by a phase in which fibrous septae and cirrhosis developed. Iron overload had no detectable effect on the hepatitis phase, but enhanced the fibrogenesis phase. The authors proposed that the lack of augmentation of ethionine-induced liver injury by iron observed by others[123, 124] might be due to the level of iron-loading and their observations of two distinct phases of hepatic injury that were not previously recognized.

Iron-induced hepatic fibrosis

Dietary and parenteral iron overload models can provoke mild or severe iron overload, depending on the form of iron, the dose used, and the duration of the treatment. As redistribution of the iron occurs in each model, both types of models are adequate for the study of certain aspects of iron overload in experimental animals. However, these regimens may not be ideal for studies of hepatic injury and fibrosis. Iron-induced hepatic fibrosis occurs in different species, including rats[26, 42, 52, 110, 111, 125], dogs[74], rabbits[126], baboons[127], and gerbils[72, 73]. However, some laboratories have not been successful in generating significant histologically demonstrable hepatic fibrosis in iron-loaded animals[43–45, 59, 62, 75, 81, 108, 128, 129]. In addition, some investigators have demonstrated an iron-induced increase in either collagen mRNA[42, 45], collagen fibrils[18, 43, 45], or indirect evidence of an effect of iron on fibrogenic processes, such as increased hepatic concentrations of hydroxyproline[18, 66, 69, 70, 72, 125], or increased prolyl hydroxylase activity[18].

In many animal models, the fibrogenic response is weak when excess exogenous iron is used alone as a hepatotoxic agent, except for the gerbil (although there are inherent complications associated with this model due to endotoxemia). One factor that possibly contributes to the poor response to iron overload in animal models is the level of α-tocopherol in the diet. There is an inverse correlation of increasing hepatic iron stores and decreasing hepatic α-tocopherol concentrations[4]. Pietrangelo reported that an α-tocopherol-enriched diet inhibited hepatic fibrosis and abrogated the development of cirrhosis in iron-loaded gerbils when compared to a standard diet[73]. However, supplemental α-tocopherol only resulted in a small decrease in hepatic hydroxyproline concentrations in rats with dietary iron overload[125]. The large capacity of the liver to store excess iron and its abundant antioxidant defenses may account for its resistance to iron-induced fibrogenesis. Although it is difficult to produce hepatic fibrosis in many animal models of iron overload, major advances have been made towards understanding the mechanisms involved in iron-induced fibrogenesis, largely due to the recognition that hepatic stellate cells play a crucial role in extracellular matrix production during fibrogenesis.

Hepatic stellate cells: the cellular source of hepatic collagen

Until recently it was unclear which cells were responsible for increased hepatic collagen production and fibrosis. Hepatocytes were considered by many to be the most likely, due to their numbers and reports that hepatocytes produce collagen in vitro[130]. With improvements in cell isolation techniques, it is now thought that hepatocyte cultures used in earlier studies may have been contaminated with a population of non-parenchymal cells called hepatic stellate cells. It is now established that hepatic stellate cells are primarily responsible for collagen production in the injured liver[130–135].

In normal liver, hepatic stellate cells are quiescent, store vitamin A, and produce basement membrane[131, 132]. Hepatic stellate cells are found in the space of Disse and may regulate sinusoidal blood flow in normal and injured liver due to their perisinusoidal location[135]. In liver injury, hepatic stellate cells are activated and transformed into myofibroblasts which are responsible for increased collagen production and hepatic fibrogenesis[131, 132]. The precise mechanisms involved in this transformation remain elusive and are the subject of extensive investigation focused on the role of cytokines and growth factors produced by different hepatic cell populations. Transforming growth factor-β_1 (TGF-β_1) and platelet-derived growth factor act as profibrogenic and proliferative cytokines, respectively, in hepatic stellate cell biology[131, 132, 134]. Insulin-like growth factor produced by hepatocytes increases hepatic stellate cell proliferation[136, 137]. Activated hepatic stellate cells are now thought to play a role in regulating sinusoidal blood flow in the diseased liver, due principally to the contractile nature of α-smooth muscle actin (SMA), the expression of which has been used as the classical marker of the activated hepatic stellate cell phenotype[131, 132].

Iron-induced activation of hepatic stellate cells

A report by Pietrangelo and colleagues documented that procollagen mRNA expression is localized to hepatic non-parenchymal cells in iron-loaded rats[138]. A prerequisite for the development of hepatic fibrosis is thought to be parenchymal iron overload, which has been confirmed by Gualdi and colleagues[139], who showed increased procollagen α_1 (I) mRNA expression in hepatic tissue from carbonyl iron-loaded rats, but not from iron dextran-loaded rats. Further studies showed that the procollagen mRNA was localized to hepatic stellate cells in iron-loaded rats using in situ hybridization and Northern blot analysis[39]. Ramm and colleagues have shown that hepatic stellate cells isolated from carbonyl iron-loaded rats express SMA and synthesize approximately twice the amount of collagen and non-collagen protein produced by hepatic stellate cells from control rats[40]. Hepatic stellate cells have also been identified as the source of increased procollagen mRNA in the iron-loaded gerbil model[73]. Activated hepatic stellate cells have been identified in hepatic biopsy specimens from patients with hemochromatosis that had hepatic iron concentrations as low as 60 μmol/g dry weight[41]. This concentration is much lower than 250–400 μmol/g dry weight (or more) at which fibrosis is commonly observed in hemochromatosis patients[20, 21]. Ramm and colleagues reported that there is a correlation between increasing hepatic iron concentration and the expression of SMA in pre-fibrotic hemochromatosis patients, and demonstrated that phlebotomy markedly decreases the number of activated hepatic stellate cells[41].

The mechanisms involved in initiating the iron-induced activation of hepatic stellate cells are unclear. Direct stimulation of collagen gene expression by products of lipid peroxidation derived from iron-loaded hepatocytes or Kupffer cells has been proposed as a profibrogenic pathway in iron overload. Houglum and colleagues demonstrated increased MDA-protein adducts and TGF-β, initiators of procollagen α_1 (I) gene expression, in the region of heaviest iron overload of the liver (acinar zone 1), in carbonyl iron-loaded rats and patients with hemochromatosis[140, 141]. Another TBA-reactive substance, 4-HNE, stimulates the expression of procollagen α_1 (I) mRNA in cultured human hepatic stellate cells[116]. A specific receptor for tissue ferritin was described on activated, but not quiescent, rat hepatic stellate cells by Ramm and colleagues[142]. Ferritin binding to this receptor is dependent on the H-ferritin subunit and ferritin is internalized by activated hepatic stellate cells. A potential biological role for ferritin or the ferritin receptor has been suggested because the expression of the ferritin receptor is inducible in culture[143], and the addition of ferritin to hepatic stellate cell cultures suppresses the expression of both SMA and the 80 kDa isoform of protein kinase C-ζ in a dose-dependent manner[144, 145]. Although these findings suggest a possible mechanism whereby ferritin might down-regulate SMA expression, the role of iron and ferritin in the iron-induced hepatic stellate cell activation cascade is not well delineated.

There is increasing evidence that interaction occurs between hepatocytes, Kupffer cells, and hepatic stellate cells in the fibrogenesis. Phagocytosed remnants of necrotic iron-loaded hepatocytes have been identified within Kupffer cells in an animal model of iron + ethanol[108]. Olynyk and colleagues showed that Kupffer cell phagocytosis of iron-loaded hepatocytes leads to the production of prostaglandin E_2 in vitro[146]. Complex interactions exist between different cell types in the iron-loaded liver involving the generation of lipid peroxidation products, the production of cytokines, and the pathways of iron sequestration and utilization. Each of these processes may influence the phenotype of the hepatic stellate cell, and therefore govern the development of hepatic fibrosis in iron overload. Although significant advances have been made toward understanding the involvement of hepatic stellate cells in the fibrogenesis associated with iron overload, the precise mechanisms involved in initiating hepatic stellate cell activation have not been delineated.

Conclusions

This chapter has discussed models of iron overload based on either dietary supplementation with excess iron or the parenteral administration of iron chelates, and the specific strengths and weaknesses of each model in reproducing the physiological and pathophysiologic manifestations of hemochromatosis have been reviewed (Table 48.1). Most models are unsatisfactory, because they preferentially iron-load Kupffer cells, or because they do not induce hepatic fibrosis when excess iron alone is present. Although the carbonyl iron-loaded rat remains the model of choice by many groups, other investigators are unable to induce significant histologic hepatic fibrosis with this model. The most clinically relevant model probably involves the iron + ethanol regimen in rats, wherein iron potentiates hepatic injury and fibrosis induced by chronic ethanol consumption. The iron dextran-loaded gerbil is another good model of the potentiating effect of excess hepatic iron on the hepatic injury produced by other hepatotoxins, especially infection or endotoxemia. One of the most promising models is the ferrocene-loaded rat in which rapid, progressive hepatocyte iron-loading is accompanied by perisinusoidal and portal fibrosis.

Table 48.1. Animal models of iron overload based on excess exogenous iron

	Model strengths	Model weaknesses
Dietary iron overload		
Carbonyl iron	Chronic iron loading with preferential hepatocyte deposition; fibrosis; intestinal absorption regulated by body iron stores[7, 26, 42, 51]	Inability to reproduce physiology and pathophysiology of hemochromatosis[43–45]
Ferrocene	Rapid, progressive hepatocyte iron loading; perisinusoidal and portal fibrosis[46, 51, 52]	Intestinal absorption not regulated by body iron stores; requires further investigation[51]
Ferric ammonium citrate	Predominantly hepatocyte iron loading[59]	No evidence of hepatic fibrosis[59]
Parenteral iron overload		
Iron-dextran		
Rat, rabbit, baboon, dog	Used to study lipid peroxidation and intracellular handling of iron and ferritin[29, 65–71]	Preferential iron loading of cells of the reticuloendothelial system[61–63]
Gerbil	Hepatic fibrosis and micronodular cirrhosis[72, 73]	Model of iron and infection or endotoxemia[75, 76]
Ferric nitrilotriacetate	Renal toxicity/tumor model; study of lipid peroxidation, transferrin receptors in liver[80–96]	Associated with pancreatic toxicity[78, 79]
Iron-sorbitol	Used to study subcellular distribution of iron and lipid peroxidation[99–102]	Preferential iron-loading of cells of the reticuloendothelial system[99]
Iron-Potentiated Injury		
Iron + ethanol	Clinically relevant model of liver injury; model of iron-potentiated ethanol-injury[111]	Need for high-fat diet; iron-potentiated fibrosis not reproducible[108, 110, 112–114]

Understanding the precise pathophysiologic mechanisms involved in the iron-induced activation of hepatic stellate cells and the role of lipid peroxidation in the development of hepatic fibrosis in hemochromatosis will require further study.

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49

Naturally occurring iron overload in animals

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Introduction

Iron metabolism has been studied extensively in humans due to two widespread diseases – iron deficiency and hemochromatosis. Because most living organisms require iron to survive, iron is also important in non-human animals. Each year, thousands of pounds of inorganic iron compounds are injected in, or fed to animals. For example, most pigs in the United States receive 200 mg of iron in the first few days of life. Iron overload might be expected to occur under non-experimental conditions. It also develops naturally from chronic hemolytic anemia and genetic, iatrogenic, dietary and unknown causes. This review discusses specific examples of these disorders.

Genetic causes of iron overload

Saler cattle

A condition occurs in the Saler breed of cattle that appears to be analogous to human hemochromatosis[1]. Originally, three animals had a history of poor growth, weight loss, and rough hair coat of several months' duration. Their condition did not improve after anthelmintic or antibiotic therapy by the owners. When they were first evaluated by veterinarians, they were small for their age and had poor body condition, poor hair coat, and diarrhea. No hematologic or abnormalities were found, but hepatic function was impaired (Table 49.1). Serum γ-glutamyl transferase was increased, sulfobromophthalein (BSP) half-time was prolonged, and hepatic iron concentration was increased.

Two animals deteriorated despite therapeutic phlebotomies and were euthanized. One heifer responded favorably to phlebotomy and gained weight. Animals that were necropsied had enlarged nodular livers. Microscopic examination showed micronodular cirrhosis with marked deposition of hemosiderin in hepatocytes, Kupffer cells, and arterioles. Hepatic iron was 30–100 times higher than reference values. Hepatic architecture was distorted by bands of fibrous connective tissue that bridged portal triads. Moderate numbers of inflammatory cells (predominantly mixed mononuclear cells with a few granulocytes) accompanied the fibrosis, and bile ductules were markedly hyperplastic. Arteriolar walls in the majority of the portal triads were pigmented and mineralized. Iron had accumulated in renal convoluted tubular cells, acinar cells throughout the pancreas, parietal cells of the abomasum, Peyer's patches, and macrophages in the lamina propria of the duodenal villi, lower small intestine, and large intestine. Abnormal pigmentation was not found in the skin, islet cells of the pancreas, or myocardium.

This disease appears to be genetically determined. The affected cattle were products of line breeding programs and shared a common ancestor (Fig 49.1.). Abnormal intake of dietary iron was not indicated because herdmates of affected animals and samples from another Saler herds had serum iron concentrations and total iron-binding capacity (TIBC) values within limits for cattle. Although a molecular defect has not been characterized in these cattle, they provide an excellent potential model for human hemochromatosis.

Hypotransferrinemic mice

Hypotransferrinemic mice provide a unique model to study massive iron overload[2]. These mice are small and pale at birth, and have hypochromic, microcytic anemia. Untreated neonatal mice die soon after birth unless they are rescued by weekly injections of mouse serum or transferrin. Serum transferrin levels in treated mice rarely exceed 1% of those in normal mice[3].

Hypotransferrinemia results from a failure to process the transferrin mRNA precursor into mature, translationally active mRNA[4]. Steady-state levels of transferrin mRNA sequences are <20% of normal and exist in the form of a 5 kb nuclear precursor, instead of the mature 2.5 kb transferrin mRNA of normal mice. The 5´ and middle introns can be removed by processing, but the last two introns of the transferrin gene are retained. The defect is inherited as an autosomal recessive. Heterozygotes have mild hypotransferrinemia and can develop mild hemosiderosis later in life[3].

Hepatic and pancreatic non-heme iron is increased greatly in hypotransferrinemic mice in comparison with normal control mice[3]. Most of the iron is deposited in parenchymal cells. Iron overload begins prenatally, before significant iron absorption has begun[5]. Despite the development of marked hepatic parenchymal iron loading, these mice continue to absorb excess iron[6].

Serum iron levels in hypotransferrinemic mice greatly exceed the binding capacity of transferrin. Serum iron measured by the bleomycin-dependent technique is surprisingly high (28 ± 9 μM (S.E.M.) for hypotransferrinemic mice vs. 3 ± 3.3 μM for control mice). Non-transferrin-bound iron consists of several distinct species (≥200 kd, 30–50 kd, and 1–5 kd), and each probably differs in toxicity and availability for tissue uptake[7]. Non-transferrin-bound iron is cleared with a half-life of <30 seconds, compared to 50 minutes for transferrin-bound iron[8]. When exogenous transferrin is injected, it is undetectable in serum after two days[8].

Histopathological evidence of iron toxicity is minimal, because the lifespan of hypotransferrinemic mice is short, or they have the capacity to sequester large amounts of iron in siderosomes as hemosiderin. Hemosiderin is less effective than ferritin in promoting oxygen-radical reactions. Mice seem to accumulate more iron in bile canaliculi than other iron-loaded species and may have enhanced biliary excretion[9].

Cirrhosis has not been observed, although severe distortion of the hepatic architecture can occur[3]. Parenchymal cells of hypotransferrinemic mice may protect themselves by forming holoferritin molecules in the cytosol until a plateau concentration is reached, forming clusters of iron-rich ferritin particles without a limiting membrane, and sequestering ferritin particles in lysosomes–siderosomes with the formation of hemosiderin[9]. Non-heme iron occurs in higher concentration in the pancreas than in the liver. Iron occurs in siderosomes and cytosolic ferritin in most, but not all, acinar cells. Centroacinar and intercalated duct cells contain massive amounts of ferritin and hemosiderin. Macrophages and other interstitial cells contain only isolated siderosomes[9]. In the heart, ferritin particles are mostly of the iron-rich variety, and siderosomes contain dense hemosiderin aggregates[9].

The following conclusions can be reached from hypotransferrinemic mice: (i) transferrin is not required by the

Table 49.1. Laboratory results from Saler cattle with hemochromatosis.

Variable	Reference values	Animals 1	2	3
Age, months	N/A	24	20	10
Serum iron, μg/dl	57–162	183	450	375
TIBC, μg/dl	165–295	102	407	383
Saturation, %	35–52	123	110	98
Hepatic iron, ppm	45–300	20100	8923	8700
BSP T½, minutes	2.5–4.1	7.5	9.4	14.7
GGT, IU/l	15–39	127	166	310

Notes:
Adapted from ref. [1]. TIBC = total serum iron-binding capacity, BSP. T½ = plasma clearance half-time of sulfiobromophthalein, GGG = gamma glutamyl transpeptidase.

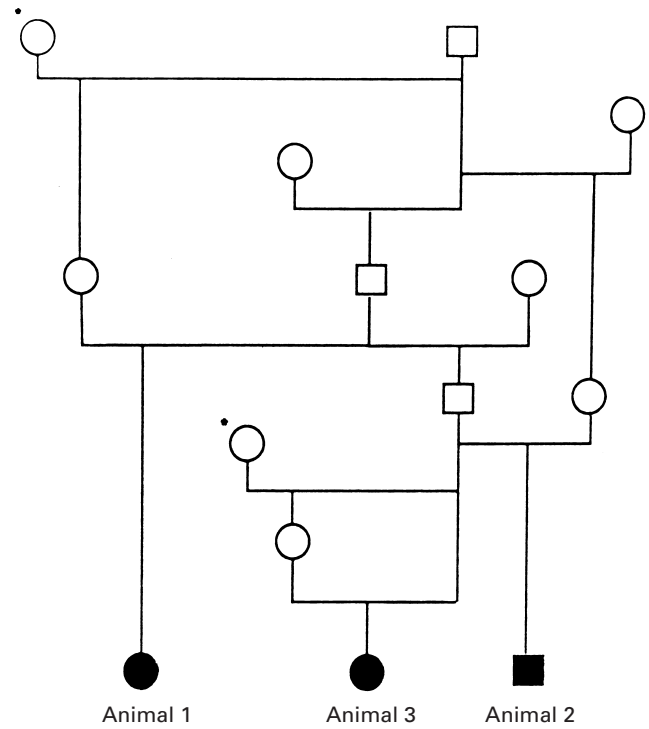

Fig. 49.1. Pedigree pattern of hemochromatosis in Saler breed of cattle (From ref.[1] with permission.)

intestinal mucosa to absorb iron; (ii) transferrin is not required for iron to be recycled by macrophages; and (iii) iron can accumulate in cells by a non-transferrin-dependent pathway[5].

Genetic knockout mice

These mice provide other models that mimic human hemochromatosis[10].

The β_2-microglobulin knockout mouse model demonstrates that a major histocompatibility complex I gene might be involved in human hemochromatosis[10]. These mice develop age-related overload manifest by hepatic iron overload, siderosis of endocrine and other tissues, and sometimes have hepatocellular carcinomas. *HFE* knockout mice have elevated transferrin saturation with iron and quickly develop siderosis of hepatocytes in a periportal distribution, although it remains to be seen whether these animals will develop arthropathy, diabetes, cardiomyopathy as do persons with hemochromatosis[11]. It seems likely that additional genetic knockout rodents will be used to study iron homeotasis as more genes pertinent to iron metabolism are discovered.

Iatrogenic causes of iron overload

Occasionally, inappropriate therapy can result in iron overload. Iron can be given intentionally or can be an ingredient in mixtures developed to provide general support.

Horses

Many horse owners, trainers, and some veterinarians believe that iron may be rate-limiting in athletic performance in horses. They provide additional iron to horses either orally or parenterally. Consequently, horses may receive an excessive amount of iron. One received 2.5 pounds of iron in two years and had serious clinical signs[12].

Neonatal foals

Foals and calves can be poisoned acutely by iron-containing vitamin and mineral mixtures. They are particularly susceptible to oral supplements containing large amounts of iron[13]. Shortly after birth, some foals were given a digestive paste orally. These foals were normal for two to three days, and then became dull and anorectic. Some were colicky; others convulsed or wandered aimlessly. Hematuria, melena, and marked icterus occurred. Ultimately, the foals became recumbent and died[14]. Signs were related to hepatic failure, and histological changes revealed marked biliary hyperplasia, hepatocellular necrosis, and fibrosis[14]. Icterus resulted from cholestasis and failure of destroyed hepatocytes to take up and conjugate bilirubin. The proportion of viable liver was correlated roughly with the length of post-natal survival[15]. The offending paste contained vitamins, iron fumarate, and viable primary cultures of fermentation products of *Lactobacillus acidophilus, L. casei, Bacterium bidfidus, Aspergillus oryzae, Torulopsis* sp., and *Streptococcus lactis*. The disorder can be reproduced by administering ferrous fumarate orally within 8 hours of birth[16].

Chronic hemolytic anemia

Hypoxia can enhance mucosal iron uptake by duodenal enterocytes[17]. Thus, chronic anemia of various etiologies results in iron overload. The severity of iron overload is related to the duration of the anemia and the degree of the oxygen-carrying deficit. Inherited defects in erythrocyte structure and function dominate the major causes in both man and animals.

Canine pyruvate kinase deficiency

Dogs of the Basenji, Beagle, Cairn terrier, and West Highland white terrier breeds can have a heritable deficiency of erythrocyte pyruvate kinase. These dogs have severe hemolytic anemia (hematocrit 15–25%), marked reticulocytosis (15–50%), bone marrow erythroid hyperplasia, and a shorted erythrocyte lifespan (1.2–3.8 days)[18, 19]. Affected animals usually die prematurely. In many cases, osteosclerosis and myelofibrosis occur terminally; bone marrow cavities are obliterated by spongy bone and fibrous connective tissue[20]. Anemia with erythroid hyperplasia is associated with increased intestinal iron absorption, hepatic parenchymal iron deposition and, eventually, cirrhosis[21].

Pyruvate kinase exists as four isotypes, L, R, M_1, and M_2. These isotypes are regulated developmentally and are tissue specific in adults. The L isotype is the major isoenzyme in liver; the R isotype, in erythrocytes; the M_1 isotype, in muscle; and the M_2 isotype, in leukocytes and platelets. Fetal tissues contain only the M_2 isotype; the change from fetal to adult isotypes occurs in the late fetal to early postnatal period. Only two genes code pyruvate kinase isotypes. The L and R isotypes are coded by the PK-L gene. In isotype L pyruvate, exon 1 is transcribed, and exon 2 is not transcribed. Isotype R pyruvate kinase results when exon 2, but not exon 1, is transcribed. Tissue-specific gene pro-

moters are responsible for differing mRNAs. The M_1 and M_2 isotypes are produced from the M gene and result from alternative splicing. The M_1 isotype contains exon 9, but not exon 10; the M_2 isotype contains exon 10, but not exon 9.

In pyruvate kinase-deficient dogs, the M_2-isotype of pyruvate kinase predominates in erythrocytes, but both the M_2-isotype and the R-isotype can be detected in reticulocytes. This suggests that aberrant isozyme expression does not result from a failure to switch from M_2 to R during erythroid maturation. The genetic defect in the Basenji breed is a single-base deletion at nucleotide position 433 (ΔC^{433}) in the R-isotype gene. This causes a translational frame shift with a premature termination, and results in a truncated, mutant protein that lacks the amino acid sequence with enzymatic activity. Transcript instability may also contribute to a lack of enzymatic activity[22, 23].

High morbidity and mortality in animals with severe, hereditary, hemolytic anemia usually are not due to anemia per se. Iron overloading of tissues, primarily the heart, liver, and endocrine glands, can occur in the absence of transfusions due to increased intestinal iron absorption[21]. When the bone marrow of dogs with pyruvate kinase deficiency is transplanted with bone marrow from a normal dog, severe anemia is corrected, and hepatic iron accumulation and subsequent cirrhosis are prevented[19]. Transplanted dogs do not develop osteosclerosis, a characteristic long-term sequela of untreated dogs[21].

Congenital dyserythropoiesis in Polled Hereford cattle

A syndrome of anemia, alopecia, and dyskeratosis occurs in the Polled Hereford breed of cattle. Anemia, present at birth, is non-progressive, and is normochromic, normocytic to macrocytic. Reticulocytosis is absent, and the bone marrow is markedly hyperplastic. Morphological abnormalities in erythroid precursors resemble those seen in type I congenital dyserythropoiesis in humans.

Calves have increased serum iron concentrations and increased saturation of serum iron-binding capacity, but the iron-binding capacity does not differ significantly from reference values. Iron levels are increased in liver, spleen, and bone marrow[24]. Marked hepatic fibrosis gives the capsular surface a distinctive mosaic appearance. Histologically, the liver is characterized by marked capsular and subcapsular fibrosis. A yellow-gold granular pigment is sometimes seen in Kupffer cells and in periportal hepatocytes. Mid-zonal and centriobular hepatocytes have mild to moderate vacuolar degeneration and swelling. Bile duct proliferation does not occur. More extensive liver lesions may not develop, because calves usually die or are euthanized before reaching 6 months of age[25].

Analyses of affected and random pedigrees are consistent with an autosomal recessive mode of inheritance. Mating of affected-to-affected animals to prove this mode of inheritance has not been possible, because affected animals do not survive to a reproductive age[26].

Changes from natural diets that cause iron overload

Most metals are maintained at relatively constant levels in vivo by regulating both uptake and loss from the body; unlike other metals, controllable export systems do not exist for iron. Consequently, body iron must be maintained by controlling absorption, and regulation of iron absorption is important. If too little iron is absorbed, anemia and deficiency of iron-containing enzymes can result. If too much iron is absorbed, tissue damage such as hepatic cirrhosis, diabetes mellitus, cardiomyopathy, and arthropathy can result.

Iron can be absorbed as either heme or non-heme compounds. Heme is found in meat or meat products and can be a source of iron for carnivores. Heme iron is absorbed independently of other dietary ingredients. In contrast, non-heme iron is found in feed/food and is largely unabsorbed, but its absorption can be affected by other dietary compounds. Certain dietary components (e.g., phosphates, tannates, and phytates) inhibit non-heme iron absorption; in contrast, meat and ascorbate enhance absorption.

Iron-deficient animals absorb more and iron-loaded animals absorb less of a particular test dose of iron. When humans and mice are given progressively increasing amounts of iron, a larger absolute quantity of iron is absorbed, although the percentage of iron absorbed progressively decreases[27]. Exposure to excessive amounts can cause acute iron poisoning. In 1994, at least 3210 children under 5 years of age were treated and two children died as result of exposure to iron-containing products[28]. Thus, bioavailability of dietary iron can be an important determinant in the amount of iron absorbed. However, the amount absorbed cannot be predicted from inorganic iron analyses, because absorption is affected by other dietary components as described above.

When non-domestic animals live in their natural environment and ingest the feed or food that is available, body iron is generally maintained at constant levels, and serum iron concentration and total iron-binding capacity values are remarkedly stable. When animals are placed in a

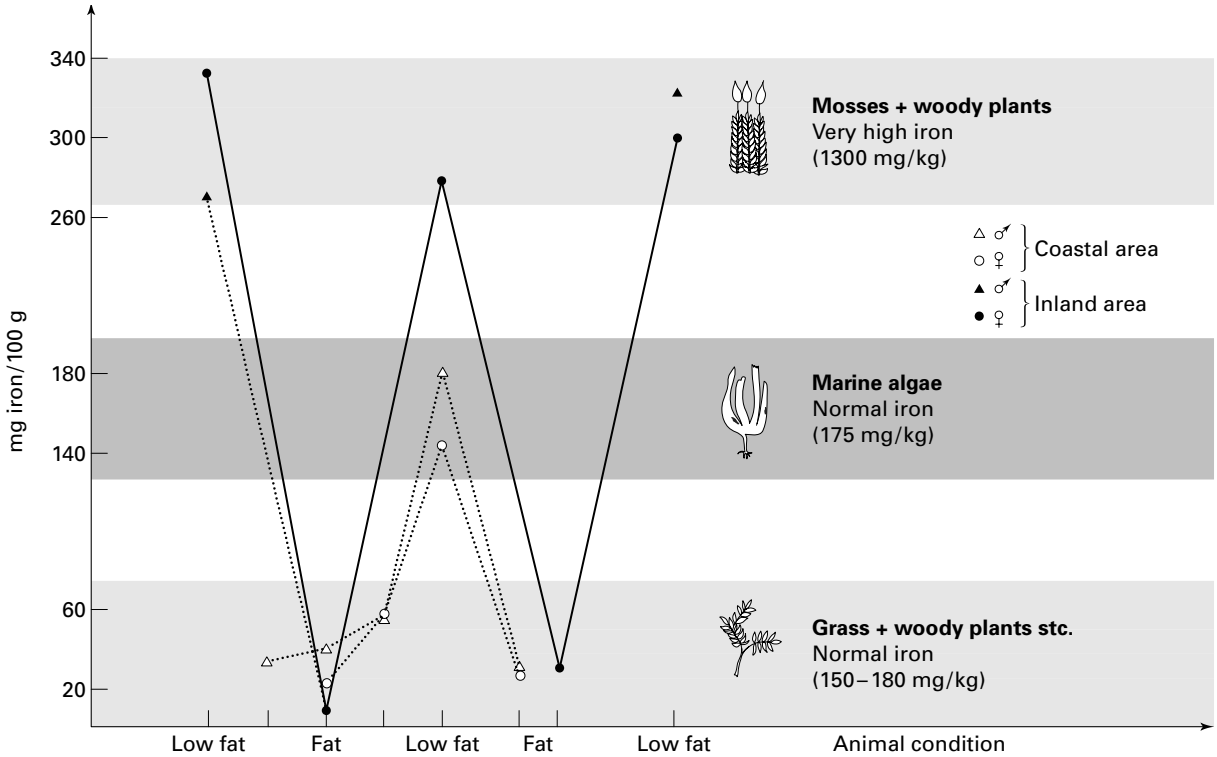

Fig. 49.2. Hepatic iron concentration related to Svalbard reindeer condition and diet, i.e., plant content and iron concentration in rumen. (From ref.[29] with permission.)

captive environment, their feed or food may change significantly. Sometimes, iron in the feed or food may be more available than that in the natural diet and is absorbed in excess. Consequently, iron overload may occur.

Svalbard reindeer

Seasonal changes in the hepatic iron of Svalbard reindeer (Rangifer tarandus platyrhynchus *Vrolik*) provide valuable insights into mammalian handling of iron[29]. Svalbard is a group of Norwegian islands in the Arctic Ocean. The winters in this area are usually harsh, and reindeer have limited access to forage. By April of each year, they have lost a quarter to one-third of their body weight. As lean tissue and erythrocytes decrease, iron accumulates, because it cannot be excreted. During this time, iron content of plants that are available to reindeer varies with the location (Fig 49.2). In coastal areas, reindeer eat marine algae with an acceptable iron content (≃175 mg/kg). Under these conditions, iron accumulates in the mononuclear-phagocyte system. Reindeer that live in non-coastal areas eat woody plants and mosses with a high iron content (≃1300 mg/kg) and have massive iron loading in both parenchymal and nonparenchymal cells. Hepatic fibrosis does not occur.

Svalbard reindeer can graze on grass and woody plants during the summer and regain the lost weight. These plants have a normal iron content, and livers from females contain very little visible iron. Livers from males have some iron-containing granules in the nonparenchymal cells. During the rutting season (September and October), males stop feeding and increase their physical activity. The resultant weight loss and breakdown of tissues may increase iron in male reindeer during the fall.

Serum iron concentration and TIBC decrease during starvation (Table 49.2). Saturation of iron binding capacity depends on dietary iron concentration. When reindeer are eating forage with a high iron content, they have a higher saturation percent.

Apparently, severe weight loss increases iron stored in nonparenchymal tissues regardless of dietary iron content. Malnourished reindeer absorb increased amounts of iron when they eat forage with high iron content despite having an increased concentration of stored iron. Several hypotheses can be advanced to explain why iron is stored in parenchymal cells. First, body stores of iron may pass some critical concentration or the iron storage capacity of nonparenchymal tissues may be exceeded. Second, near saturation of serum iron binding capacity may increase uptake

of iron by parenchymal cells. Transferrin receptors have a higher affinity for transferrin with both iron binding sites occupied versus transferrin with only one site occupied[30]. Third, when iron is absorbed from dietary sources with high iron, it may be stored preferentially in parenchymal cells. If transferrin is nearly saturated, additional absorbed iron might exceed the iron binding capacity in the portal circulation.

Black rhinoceros

Black rhinoceros, but not white rhinoceros, accumulate hepatic iron during captivity (Fig 49.3)[31]. Serum iron concentration and percentage of transferrin saturation also increase in black rhinoceroses compared to white rhinoceroses. Serum ferritin can be measured with an enzyme-linked immunoabsorbent assay that uses an anti-equine ferritin antibody. The serum ferritin concentration in black rhinoceroses is significantly higher than in white rhinoceroses and increases with time in captivity.

Two mechanisms have been hypothesized for this accumulation. First, more iron may be absorbed from the feed provided in captivity than that available under natural conditions. Black rhinoceroses browse on shrubs, forbs, and trees. The low quality of the forage necessitates that they spend most of their time ingesting food. In contrast, white rhinoceroses have broad lips, live in a grassland savannah, and graze on short grasses. When rhinoceroses are translocated, they are fed alfalfa or grass hay which is closer to the diet of a grazer than that of a browser.

Second, iron absorption can increase with increased erythropoietic activity. Erythrocytanemic hypoxia can enhance mucosal iron uptake by duodenal enterocytes[17]. Severe hemolytic anemia is a leading cause of death among captive black rhinoceroses and may account for as many as 40% of the deaths of these animals. This hemolytic disease is not seen in white rhinoceroses. Erythrocyte metabolism in rhinoceroses differs significantly from that in most species in that their erythrocyte adenine nucleotides are unusually low[32]. Black rhinoceroses have catalase activities that are 2–4% of the activities found in human erythrocytes. Erythrocytes from other rhinoceros species have activities that are intermediate between those of black rhinoceros and human erythrocytes[33]. Some investigators[34] have suggested that the iron accumulation is related to the hemolytic anemia seen in captivity. Unfortunately, because rhinoceros are an endangered species, definitive experiments to determine the correct hypothesis are not possible.

Table 49.2. Serum iron analytes in Svalbard reindeer[a]

Animal condition	Dietary iron content, mg/kg	Serum iron, μg/dl	TIBC, μg/dl	Transferrin saturation, %
Low fat	Mosses, woody plants (1300)	178 ± 39	203 ± 50	91 ± 18
Low fat	Marine algae (≃175)	128 ± 61	186 ± 22	68 ± 30
Fat	Grass, woody plants (150–180)	263 ± 61	422 ± 84	61 ± 15

Notes:
[a] Adapted from ref. [29]. Data represent mean ± S.D. TIBC = total serum iron-binding capacity.

Primates

Common marmosets often have hepatic hemosiderosis that is generally considered to be without clinical or pathologic relevance. The pigment occurs principally within hepatocytes; small amounts occur in Kupffer cells. More pigment is deposited in centrolobular and midzonal hepatocytes than in the periportal region. A diet with high concentrations of iron fed to captive marmosets can cause hepatic hemosiderosis, debility, and premature mortality. A subgroup of marmosets may be at increased risk of iron overload. Many marmosets fed a high-iron diet accumulate hepatic iron in a pattern similar to that of human hemochromatosis[35].

African rock hyrax

A single African rock hyrax (*Procavia capensis)* was diagnosed with hemosiderosis. He showed only minor neurological signs prior to death. Liver and jejunum contained the most significant histopathological findings. Hepatocytes contained numerous dark yellow to brown pigment granules. The animal's diet consisted of green leafy vegetables, fruit, and commercial primate chow with supplemental iron[36].

Avian species

Mynahs (*Gracula religiosa)*, birds of paradise (Family Paradisaeidae), quetzals (*Pharmonachrus monino*), blackbirds (*Turdus merula*), touracos (Family Musophagidae), ducks (*Anas platyrhynchos)*, toucans (Family Ramphastidae), crows (*Corvus brachyrhynochos*), starlings (*Sturnus vulgaris*), rails (Family Rallidae), green aracaris (*Pteroglossus viridis*), Australian bittern (*Botaurus poiciloptilus)*, Green Cat Birds, tanagers (Family Thraupidae),

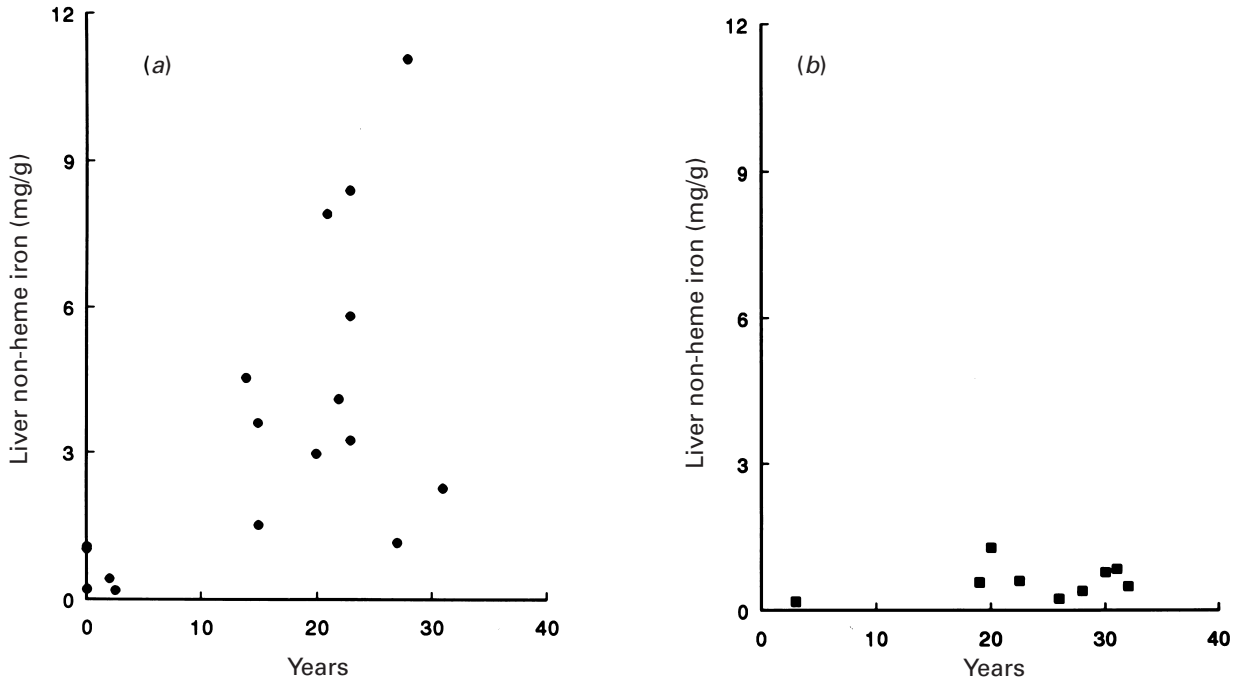

Fig. 49.3. Relationship of hepatic non-heme iron to age or time in captivity in black (*a*) and white (*b*) rhinoceroses. (From ref.[35] with permission.)

and hornbills (*Buceros bicornis*) are affected frequently with iron overload. Most of the iron accumulates in the hepatocytes with variable amounts in Kupffer cells. Fibrosis is variable but some birds also develop cirrhotic livers. Birds react to iron overload with the same defensive mechanisms that occur in mammals[37]. Gosselin and Kramer[38] hypothesized that excessive iron storage in the liver of mynah birds is not diet induced. They supported this conclusion by the dissimilarity of hepatic and splenic iron distributions between mynah birds and other birds receiving an identical or similar diet[38]. Unfortunately, the authors did not indicate how mynah birds received the excessive iron and did not consider that they may have absorbed a higher percentage of the dietary iron.

Eider (*Somateria mollissima*) in Svalbard exhibit a phenomenon similar to that seen in Svalbard reindeer. Female eider fast completely from the beginning of egg laying to the end of hatching. Blood and lean tissue are catabolized during the fast, and the liberated iron is translocated to the liver. This is complicated by an increase in iron absorption prior to the egg laying period. Total hepatic iron content increases about three-fold from prelaying to the end of brooding. Fibrosis does not occur. After hatching, the birds regain their weight, and the hepatic iron is recycled or redistributed[39].

Unknown

Several horses of different breeds have a syndrome with hepatic cirrhosis and abnormal increase in hepatic iron. Three horses had a history of weight loss, lethargy, and ventral edema. Serum γ-glutamyltransferase and aspartate aminotransferase activities were high. Serum total bilirubin, plasma fibrinogen, and blood ammonia concentrations also were increased. Liver samples obtained by biopsy had diffuse bile duct hyperplasia, portal fibrosis, and abundant iron in hepatocytes and Kupffer cells. Hepatic iron concentrations were about 20 times the reference range[40]. Other horses have had severe coagulopathy[41]. Subsequent horses have had increased serum iron, nearly saturated iron binding capacity, and elevated serum ferritin levels. In some cases, a readily apparent source of iron could be located in feed or surroundings, but some horses have had histories of excessive oral or parental iron[41, 42] Currently, whether the elevated hepatic iron precipitated liver disease and fibrosis or the reverse is unclear. Because cases are sporadic and involve several breeds, the condition is unlikely to be genetically determined. Horses might provide a model to study fibrosis secondary to elevated hepatic iron.

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Part XI

Screening for hemochromatosis

50

Screening tests for hemochromatosis

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Introduction

Tests to identify occult hemochromatosis must be considered in the context of accepted practices for screening laboratory programs and have thoughtful goals and evaluation procedures to yield acceptable outcomes for both screen-positive and screen-negative individuals.

The clinical course of hemochromatosis (Fig. 50.1) is consistent with the recommendations of the World Health Organization[1] and others[2] for conditions appropriate for screening programs. Hemochromatosis is prevalent (at least 5 per 1000) and has a prolonged presymptomatic phase. Treatment during the early phase prevents future organ failure and restores the length and quality of life to match that of unaffected controls[3, 4]. The total burden of illness is not precisely known. Data suggest that 50% of male and 10% of female homozygotes will develop serious symptoms[5]. Therefore, the burden of ilness is high enough to warrant medical and public attention. The costs of testing and preventative treatment are favorable compared to awaiting the development of symptoms[6].

Why has there been no widespread, publicly supported screening program for hemochromatosis? Three explanations seem plausible. First, the burden of illness is neither obvious to the general public nor completely documented by medical science. Second, hemochromatosis has not yet been shown to cause or have the potential to generate a high dollar volume in either diagnostic testing or sale of therapeutic drugs. Finally, the evidence regarding cost-effectiveness for early detection and treatment of hemochromatosis has not been understood in the context of other disease care or wellness interventions.

The economic and epidemiological evidence is presented throughout this volume. Phatak[6] and Buffone[7] have shown that laboratory screening with an initial screening test cost of $12US is both more effective and less expensive than waiting for symptoms to develop. The Canadian recommendations label this a 'grade A' process, the most cost beneficial, because screening for hemochromatosis saves money and life-years[8]. The models of Phatak indicate a quality-adjusted life-year (QALY) gained from hemochromatosis screening and treatment costs from $0 to $600US. This is much less expensive than the $7,000US to $36,000US for an additional QALY from prevention of myocardial infarctions using cholesterol lowering HMG-CoA reductase inhibitors[9, 10]. Early detection of hemochromatosis is an excellent mechanism to postpone morbidity and reduce societal care costs[11].

Epidemiologists carefully examine screening programs purported to lengthen survival, looking for lead-time bias, length bias, and volunteer effects[2, 12]. Lead-time bias is the movement of detection to an earlier time without postponing morbidity or mortality. The survival data of Niederau[4] and Adams[3] suggest that lead-time bias is not responsible for the lengthened survival observed in early detection of hemochromatosis. Length bias is the preferential detection of indolent, slowly progressing cases by screening programs. It is no longer ethical to conduct a trial without intervention, and the data suggest length bias could alter actual survival estimates, but is unlikely to change the conclusion that morbidity and mortality are postponed by early detection. Volunteer bias is widespread in early disease detection and survival studies. The unexplainable better survival of those volunteering to be screened seems to lack a clear pathophysiologic explanation[2]. The volunteer effect seems absent in the Adams and Neiderau survival data. None of these common biases in screening studies negate the economic benefits projected.

There is growing support for comparing intervention and prevention programs on the basis of the number of persons who must be treated to cause one positive outcome. The primary prevention of acute myocardial

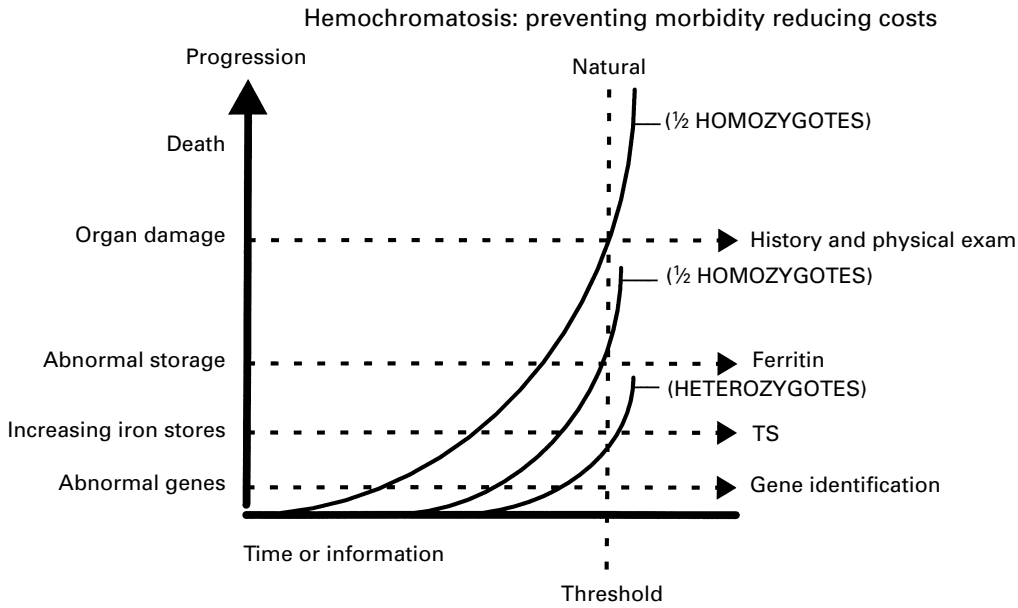


Fig. 50.1. Hemochromatosis natural history for 50% of homozygotes who develop more serious symptoms, homozygotes with less serious symptoms, and heterozygotes without any other risk for iron loading. The upper one-half of the homozygotes are the more seriously affected, the lower one-half are less affected. Historical information or physical examination is not effective earlier in the progression. The threshold refers to the presence of enough information to confirm the diagnosis and/or initiate treatment[29].

infarction (AMI) through reduction of serum cholesterol concentrations provides an instructive comparison. Rembold[13] reports 71 hypercholesterolemic individuals need to be treated with a HMG-CoA reductase inhibitor to prevent one first AMI. While treating these 71, two persons will die of AMI while the net prevention of one is occurring. These seemingly counter-intuitive results are attributable to the high degree of overlap in the distributions of serum cholesterol concentration values in those who develop AMI and those who do not. To summarize the anti-cholesterol AMI prevention strategy: (i) three persons need to be tested to find one candidate for treatment; (ii) it might be necessary to re-test ~10% to clarify who is eligible for treatment; and (iii) then treat 71 persons to prevent one AMI, while two of 71 die of AMI. Contrast a hemochromatosis screening strategy: (i) 200–300 persons need to be tested to find one candidate for treatment; (ii) we may need to re-test 10–15% to clarify who to treat; and (iiii) then treat at most one or two to prevent one premature death due to hemochromatosis. Widespread screening may not begin until these facts are better understood in the context of other health interventions[14].

Screening goals

Clinical, social and economic goals should be well-defined. The screening program must be carefully considered by all stakeholders: patients, providers and payors[15, 16]. Labeling must avoid adverse psychological effects[17] and create the positive clinical effects of more QALY. Two goals of screening seem most accepted. The first is to identify individuals who will be helped, i.e., those for whom intervention is beneficial. This implies avoidance of labeling individuals who may or may not ever need intervention or risk serious morbidity. Second, the evaluation and management of those with screen-positive results must be clear.

Laboratory tests

The variable progression of hemochromatosis is depicted in Fig. 50.1. Signs and symptoms may be useful in screening for hemochromatosis only if early signs, such as fatigue and arthralgia, lead to biochemical testing. Unfortunately, most persons who present with early complaints are not tested or diagnosed; late manifestations are less helpful in finding cases before irreversible change occurs. The serum

ferritin concentration is a manifestation of the pathophysiologic accumulation of excess iron. Serum ferritin concentration is related to the size of accumulated stores, and adds to the diagnostic evaluation. However, this parameter is not regarded as the best first screening test because an elevated ferritin concentration can occur in many other conditions and the charges for measuring the serum ferritin concentration are often higher than charges to measure transferrin saturation[18].

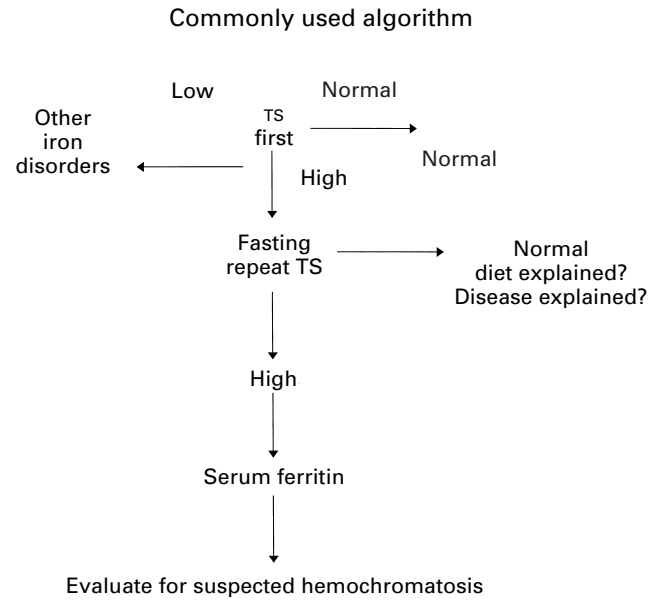
Elevated serum transferrin saturation (TS) is a reflection of the abnormal metabolism that creates the propensity for abnormal accumulation of storage iron in hemochromatosis and therefore is detectable early. TS is widely accepted as the preferred first test[5, 18–20].

Direct measurement of genes may be useful to sort cases that are difficult to interpret biochemically, and, perhaps to identify heterozygotes. However, the relationships of genes to iron overload status grows in complexity[21], and does not currently meet the inexpensiveness criterion for the first screening test. Adams[21] reports family studies with abnormal *HFE* homozygotes without iron overload, and iron overload in persons without *HFE* abnormalities. The predictive value of genetic measurements is probably high, but genetic testing may not be useful to identify those needing medical intervention. It may be better to measure TS first, then perform genetic tests. However, gene measurements may help predict the degree of manifestations in iron overload[22], and may be included for the prognostic evaluation of probands or evaluation of relatives of a proband.

Currently, TS is the first test of choice (Fig. 50.2). The disadvantages of TS are like those of other blood biochemical tests[18]. The most important pre-analytical factors are ingestion of iron-containing medications, supplements, and foods, and inherent biological variability in the test results. Between-day and within-day variability of serum iron concentration, total iron-binding capacity (TIBC) TS, and serum ferritin concentration in normal volunteers has been reviewed[18]. The intra-individual CV for serum iron concentration and TIBC were 28.5 and 4.8%, respectively, in 13 healthy volunteers studied daily using morning samples during a three-week interval. Other reports confirm the wide intra-person, day-to-day variation in serum iron concentration and lesser variation in TIBC. Much larger variability has been observed in samples collected within a 24-h period. Published data disagree regarding the pattern of changes between 08:00 and 17:30. Data in 62 volunteers did not show a systematic pattern among values obtained within a single day.

The within-day variability of serum iron concentration, TIBC, and TS measures has also been studied in 43 hemochromatosis patients and normal controls who were sampled at 2-h intervals for one 24-h period. Serum iron concentration and TS results were constantly abnormal in the hemochromatosis patients, and revealed little variation between the mean values observed at each sample time. These data support the conclusion that sample timing is unimportant for individuals who are hemochromatosis homozygotes and do not negate the utility of a random timed measurement of TS. If all elevated TS values are followed by a retest after an overnight fast and avoidance of iron supplements and vitamin C for several days, preanalytical concerns are minimized.

The analytical concerns of standardization, chemical specificity, and precision have been addressed in the laboratories of most countries with the highest populations of Caucasians who have the highest prevalence of hemochromatosis[14, 18]. Significant opportunity to improve analyses does not preclude starting testing or screening now. The postanalytical decision making and the pre-analytical decision to order the test are major areas that need improvement[14]. Appreciation of the prevalence of hemochromatosis by clinicians is poor enough that iron parameter tests are rarely ordered; test results obtained are frequently ignored.



Choose from:
(i) follow-up if young, ferritin low and ALT normal
(ii) gene testing
(iii) phlebotomy trial
(iv) hepatic biopsy and iron quantitation.

Fig. 50.2. Algorithm for evaluation of initial transferrin saturation result above the decision point. Choice of follow-up depends on amount of iron accumulation as well as the number and severity of symptoms.

Table 50.1. Saturation decision points and predictive value[a]

					Prevalence is 5 per 1 000		Prevalence is 5 per 10 000	
	TS decision level (%)	Detection rate	False positive rate	Likelihood ratio	PV Pos	PV Neg	PV Pos	PV Neg
Males	50	0.94	0.0700	13	0.0632	0.9997	0.0067	0.9999
	55	0.91	0.0320	28	0.1250	0.9995	0.0140	0.9999
	60	0.86	0.0150	57	0.2237	0.9993	0.0279	0.9999
	65	0.79	0.0066	120	0.3756	0.9989	0.0565	0.9999
	70	0.72	0.0027	267	0.5727	0.9986	0.1177	0.9999
	75	0.64	<0.0010	640	>0.7628	0.9982	0.2425	0.9998
	no test	0.00	—	—	—	0.9950	—	0.9995
Females	50	0.82	0.0500	16	0.0761	0.9990	0.0081	0.9999
	55	0.75	0.0180	42	0.1731	0.9987	0.0204	0.9999
	60	0.67	0.0059	113	0.3633	0.9983	0.0538	0.9998
	65	0.58	0.0018	322	0.6182	0.9979	0.1388	0.9998
	70	0.48	<0.0010	480	>0.7069	0.9974	0.1936	0.9997
	no test	—	0.00	—	—	0.9950	—	0.9995

Notes:
Adapted from ref. [19].
[a] TS = transferrin saturation; PV = predictive value.

Decision threshold for TS interpretation varies in reported studies. There is no perfect decision level and every program designer must balance needs for sensitivity with that for specificity. Acknowledging the goal of seeking positive interventions and enhancement of specificity through repeat TS testing and serum ferritin concentration measurement as shown in Fig. 50.2, many investigators favor a lower decision level for the initial TS test, i.e., 50%, 55%, or 60%. If two elevated values of TS are measured on fasting serum samples, the specificity of the screening activity will be increased, and the false-positive rate is expected to be very small. In cases in which the second TS measurements are in the normal range, and the explanation for the discrepancy is not apparent, a third fasting specimen for measurement of TS may be needed. If the algorithm in Fig. 50.2 is followed and the undetected subject with hemochromatosis has had an opportunity to accumulate iron, an elevated serum ferritin concentration will further decrease the likelihood of a false-positive testing result.

Serum ferritin concentrations must be interpreted cautiously. Serum ferritin is an acute-phase reactant, and its concentrations are elevated in inflammatory disease. Hepatocellular diseases cause leakage of both iron and ferritin from hepatocytes, further complicating interpretation of both TS and serum ferritin concentration. In the absence of either inflammatory disease or significantly elevated serum concentrations of transaminases, the 97.5 percentile serum ferritin concentration value is ~200 ng/ml for young women and ~400 ng/ml for postmenopausal women and men[23]. Each laboratory or method may have slightly different reference ranges for serum ferritin concentration. However, methods have become more similar in the 1990s and the decision points of 200 and 400 ng/ml, respectively, are appropriate for most methods.

When Edwards[24] followed the strategy in Fig. 50.2 with 11 065 blood donors, those who underwent hepatic biopsy included 69% hemochromatosis homozygotes, 31% hemochromatosis heterozygotes and 0% normal individuals. When Olsson[25] followed such a strategy, those who underwent biopsy included 100% hemochromatosis homozygotes and 0% normal individuals. Following the algorithm in Fig. 50.2 should provide effective case finding and prevent unneeded morbidity and mortality from hemochromatosis without imposing the risks of hepatic biopsy on many normal individuals. Care and thought in the subsequent evaluations will help avoid excessive use of invasive procedures (Fig. 50.2).

The choice for follow-up evaluation is complex. Patient desires are important. Some patients demand that a hepatic biopsy be performed for prognostic information. Others will shun the 'painful and risky' procedure in favor

of therapeutic phlebotomy. The utility of patient age and serum ferritin concentration to guide advice for hepatic biopsy is being investigated [26, 27].

What TS value should constitute a screen-positive result? Bradley[19] has performed a meta-analysis of five hemochromatosis detection studies that employed HLA typing to help clarify the genotype of those studied. Multiple laboratories are currently evaluating other genetic tests, along with TS results. New data may modify current estimates of predictive value. Bradley's data are reproduced in the first three columns of Table 50.1 with additional predictive value calculations. An additional line labeled 'no test' illustrates the negative predictive value of not testing anyone and calling all the people tested 'negative'. This illustrates the small improvement in negative predictive value attributable to testing[28]. The improvement in positive predictive value illustrates the gains in information occurring from testing. Bradley's meta-analysis confirms the number needed to test and treat analysis presented earlier for Caucasians. The far left columns illustrate predictive values in ethnic groups where the prevalence may be one-tenth as high.

Each local laboratory has specific resources available. TS measurement requires an estimate of TIBC or transferrin. Direct manual TIBC measurement is laborious and usually less precise. Immunologic transferrin measurement is more precise, but is associated with higher reagent costs. Unsaturated iron-binding capacity indirectly measures empty iron sites on transferrin and is easily and inexpensively automated[29]. Some laboratories may choose to use serum iron concentration as the first test. Serum iron concentration is likely to have lesser sensitivity[30]. Other laboratories may choose to start with serum ferritin concentration. Neither are as effective as TS as the first test, but both could provide some benefit over not testing.

Bradley's results illustrate the putative 'efficacy' of screening in a research environment. Multiple studies are on-going to evaluate the 'effectiveness' of TS-based screening programs in local health care settings. Preliminary reports of these programs indicate the predictable results (i.e., the prevalence is 5/1000; 50% of males and 10% of females progress to organ-threatening accumulation of iron; and community programs find 2–3 cases per 1000 screened). One study using an unsaturated iron-binding capacity initial decision level of 125 μg/dl for 8939 samples from 7073 people identified nine persons requiring intervention and nine pending evaluation with a reasonable likelihood to have hemochromatosis[29]. This yield is between 1.3 and 2.6 per 1000 screened.

This discussion of TS testing has focused on the usual hemochromatosis case. Other conditions with iron overload and normal TS have been reported[31]. Iron overload in those with sub-Saharan black ancestry is associated with lesser elevations of TS[32]. Efficacy of TS screening for these conditions has been less extensively studied and is discussed elsewhere in this volume.

Screening program characteristics

TS screening of individuals presenting to health care facilities could be classified as 'case finding'[2, 15]. Regardless of what it is called, testing for asymptomatic conditions should be associated with adequate assistance for test interpretation and management of both test-positive and test-negative persons.

Hemochromatosis is a genetic disorder and issues such as stigmatization and discrimination require consideration of informed consent before testing. These issues are even more important when gene tests are employed. Most feel that TS testing as part of the healthcare process bears less negative connotations. Management and therapeutic guidance is essential. Therapy end-points for initial iron depletion therapy and maintenance phlebotomy should be delineated. Counseling of patients and family members regarding diet, alcohol consumption, avoidance of raw shellfish, and other lifestyle issues must be available to primary care givers[14, 18].

Future of screening

In the near future, screening will depend upon TS as the first test. The issues will be test performance improvement and education of patients, payers and providers about the benefits of screening for hemochromatosis. In the United States, the 'know your number' campaign to encourage screening for hypercholesterolemia could be emulated. If individuals were to 'learn their iron status,' the morbidity and premature mortality of 3–7 per thousand attributable to iron overload and 80–150 per thousand attributable to iron deficiency could be decreased or eliminated.

In the distant future, additional detailed genetic information will become available. Whether this will alter our initial screening tests is unclear. The genotype–phenotype relationship will need to be much clearer[21] before gene tests can be suggested as the first test. The cost of gene testing will be driven up by issues of intellectual property rights[33] and down by automation. Issues of confidentiality, labeling, and discrimination seem more focused on gene tests than on the phenotype-identifying tests like TS, and will not be resolved soon.

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Cost-effectiveness of screening for hemochromatosis

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Introduction

Hemochromatosis is an inherited disease characterized by excessive absorption of dietary iron leading to progressive tissue iron accumulation and ultimately organ damage. Early diagnosis and appropriate management can prevent disease manifestations. The high prevalence of this disorder makes it a logical target for routine screening[1, 2]. Hemochromatosis is generally viewed as a relatively uncommon disorder. However, several recent studies estimate its prevalence to be 3–8 per 1000, making it one of the most common genetic disorders[3–12]. These high prevalence figures have been derived largely by screening asymptomatic individuals using serum transferrin saturation (TS), serum ferritin (SF) concentrations, or a combination of these tests. Hemochromatosis is an autosomal recessive disorder, the gene for which is linked to the histocompatibility (HLA) loci on the short arm of chromosome 6. Recently, a single amino acid mutation (C282Y) in a gene on chromosome 6, designated *HFE*, has been described, and appears to account for approximately 83% of cases of hemochromatosis[13–15]. Another 4% of affected individuals are compound heterozygotes for the C282Y mutation and for another mutation in the same gene (H63D)[13]. The mechanism by which these mutations result in iron overload is unclear (see Fig. 51.1).

Hemochromatosis has been defined as homozygosity for the mutant allele[1]. With the description of the *HFE* gene, it is clear that there may be genetic defects that result in iron overload in some cases. Persons homozygous for the hemochromatosis allele(s) do not have increased iron stores early in life. Iron is absorbed from the diet in excessive amounts and this results in progressive accumulation of tissue iron stores. There is significant variation among persons with hemochromatosis with regard to the rate of accumulation of iron stores and appearances of organ dysfunction. Some authorities contend that only a minority of affected individuals develop disease manifestations. However, family studies using HLA typing to define homozygosity have shown that most homozygotes have significantly increased tissue iron stores[2]. It is possible that the latter reflects the propensity of a particular family to develop disease manifestations.

Even when liberal phenotypic standards are used to diagnose hemochromatosis, there remains a sex difference in prevalence rates of detectable hemochromatosis. The autosomal recessive nature of the genetic defect should lead to an equal prevalence in males and females. The likely explanation for the observed sex difference is that the cut-points used for screening blood iron studies fail to detect the defect in some females. It is not possible to measure the magnitude of this shortfall at present.

The prevalence of the genetic predisposition to develop iron overload is at least 3.9 per 1000, the prevalence of proven hemochromatosis in males, but this could be higher. Affected individuals accumulate iron at varying rates depending on their sex, dietary intake of iron, alcohol intake, blood donations and other factors that remain to be identified. This variability is depicted in the Fig. 51.1. It is likely that many never accumulate clinically significant amounts of iron. The possible range of variability and the factors that account for it need to be determined more accurately.

The treatment of hemochromatosis involves the removal of body iron by periodic phlebotomy treatments. If the disorder is detected prior to the onset of organ damage, particularly hepatic cirrhosis, then phlebotomy treatments prevent organ damage and life expectancy is normal[16–26]. Once significant organ damage has occurred, progression can be slowed by phlebotomy treatments, but some irreversible manifestations persist[16]. The presence of hepatic cirrhosis shortens life expectancy substantially.

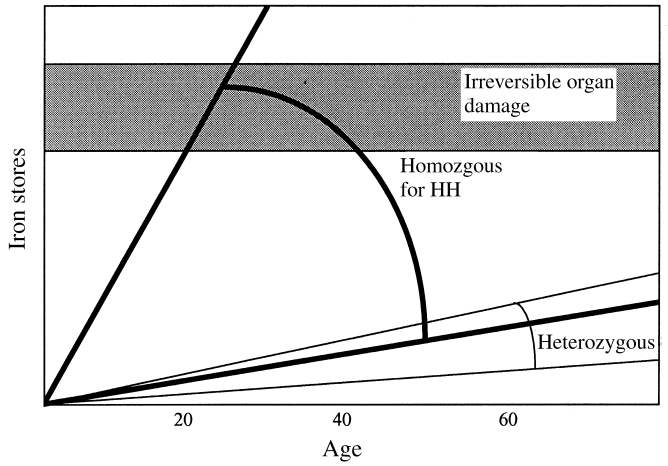

Fig. 51.1. Natural history of hemochromatosis. Iron stores increase progressively during an individual's lifetime. In heterozygotes, the levels probably never reach a clinically significant threshold. Some homozygotes develop irreversible organ damage. All the factors that determine whether this will occur have not been defined.

Congestive heart failure and diabetes mellitus are partly reversible by therapeutic phlebotomy. Arthropathy, once established, tends to persist in spite of therapy. Impotence and sterility may also not be reversible. Early diagnosis is crucial in individuals with hemochromatosis, but early manifestations are subtle and non-specific. Therefore, it has been suggested that general screening of asymptomatic individuals be undertaken to detect the disorder early and prevent irreversible organ damage.

Should we screen for hemochromatosis? Principles for defining diseases that are good candidates for general population screening have been formulated by epidemiologists and public health officials[27, 28]. The disease must have a significant impact on the quality or length of life and be sufficiently prevalent to justify the cost of screening. The disease should have an asymptomatic phase during which detection and treatment lead to reductions in morbidity and mortality. Treatment in the asymptomatic phase must yield better outcomes than treatment delayed until the onset of symptoms. Treatment and testing strategies must be acceptable and available to the population. The tests used for screening must be sensitive enough to detect asymptomatic disease and specific enough to avoid initiating evaluations on patients without actual disease.

Based on these criteria, hemochromatosis is an extremely strong candidate for routine screening. It is prevalent, and the early detection of disease improves outcomes by preventing irreversible end organ damage and thereby prolong life expectancy. TS and SF testing and phlebotomy treatments are easily tolerated and widely available. The need for hepatic biopsy is a major source of concern, but is generally well tolerated and safe. The morbidity and mortality caused by the disorder impose a high degree of disability in affected individuals. The tests currently used for screening are accurate enough when used in combination and the advent of genetic testing may reduce inaccuracy in testing. Therefore, the remaining question is whether screening is cost-effective, that is, do reductions in morbidity and mortality justify the costs incurred during the evaluation.

Screening strategies

TS is considered the most sensitive test for the detection of hemochromatosis[29–31]. The cut-point for TS above which evaluation for hemochromatosis is appropriate is not clear. Although using a higher cut-point (such as 62%)[8] increases the specificity of the test, the sensitivity decreases. We used TS as the initial screen and used a cut-point of 45% to optimize sensitivity, realizing that specificity would be lost and further confirmatory testing would be required[12]. The SF correlates better with body iron stores, but is relatively insensitive for the detection of early hemochromatosis. Several probands detected in the Utah studies[8] and in our study[12] had normal or relatively marginal elevations of SF. No study has compared SF with TS as a screening test for hemochromatosis. The College of American Pathologists recently advocated screening guidelines using the schema shown in Fig. 51.2[32]. In using TS for screening, there is inherent variability of the result and the possibility of false-positive results with consumption of iron-rich meals, iron supplements, and alcohol.

Description of the *HFE* gene raises the question of whether genetic testing might be used to screen for the predominant mutant genotype. However, several questions need to be answered before this determination can be made. It seems likely that homozygosity for the C282Y mutation results in a high likelihood of iron-loading. The significance of the H63D mutation, however, is not clear and the likelihood of iron-overload with this mutation appears to be low[33].

The appropriate place of genetic testing in the screening algorithm needs to be developed. There are three potential strategies:

(i) The least expensive would be to use TS first, with subsequent confirmation using genetic testing rather than hepatic biopsy. Persons that are divergent (i.e., TS/SF test positive but genetic test negative may still need to

undergo hepatic biopsy. The advantages of this strategy are to identify those individuals with hemochromatosis who do not have *HFE* mutations. In addition, iron deficiency would be detected. The major disadvantage would be that this strategy may fail to identify some homozygous individuals. This is supported by the 2:1 male:female ratio observed in screening studies that used TS. However, homozygosity without phenotypic expression may not be clinically important to detect.

(ii) A second approach would be to use genetic testing for the *HFE* mutations, and test positive persons on a regular basis for iron overload. If confirmed, therapy with phlebotomy would begin. This approach is more expensive than the first, and would not identify 15% of iron-loaded hemochromatosis persons. However, the sensitivity for detection of C282Y homozygosity would be higher.

(iii) Lastly, all persons could undergo TS and genetic testing to optimize detection. This combines the advantages of the two tests and increases the sensitivity of screening, and would also detect iron deficiency. However, this will increase the cost of screening and is likely to increase the false-positive rate. Further studies and decision models that incorporate genetic screening into the screening algorithm are necessary before firm recommendations about the optimal screening strategy can be made. In the meantime, it seems logical to use TS as the initial screening test in populations because its value has been most clearly demonstrated.

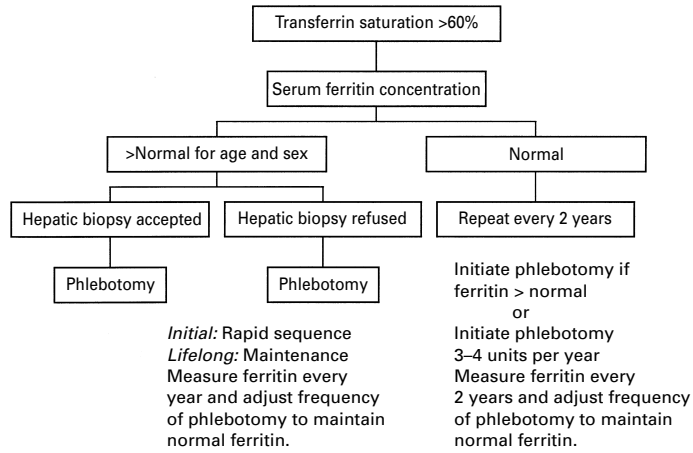

Fig. 51.2. Stepwise strategy for screening and treatment of hemochromatosis. This strategy was proposed in the practice guidelines of the College of American Pathologists[32].

Economic considerations

Economic analyses have a variety of formats and yield different conclusions. Many focus on cost; others evaluate costs and potential benefits. Cost-effectiveness analysis involves choosing an action after weighing the costs and benefits of alternative strategies, and makes explicit the alternatives that can be used and the outcomes of each. Cost-effectiveness analyses are structured around a clinical choice; in the case of hemochromatosis whether to screen for iron overload. Each alternative has a series of consequences and the probability of each consequence and its outcome are used to determine its overall utility. Each branch terminates in an outcome associated with a cost, and a utility measure such as years of life saved or quality-adjusted years of life saved. Finally, a distinction between costs and charges is made. Often charges are used in economic analysis because they are easier to determine. When costs are available, they are preferred because they represent the actual societal resources spent rather than the amount paid by the consumer[34].

Five groups have analyzed various aspects of the costs, benefits, and effectiveness of screening for hemochromatosis. No group has fully evaluated all of the economic considerations, but all add weight to the evidence that screening for hemochromatosis will improve health status. Two groups primarily assessed case-acquisition costs, whereas three conducted more complete cost-effectiveness analyses. The findings of all five are summarized below.

Balan et al. screened > 12 000 patients at the Mayo Clinic for hemochromatosis[35]. Fasting serum iron concentration was used as the initial screen. Any patient with a serum iron concentration > 180 mg/dl underwent further evaluation, including TS and SF determinations. Eight of 44 patients who had TS > 62% and SF > 400 mg/l did not have a clinical explanation for iron overload. Six of the eight cases were determined to have hemochromatosis by hepatic biopsy or mobilizable iron measurement. Costs of screening were determined by adding the costs of all laboratory tests, hepatic biopsies, and physician consultation for the eight patients. Total costs were $33 787 US for the program in which the prevalence of hemochromatosis detected was 0.33/1000, much lower than that found by others. Based on the discovery of six cases, the cost per case was $5621 US. No discussion of long-term benefits or costs of ongoing therapy were considered.

Baer et al. performed a similar analysis of patients enrolled in an HMO in the San Francisco area[36]. They screened 3977 men > 30 years old using TS. Those with a TS ≥ 62% and SF ≥ 500 mg/l were referred for hepatic biopsy.

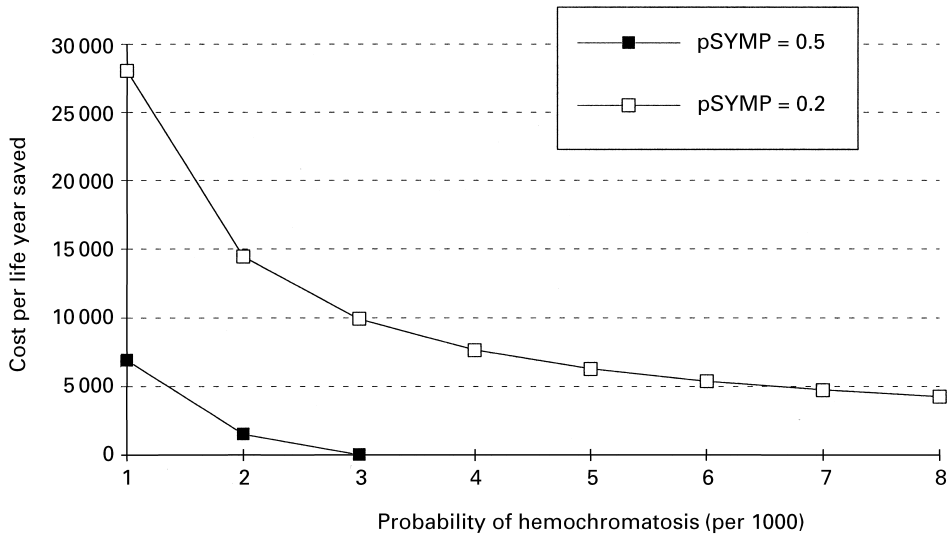

Fig. 51.3. Cost-effectiveness of screening for HH. The cost per life–year saved in thousands of dollars US is plotted against the prevalence of hemochromatosis. A TS cost of $12 US is assumed. The plots represent different probabilities of developing disease manifestations: 50% (pSYMP = 0.5, closed squares and 20% (pSYMP = 0.2, open squares). A screening strategy is dominant when the disease prevalence exceeds 0.005 and the probability of disease manifestations is 50%.

After a complete evaluation, a chart review revealed outcomes and procedures utilized to obtain resolution of the abnormal iron studies. Costs were estimated based on charges used in the San Francisco region. Forty patients had TS ≥62% and eight were subsequently determined to have hemochromatosis. Only three of eight cases were felt to be at risk for serious complications of iron overload. 172 patients had TS ≤15%, and four of these had malignancy that was under prior investigation. The cost of screening was $49.02US per patient, and ~$17000 US per case of hemochromatosis detected, if first-degree relatives are included. If only the three severe cases are included as clinically significant hemochromatosis, the cost rises to $65000 US per case detected; initial screening tests account for 83% of the costs. This study adds an important consideration: the cost of evaluating low iron states that are detected incidentally. Like the Mayo Clinic study, it only reports detection costs, and does not permit comparison to the unscreened state. Furthermore, the additional costs or savings associated with screening and the improvement in patient status are not considered. Both of these studies concluded that screening should be performed on the basis of the low cost of case-acquisition[35, 36].

Three studies have used a more complete cost-effectiveness analysis. Our group constructed a decision tree comparing a strategy of routinely screening cohorts of 30-year-old males using TS followed by fasting TS and SF, with that of awaiting the development of symptomatic disease[37]. Probabilities and costs were assigned to various outcomes and interventions based on the literature and local hospital charges. Persons homozygous for the hemochromatosis allele(s), were assumed to develop symptoms at age 50 years. It was assumed that the diagnosis would be made after a median of three years (age 53 years) after which therapeutic phlebotomy would commence. When the disorder is detected by screening at age 30 years, therapeutic phlebotomies are instituted immediately. Sensitivity analyses were conducted to test which of the variables had the most influence on the decision to screen. Four variables had a significant effect on the outcome: the prevalence rate of hemochromatosis, the probability that persons homozygous for hemochromatosis will develop disease manifestations, the cost of the screening test, and the discount rate (the discount rate adjusts for the fact that a dollar or a year in the future is not worth as much as one today). Using the base case estimates (prevalence 3/1000, probability of symptoms 50%, test cost $12 US and discount rate 3%), the decision to screen for hemochromatosis was found to be a dominant strategy, i.e., both lives and money were saved. Using more pessimistic estimates for the above variables, the cost per life-year saved varied in a range that is considered to be reasonable for other well-accepted medical screening strategies and therapeutic maneuvers (Fig. 51.3).

The analysis was limited, because it considered only complications that reduced life expectancy. The quality of

life in patients suffering these life-threatening complications and other morbidity such as hypothyroidism, arthritis and impotence were not included in this model, although inclusion of these features would make a screening strategy more attractive. Once a case is found by screening, family studies are likely to reveal further cases in a cost-effective manner. The study concluded that screening for hemochromatosis using TS should be performed on all males at the approximate age of 30 years. The optimal age for screening and the advisability of screening women could not be adequately assessed using the available data.

Adams et al. constructed a cost-effectiveness model based on a database of blood donors in Canada and applied a screening strategy initiated by measuring unsaturated iron binding capacity (UIBC)[38]. Patients with low UIBC values underwent sequential testing with TS, followed by SF. Those with abnormal results underwent hepatic biopsy or empiric phlebotomy treatments. Estimates of the prevalence of hemochromatosis, asymptomatic intervals, probabilities of life-threatening complications, life expectancy, and operating characteristics of the screening tests were based on the authors' database of 170 hemochromatosis patients and the medical literature. Males and females of all ages were evaluated, and the effect of pursuing family studies in identified cases is included in the decision structure.

This strategy led to a dominant result. Patients undergoing screening had an incremental gain of 0.84 quality-adjusted life-days at a cost savings of $3.19 US per blood donor screened. When the effect of including siblings of identified cases is considered, these values improve to 1.14 quality-adjusted life-days, and $12.57 US per person screened. In either case, the screening strategy saved both days of life and money. Sensitivity analysis identified eight important variables in the decision to screen; the prevalence of hemochromatosis, the cost of the initial screening test, the inclusion of siblings of identified cases, the probability of life-threatening complications, sex of the blood donors, sensitivity and specificity of the initial screening test, the cost of phlebotomy, and the discount rate. The screening strategy remains dominant through a wide range of values for these variables. In a population of females, the screening strategy loses dominance, but maintains a favorable value of $4082 US per quality-adjusted life-year. The authors recommended screening as a cost-effective maneuver, but raised concerns about its broad application centered on each of the variables identified in the sensitivity analysis.

Buffone and Beck performed a detailed cost-effectiveness analysis incorporating a Markov model to simulate the various health care states or conditions that can occur as a result of hemochromatosis in 25-year-old males[39]. Markov models simulate the passage of time, and as time progressed in the model, patients can remain in non-death states or die. The basic model compared three alternatives: no screening, screening with TS followed by SF, and treatment of the whole population with phlebotomy. All the branches terminated in a Markov model that included six states: no health care problems, hemochromatosis under treatment with phlebotomy, hemochromatosis without treatment and without complications, hemochromatosis with complications, death from non-hemochromatosis causes, and death from hemochromatosis. Logical transititions between these states were presumed to occur.

The combined decision analysis determined that it would cost $605 US per year of life saved to screen the target population (compared to no screening). Treating without testing would cost $>$ $31 000 000 US per year of life gained and is obviously not a viable alternative. When tested in a range considered to be clinically realistic variables including the cost of TS testing, the cost of phlebotomy, the cost of disease treatment once symptoms appeared, the prevalence of hemochromatosis, the specificity of TS, the specificity of SF, and the probability of dying from hemochromatosis, were determined to have the most influence on the result; no discounting was employed. The authors concluded that there is a reasonable likelihood that screening is cost-effective.

Should these economic evaluations be taken seriously? Recently, guidelines for evaluating economic analyses have been proposed[40]. Three basic questions need to be addressed: (i) Are the results valid? (ii) What were the results? (iii) Will the results help in patient care? These can be used to evaluate the published cost-effectiveness analyses.

Are the results valid?

First, was a full economic comparison presented? The three cost-effectiveness studies present a realistic accounting of the costs associated with either diagnosing hemochromatosis early or late, and treating it appropriately. However, none adequately account for the problem of uncovering iron deficiency and the indicated evaluation. Only one of the acquisition-cost studies considers the issues of low TS. Screening for hemochromatosis using TS will identify a large population of patients with previously unsuspected low TS. The literature on causes and prevalence of iron-deficiency anemia is extensive, but little is known about the causes and appropriate evaluation of incidentally detected iron deficiency or iron deficiency in

the absence of anemia. This may result in some potential health benefits such as detection of early colon cancer, but this will come at an additional cost. Second, were the costs and outcomes properly measured and valued? Although the studies come to similar conclusions, they do so in a variety of ways. Some used discounted costs and benefits, others did not. Charges are often employed rather than costs; thus, the true measure of resources utilized, i.e., not available for other activities, is not clear. Most authorities favor use of quality-adjusted life-years, but two of the studies did not employ this technique. Third, were appropriate allowances made for uncertainties in the model? All of the studies make clear the variables around which uncertainty exists. The true value of many clinical variables needs to be determined more accurately before the results of analyses can be applied. The most controversial variable is the likelihood that an untreated homozygous person will develop organ dysfunction. Finally, were comparisons made to baseline risk? In all of the cost-effectiveness analyses, the comparison was made to the unscreened population to determine the relative improvement over baseline.

What were the results?

Next, the actual results must be examined carefully. What were the incremental cost and outcomes for each strategy? Two of the studies showed dominance for screening, and the third could easily do so with minor changes in the values of some variables. Within the studies, did subgroups have different results? In the study by Adams et al. one subgroup (women) had a low cost, but a non-dominant result, when screening was performed. The other two studies only studied men. Lastly, how much does uncertainty change the results? By varying critical values, the results can be changed from dominant to non-dominant strategies, but generally with costs per life-year that are still acceptable in comparison to other health care interventions.

Will the results help in patient care?

Are the treatment benefits worth the risks? The results of all these studies seem to support this premise. The complications associated with the screening strategies are minimal, whereas the benefits of preventing disease seem substantial. Populations screened for hemochromatosis could experience better health care outcomes at a reasonable cost.

Based on the available economic data, screening for hemochromatosis appears to be defensible. The recent development of a genetic test will probably reduce the need for costly and invasive hepatic biopsies, thus improving the incremental gains in screening strategies further. New decision models must incorporate the genetic test. However, several important questions about the place of genetic testing in the screening algorithm need to be addressed, and the answers obtained will influence the cost-effectiveness of screening. Ultimately, the cost of genetic testing may determine whether it is used early in a screening strategy or only after iron testing. Decision models attempt to simulate real world circumstances, but many variables such as physician education, patient compliance, and the psychological and social impact of stigma are practical obstacles to screening that cannot be easily quantified in a theoretical model. The ultimate application of a general screening policy must be preceded by pilot programs and demonstration projects to identify and address the problems involved. The application of screening must be tailored by each health care organization and practice to suit their own circumstances.

Conclusions

Hemochromatosis is a common inherited disorder that is a good candidate for general population screening based on established criteria. The available analyses conclude that screening is cost-effective under a wide range of assumptions. However, answers to several questions regarding disease expression and penetrance of hemochromatosis remain to be defined. Moreover, the place of genetic testing in the screening algorithm is unclear. For now, TS is the most appropriate initial screening test, although initial genetic testing may be the superior strategy. Ongoing pilot studies and demonstration projects are needed. In the meanwhile, clinicians may consider the judicious implementation of screening for hemochromatosis in their practices.

Acknowledgment

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Part XII

Hemochromatosis: societal and ethical issues

52

Hemochromatosis: effect of iron fortification of foods

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Introduction

It has been estimated that more than one billion inhabitants of the world are anemic and that the predominant cause is iron deficiency. The prevalence of anemia varies among different geographic regions from less than 10% to more than 50%, depending mainly on the socioeconomic status of the population[1]. The prevalence also varies greatly within a population and is much higher with the increased physiological iron requirements associated with growth, menstruation, and childbearing. The term 'nutritional' has been applied to the iron deficiency identified in population surveys on the assumption that the predominant cause is inadequate iron absorption due to the low bioavailability of dietary iron.

In recent years there has been an effort to define the liabilities associated with iron deficiency more precisely and thereby assess the cost-effectiveness of various intervention strategies. The most important deficit is the impairment in cognitive performance in infants and toddlers who develop iron-deficiency anemia[2, 3]. Even a brief period of iron deficiency in persons less than two years of age can lead to lasting deficits in mental and physical performance[4]. Another significant liability is the limitation in work output in adults who become iron deficient. Studies in manual workers whose work output can be measured accurately have indicated that the repair of iron-deficiency anemia can lead to a significant increase in work productivity[5]. Finally, there is evidence that iron-deficiency anemia during pregnancy is associated with low birth weight and preterm delivery[6], although the need for routine iron supplementation during pregnancy is still debated[7]. However, these liabilities have only been demonstrated when iron deficiency is severe enough to produce anemia.

The major strategy to control iron deficiency in a population has been to increase iron intake either by providing medicinal iron (supplementation) or by increasing the iron content of the diet (fortification). Iron supplementation has had little success beyond the stage of pilot studies due to poor compliance in the targeted population. Consequently, most authorities in the field of iron nutrition advocate iron fortification as the optimal long-term approach for controlling iron deficiency. There are two broad categories of iron fortification: universal fortification, in which iron is added to a staple food vehicle with the goal of reaching all segments of the population, and targeted fortification, in which iron is added to food items that are consumed predominantly by individuals at greatest risk of iron deficiency.

Variables affecting the impact of iron fortification

The two most important variables are the bioavailability of the fortified diet and the availability of the iron added to it. Other significant variables are the consumption pattern of the food vehicle, the amount of iron added, and the iron status of the individual or population segment consuming the fortified diet (Table 52.1).

Food iron bioavailability

Radioisotopic measurements of iron absorption in human subjects have produced valuable information about the key biochemical determinants of food iron absorption. There are two discrete dietary pools of heme and nonheme iron that differ markedly in their bioavailability for absorption and thus their impact on iron balance[8]. The more bioavailable fraction is heme iron that represents 40–50% of the iron in beef and somewhat less in poultry and fish. Heme iron is absorbed as an intact porphyrin

Table 52.1. Factors affecting the efficacy of iron fortification

1. Bioavailability of added iron
2. Bioavailability of basal diet
3. Consumption pattern of vehicle for fortification
4. Increment in dietary iron
5. Iron status of population or individual

complex and is therefore unaffected by foods consumed in the same meal. Because its assimilation is threefold greater than non-heme iron, it can contribute as much as one-third of iron absorbed from the diet in industrialized countries, even though it typically represents only 10–15% of dietary iron[9].

Absorption of the large remaining fraction of non-heme food iron is highly variable and hence most investigations of food iron bioavailability have focused on this component. Studies with an extrinsic tag have demonstrated that the non-heme iron in foods consumed in the same meal form a common pool within the gastrointestinal tract[8]. Absorption of this iron is determined by the net effect of inhibitory and facilitating factors in the meal that influence absorption by affecting the solubility of iron in the lumen of the gastrointestinal tract. Although many factors have been reported to influence non-heme iron absorption[10, 11], only a few are of key importance. The major facilitators are ascorbic acid and tissue foods such as meat, fish, and poultry. The main inhibitors are phytic acid in cereals and grains, and phenolic compounds in tea, coffee, legumes, and certain vegetables.

The nutritional importance of these biochemical determinants of food iron bioavailability has been exaggerated by measuring absorption from single meals rather than from a complete diet. When diets or single meals were chosen to represent the extremes in non-heme iron absorption, mean percentage absorption from single meals varied from 2.5 to 13.5%, whereas absorption from comparable diets varied from only 3.2 to 8%[12]. Later studies have confirmed that the extremes in absorption from a complete diet are significantly less than from single meals[13, 14]. Thus, in countries where the diet is highly varied, the influence of the diet on the absorption of fortification iron is less pronounced than absorption studies with single meals would suggest.

Bioavailability of fortification iron

The most important determinant of the efficacy of iron fortification is the availability of the added iron relative to the common pool of dietary non-heme iron. Ideally, the fortification iron should be fully soluble and therefore absorbed to the same extent as the native non-heme iron in the diet. Iron (II) sulfate is the most available iron source in widespread use and is consequently used as the reference material with which the availability of other forms of fortification iron is judged. Unfortunately, highly soluble iron compounds such as ferrous sulfate frequently produce oxidative reactions with the food vehicle leading to undesirable changes in color or taste. These reactions are especially troublesome when the product is stored for extended periods in the warehouse, grocery store, or home. Organoleptic problems can be circumvented by using more inert iron compounds, but, unfortunately, these are less well absorbed.

There are significant technical difficulties in determining the relative availability of iron compounds used for fortification. The optimal approach is to label the iron compound with radioactive iron and to compare its absorption with the non-heme iron in a meal tagged extrinsically with a second radioiron tracer. The major drawback with this approach is the difficulty in labeling the commercially available iron sources that are produced on a large industrial scale. This problem has necessitated the development of animal models such as the hemoglobin repletion method in rats or in vitro methods such as solubility in dilute acid or simulated gastrointestinal digestion. When these various methods were compared with absorption measurements in human subjects, the in vitro digestion technique and the hemoglobin repletion assay gave comparable results[15]. Nevertheless, there have been some major disparities between human and animal studies. For example, carbonyl iron was used for many years in Sweden because it had high bioavailability in animal studies. However, the absorption in humans of a commercial source labeled by neutron activation absorption was only 20% that of ferrous sulfate[16]. The routine assessment of the relative bioavailability of iron compounds used for fortification is an important unsolved problem.

The first of two main categories of iron compounds used currently for fortification are the elemental iron powders: reduced iron, electrolytic iron, and carbonyl iron. These are ground mechanically to produce iron powders of varying particle size that is a key determinant of bioavailability. The relative availability of elemental iron of small particle size approaches that of iron (II) sulfate, but absorption falls progressively as the particle size increases. The second major category of commercially available iron sources is the iron salts. The more soluble forms are iron (II) sulfate, iron (II) gluconate, iron (II)

fumarate, and ferric ammonium citrate, all of which are absorbed to the same extent as dietary non-heme iron. The less soluble iron salts contain phosphate and include iron (III) orthophosphate, sodium iron pyrophosphate, and iron (III) pyrophosphate. These relatively inert forms of iron are used commonly to fortify infant foods and ready-to-eat breakfast cereals.

Food vehicles

The nature of the food vehicle used to deliver the iron is important in determining the impact on the iron status of the population. Universal fortification, the most common approach in industrialized countries, is designed to reach all segments of the population, irrespective of iron status. By adding iron to a staple food such as wheat, maize, or rice, delivery of the iron is roughly proportional to caloric intake. The addition of iron to wheat flour that was introduced in the United States in the early 1940s is sometimes referred to as enrichment, rather than fortification. This term indicates that sufficient iron is added only to restore that lost in the milling of wheat, rather than to increase the native iron content of unmilled wheat. A typical extraction rate for wheat flour in industrialized countries is about 75% that reduces the native iron content of whole wheat from 36 to 12–13 ppm iron for unenriched wheat flour. Iron (II) sulfate is the preferred iron source when iron is added at the bakery and allows storage of the product for up to three months. Elemental iron powders are used when storage requirements are longer. Other staple foods that have been used, many under experimental conditions, include common salt in India, sugar in Guatemala, and condiments such as curry powder in South Africa and fish sauce in Thailand[17].

Targeted fortification is an effective means of delivering iron to groups at high risk of developing iron deficiency. In most industrialized countries, infant milk powders and infant cereals are routinely fortified with iron. Although not strictly targeted fortification, breakfast foods are usually fortified with iron and other minerals and vitamins. Several ready-to-eat breakfast cereals contain 50% or more of the Recommended Dietary Allowance (RDA) for iron in a single one ounce serving. In some cases, this large quantity of iron is required for certain foods to be eligible for purchase by food vouchers distributed in the US Department of Agriculture Special Supplement Food Program for Women, Infants and Children. Because the nature, amount and type of iron used to fortify specialty food products are constantly changing, it is difficult, if not impossible, to assess their impact on iron status.

Iron status

Body iron levels are tightly regulated in normal subjects by adjusting the absorption rate of dietary iron from the gastrointestinal tract. Consequently, there is a highly predictable inverse relationship between the absorption of non-heme iron from a particular meal or diet and the level of storage iron as measured indirectly by the serum ferritin concentration. Within the normal concentration range of 10–200 μg/l serum ferritin, there is a precise inverse relationship between log percentage absorption and log serum ferritin with a slope of unity[12, 18]. For example, percentage absorption is halved when the serum ferritin doubles. Absorption studies using a standard hamburger meal have demonstrated that when a representative serum ferritin concentration of 100 μg/l for an adult male falls to 10 μg/l with the development of storage iron deficiency, non-heme iron absorption increases from 2.5 to 22%[19]. This tenfold difference in the absorption of both non-heme and fortification iron indicates that the basal iron status of an individual is of primary importance in predicting the effect of iron fortification. However, little is known about the extent of the further increase in absorption when iron deficiency becomes more severe, or further reduction when the serum ferritin rises above 200 μg/l with expansion of body iron stores.

Amount of fortification iron

As the iron content of the meal increases, percentage absorption falls while the absolute amount of absorbed iron increases[8]. With universal fortification that typically increases dietary iron content by 10–20%, the effect on percentage absorption of the added iron is small, but becomes significant when specialty foods are heavily fortified with iron. In a detailed study by Layrisse and co-workers, from 0.5 to 60 mg iron as iron (II) chloride was added to three types of meals containing 2–3 mg dietary iron: maize alone, meat alone, and a mixture of the two foods[20]. The relative decrease in percentage absorption was similar for all three meals: a three-fold increase in iron content of the meal reducing basal percentage absorption by roughly 50%. The relative increase in absorbed iron as more iron was added was similar for the three meals, but the amount differed dramatically depending on the type of meal. For example, regression analysis indicated that as the non-heme iron content of the meal was increased from 5 to 10 mg, only 0.04 mg additional iron was absorbed from the maize meal as compared to 0.16 mg from the meat/maize meal and 0.60 mg from the meat meal. In a similar study reported by Bezowoda[21], percentage absorption of non-heme iron fell

from 18 to 6% when non-heme iron was increased from 1.5 to 6 mg by fortification. These studies indicate that with heavy fortification, one cannot use percentage absorption values from the unfortified diet to predict the impact of fortification.

Effect of iron fortification on iron deficiency

Definitions of iron deficiency

The prevalence of iron deficiency depends on the criteria used to define it. It is common practice to define two levels of severity depending on the presence or absence of anemia. The milder form, often called iron deficiency without anemia or iron-deficient erythropoiesis, will be referred to in this review simply as iron deficiency. The earliest laboratory evidence of iron deficiency is a serum ferritin concentration ≤ 12 μg/l that reflects the exhaustion of iron stores. Most investigators require additional laboratory criteria to identify iron deficiency such as a low serum iron concentration, low transferrin saturation, elevated red cell distribution width, low mean corpuscular volume, or elevated free erythrocyte protoporphyrin (FEP). For example, in the National Health and Nutrition Examination Survey (NHANES III, 1988–1994), the definition of iron deficiency was based on three laboratory tests of iron status: transferrin saturation, FEP and serum ferritin concentration[22]. An individual was considered to be iron-deficient if two or more of these measurements were abnormal. However, these criteria are not specific for iron deficiency, because the combination of a low transferrin saturation and elevated FEP, with or without anemia, also occurs in chronic inflammation.

In assessing the impact of iron fortification, it is preferable to use the prevalence of iron-deficiency anemia, because liabilities due to iron deficiency have rarely, if ever, been detected in the absence of anemia. Most authorities still employ the well-established WHO criteria of anemia: a hemoglobin concentration <130 g/l in men, <120 g/l in women, and <110 g/l during pregnancy. In NHANES III, age and sex-specific hemoglobin criteria of anemia were derived from the survey population after excluding all individuals with an abnormal laboratory measurement of iron status. Because there are many causes of anemia other than iron deficiency that can be detected in population surveys, additional laboratory evidence is required to identify iron-deficiency anemia. A low serum ferritin concentration in the presence of anemia is a reliable definition, but additional laboratory criteria are often included due to the tendency of mild inflammation to elevate the serum ferritin concentration falsely. In the NHANES III survey, iron-deficiency anemia was defined as anemia combined with at least two abnormal iron measurements as listed above.

Recent studies indicate that the serum transferrin receptor will be useful in assessing the prevalence of iron-deficiency anemia[23, 24]. The concentration of serum transferrin receptor is roughly proportional to the total body mass of tissue receptor and is consequently elevated with either enhanced erythropoeisis or tissue iron deficiency. Serial phlebotomies in normal subjects have shown that as body iron declines, the ratio of the transferrin receptor/serum ferritin concentration measures the change quantitatively over a wide spectrum of iron status[25]. The combined use of hemoglobin concentration, serum ferritin concentration, and serum transferrin receptor will greatly enhance the sensitivity of future prevalence surveys by providing a quantitative measure of iron status in each sampled individual. An important additional advantage of using the serum transferrin receptor is that it is not affected by chronic inflammation and can therefore distinguish iron-deficiency anemia from the anemia of chronic disease and even identify iron-deficiency anemia when it occurs in individuals with chronic inflammation or infection[26].

Efficacy of targeted iron fortification

One of the important advances in iron nutrition in many industrialized countries has been the virtual elimination of iron deficiency in infants and preschool children by means of targeted fortification. This occurred in the United States in response to recommendations by the Committee on Nutrition of the American Academy of Pediatrics published originally in 1969. It is presently recommended that all formula-fed infants should receive an iron-fortified formula until 12 months of age, and that breast-fed infants should be given an iron-fortified formula if weaned before 12 months. Iron-fortified infant cereal is recommended when infants begin solid foods. These recommendations have been widely applied in the United States and undoubtedly account for the dramatic reduction in the prevalence of iron-deficiency anemia in this highly susceptible age group. In one report, anemia in middle-class children surveyed between 1982 and 1987 had been virtually eliminated during the prior decade[27]. These findings leave little doubt about the effectiveness of delivering adequate amounts of fortification iron to a population in greatest need.

Table 52.2. Comparison of non-heme and heme iron absorption in hemochromatosis with predicted absorption in normal subjects of comparable iron status

				Iron absorption					
		Serum ferritin, μg/l		Non-heme, %			Heme, %		
Treatment status	N	Mean	Range	Hemochromatosis	Normal	Relative increase	Hemochromatosis	Normal	Relative increase
Treated	5	14	10–21	42	16	2.6	39	42	1.1
Partial	3	538	(210–786)	12	0.5	24	50	26	1.9
Untreated	7	>1000		9	0.2	45	41	9	4.6

Effect of universal fortification

Many of the existing programs of universal iron fortification in which iron is supplied to the entire population were introduced in the early 1940s with little or no subsequent change in the quantity, if not the quality, of the added iron. It is impossible to assess the effect of these programs because reliable methods for evaluating iron status of a population were not available at the time they were introduced. One possible exception is the fortification of wheat flour in Sweden where there has been a stepwise increase in the level of added iron from 30 ppm in 1944 to 50 ppm in 1963 and to 65 ppm since 1970. Hallberg used the reduction in the prevalence of anemia in menstruating women during the 1960s to estimate the relative contribution of the enhanced level of iron fortification during the same period of time[28]. He assumed that the bioavailability of the carbonyl iron used for fortification was approximately 50% that of non-heme dietary iron. He also estimated that in iron-deficient women, the increment in fortification produced an increase in absorbed iron from 1.7 to 1.9 mg, or 0.2 mg daily. Anemia prevalence in women over this period decreased from 30 to 7%, but only one-third of this reduction was believed to be due to the increase in fortification iron. The remaining 22% decline in anemia prevalence was attributed to increased use of iron supplements (10%), wider use of birth control pills (3%), and increased intake of ascorbic acid supplements. These calculations are of interest in assessing the impact of universal fortification of wheat flour, but underscore the difficulty in defining precisely the relative contribution of factors that can affect the iron status of a population and often vary over time: caloric intake, dietary iron bioavailability, and use of iron supplements and medications that influence iron losses such as aspirin and birth control pills.

The recent introduction of universal fortification in Venezuela provides valuable information about its potential impact on the prevalence of iron deficiency[29]. A severe economic crisis in this country beginning in the mid-1980s led to a progressive decline in the quality and quantity of food consumed by the lower socioeconomic strata of the population. Surveys from 1989–1990 and in 1992 in children 7 to 15 years of age showed a marked increase in the prevalence of iron deficiency (serum ferritin <12 μg/l) from 13.5 to 30.5%, of anemia from 5.6 to 13.2%, and of iron-deficiency anemia (anemia and serum ferritin <12 μg/l) from 2.0 to 5.5%. As in other surveys, only about one-third of the anemia could be attributed to iron deficiency although iron deficiency, and co-existing inflammation presumably explained most of the remaining cases of anemia.

This rapid deterioration in iron status prompted the introduction of a national fortification program in 1993. All precooked maize flour was fortified with 50 ppm iron as ferrous fumarate and all wheat flour with 20 ppm iron using the same iron compound. These two staple foods represented 45% of the energy consumed daily by persons in the low socioeconomic strata. The fortification program was estimated to have increased the mean total per capita iron intake from 12.1 to 17.5 mg daily, or by approximately 30%. The organoleptic problems were negligible despite the use of a form of iron that is roughly equivalent to ferrous sulfate in its relative bioavailability. A limited survey of 5–15-year-old children living in Caracas showed a reduction between 1992 and 1994 in the prevalence of iron-deficiency from 36.0 to 15.8%, of anemia from 19.0 to 9.3% and of iron-deficiency anemia from 6.3 to 4.6%. These preliminary findings provide convincing evidence that a well-designed national fortification program can have a major impact on the prevalence of iron deficiency. Follow-up data on changes in iron status in other segments of this population including iron-replete adults should answer important questions regarding the efficacy and safety of national programs of iron fortification.

In judging the pros and cons of continuing mandatory fortification of wheat flour and bakery products in the United States, it is relevant to examine current estimates of iron deficiency prevalence. Data on the prevalence of iron deficiency based on NHANES III (1988–1994) has recently been published[22]. A total of 24 894 persons aged 1 year and older were examined using a national, stratified, probability sample of the civilian population. As discussed previously, the criteria used to define iron deficiency were developed for NHANES II (1976–1980) on the assumption that the more laboratory indices used to define iron deficiency, the more reliable is the estimate. Unfortunately, nearly all the cutoff values for laboratory measurements of iron status used in NHANES II were modified for the NHANES III study that limited the ability to assess temporal changes in iron status. Furthermore, the twentieth rather than the customary fifth percentile was used in many instances due to concern by the authors about missing individuals with iron deficiency. Finally, certain criteria of iron-deficiency anemia did not distinguish it from the anemia of chronic inflammation. The prevalence of iron-deficiency anemia was $<1\%$ in children 3–11 years of age, and all males under 70 years of age. Even in women 20–49 years of age, the prevalence was $<5\%$ in the total sample and 4% in non-blacks. Many of these estimated prevalence rates of iron-deficiency anemia appear lower than the statistical threshold of detection, and in all age and sex categories, the prevalence rates were at or below those observed in NHANES II. Consequently, the most dramatic change in iron status between NHANES III and prior NHANES is a marked rise in the proportion of adult men with an abnormally high serum ferritin concentration[30].

Experimental iron fortification

There has been a long-standing interest in the use of sodium iron ethylenediamenetetracetic acid (EDTA) a multidentate chelating agent that satisfies most, if not all, of the desirable attributes of a fortifying agent. The complex is highly stable and is therefore particularly useful for fortifying foods that require prolonged storage or are prepared at high temperature. The most important property of iron EDTA is its unique absorption characteristics when given with food. With a meal that strongly inhibits non-heme iron absorption, iron EDTA is absorbed 2–3 times more efficiently than iron (II) sulfate[31]. Importantly, it also enhances absorption of the non-heme iron consumed in the same meal. Mean absorption of iron EDTA in iron-deficient individuals typically exceeds 10%, regardless of the nature of the diet. Iron EDTA has little effect when added to highly bioavailable meals containing meat and/or ascorbic acid and can actually inhibit absorption from such meals.

The efficacy of fortification using iron EDTA has been examined in several large field trials. When iron EDTA was added to sugar in two Guatemalan communities in amounts that increased iron intake by 4.3 mg iron/person/day for 20 months, a significant improvement in iron status was observed[32] In another trial, sufficient iron EDTA was added to a curry powder in an Indian community in South Africa to provide an additional 7.7 mg iron/person/day. The prevalence of iron deficiency dropped from 22 to 4% in women who consumed the fortified product for 24 months[33]. These trials are of interest because they define the efficacy of iron fortification under carefully controlled conditions and with reliable laboratory indices of iron status.

Effect of iron fortification in hereditary hemochromatosis

Iron absorption in hereditary hemochromatosis

The fundamental disturbance in iron metabolism in hemochromatosis is an excessive absorption of dietary iron. Estimates of the latter were originally based on the rate of reaccumulation of iron stores in patients who had been rendered iron-deficient by repeated phlebotomy. Typical values based usually on individual case reports ranged between 3 and 5 mg of iron daily, although values as high as 8–10 mg daily were sometimes reported[34,35]. The advent of serum ferritin measurements for monitoring iron stores increased the reliability of this approach, and has the advantage that it integrates the rate of total iron absorption from the diet over an extended period of time. On the other hand, it lacks the precision of radioactive measurements of iron absorption.

In initial reports of iron absorption in hemochromatosis, test doses of inorganic iron were used that do not portray iron absorption from the diet accurately. More reliable estimates awaited the development of extrinsic radioiron tagging for measurements of heme and non-heme dietary iron. This technology was used in a key investigation of the impact of iron fortification in hemochromatosis reported by Bezwoda and colleagues[36]. They performed absorption studies in seven treated patients, all of whom had serum ferritin concentrations <33 μg/l. The basal test meal contained maize porridge, milk and orange juice and a total of 1.2 mg non-heme iron. Non-heme iron absorption was measured by extrinsic radioiron tagging in the same subjects after adding first 5 and then 10 mg additional iron as

ferric chloride. Percentage absorption averaged 25.2% from the meal containing 6.2 mg iron, and 19.7% from the meal containing 11.2 mg iron. Thus, a doubling of iron fortification produced a 43% increase in absorbed iron from 1.56 to 2.2 mg, similar to that reported earlier in normal subjects by Layrisse and co-workers[20]. The South African investigators concluded that the rate of iron accumulation in subjects with hemochromatosis is faster with iron fortification, and that clinical manifestations occur at a younger age. However, the ultimate size of the body iron burden is not necessarily higher.

In a later study, the relationship between iron status and food iron absorption was evaluated in normal subjects and 15 patients with hemochromatosis who had a wide range of iron status[37]. Double extrinsic radioiron tags were used to measure iron absorption separately from a standard hamburger meal containing 1.4 mg heme, and from a 3.4 mg non-heme iron. Consistent with many prior studies, a highly significant, inverse correlation was observed in normal subjects between iron stores measured by the serum ferritin concentration and the absorption of both heme and non-heme iron. The absorption of heme iron was much higher than non-heme iron over a wide range of iron stores, and it was also less inhibited than non-heme iron at higher levels of storage iron resulting in a three-fold difference in regression slopes. Using serum ferritin concentrations of 100 μg/l, 30 μg/l and 10 μg/l as representative values for normal adult males, females, and iron-deficient subjects, respectively, estimated absorption was 2.5%, 7.7% and 21.5% for non-heme and 21%, 32% and 47% for heme iron, respectively. Thus, non-heme iron contributed approximately 20% of absorbed iron in normal men and 50% in iron-deficient individuals.

The serum ferritin concentration varied widely in the 15 patients with hemochromatosis, from 10 μg/l to nearly 5000 μg/l. A significant inverse correlation between non-heme iron absorption and serum ferritin concentration was observed, but the decrease in absorption for a given increase in serum ferritin concentration was far less than in normal subjects. No correlation was observed between serum ferritin concentration and heme iron absorption. Because absorption of heme and non-heme iron can be estimated in normal subjects with different serum ferritin concentrations, it is possible to compare absorption in normal individuals and patients with hereditary hemochromatosis(Table 52.2). In five treated patients with a mean serum ferritin of 14 μg/l (range 10–21 μg/l), non-heme iron absorption averaged 42% as compared with 16% in normal subjects with the same ferritin concentration, giving a relative increase in absorption in hemochromatosis of 2.6 times normal. In three patients with a moderate increase in iron stores (mean serum ferritin 538 μg/l; range 210–786 μg/l), mean non-heme was 12% as compared to a predicted value of 0.5% in normal subjects, a 24-fold relative excess in absorption. In seven untreated patients with serum ferritin concentrations $>1000\,\mu g/l$, mean absorption of non-heme iron fell to only 9% as compared to 0.2% in normal subjects, a 45-fold relative difference. The disparity in the absorption of heme iron was much less. In phlebotomized patients, heme iron absorption was similar to normal subjects, but did not fall significantly in untreated patients with hemochromatosis, remaining 4.6 times greater than normal individuals. These observations indicate that one of the characteristic disturbances in patients with hemochromatosis is the inability to reduce iron absorption as iron stores expand.

Models for predicting the impact of iron fortification in hemochromatosis

A series of reports has been published by Bothwell and colleagues in which detailed calculations have been made about the potential impact of iron fortification on patients with hemochromatosis. The initial report was published in response to concerns about the safety of iron fortification[38] in the early 1970s when it was proposed to increase the level of iron fortification of wheat flour in the United States from 7 to 18 mg/kg. It was estimated that this would increase daily iron intake in adult men and women by approximately 20%. The authors assumed that the relative decrease in iron absorption in hemochromatosis with expansion of iron stores is similar to that in normal subjects, but that the absorption rate is set at a much higher level. It was assumed that in a patient with hemochromatosis who absorbs 4 mg dietary iron daily early in the disease when iron stores are normal, 18 g of body iron would accumulate after 40 years. It was estimated that if fortification increased the daily iron intake by 20 %, the same degree of body iron excess would develop approximately 10 years sooner.

These predictions are entirely consistent with the absorption data reported by Lynch et al.[37] at various stages of iron-loading in hemochromatosis (Table 52.2). Because there is recent evidence that fecal ferritin losses are not increased in hemochromatosis[39], it is more appropriate to assume a normal iron excretion of 1 mg daily in patients with hemochromatosis, rather than a higher excretion of 1.5 mg assumed in the prior model. Based on a daily iron intake from an unfortified diet of 14 mg/day, of which 2 mg is heme iron, the initial rate of iron-loading in hemochromatosis is 4.8 mg non-heme iron ($12\,mg \times 0.40$) and 0.8 mg heme iron ($2\,mg \times 0.39$), resulting in a net accumulation of

4.6 mg iron daily. Wheat fortification at the current level increases non-heme iron intake to 15 mg daily, resulting in a net accumulation of 5.8 mg iron daily. Thus, fortification initially accelerates the rate of iron loading from 1.7 to 2.1 g iron annually, a difference of over 0.4 g. If these same calculations are applied to the untreated patients with serum ferritin concentrations >1000 μg/l (Table 52.2), the difference in absorption between an unfortified and fortified diet falls to ~0.3 mg daily or 100 mg annually. These estimates agree with the earlier predictions[36, 38] that the greatest impact of iron fortification is at the early stages of the disease, and that the accelerated evolution of clinical disease is directly proportional to the amount of fortification iron added. If dietary iron intake is increased by 20% with fortification, the time required to develop clinical manifestations is likewise shortened by 20%.

Another influence of iron fortification is its effect on the frequency of maintenance phlebotomies required to maintain low iron reserves in fully treated patients. With a typical phlebotomy rate of 4–6 times annually, a 20% increase in dietary iron by fortification of wheat flour would necessitate 1–2 additional bleedings per year. On the other hand, the regular consumption of a breakfast cereal containing one RDA for iron per serving could double the rate of maintenance phlebotomies. This concern is obviously less than the accelerated rate of iron accumulation in the undiagnosed patient with hemochromatosis.

Summary and conclusions

The purpose of iron fortification is to reduce the prevalence of iron-deficiency anemia. Lesser degrees of iron lack are not relevant because no liabilities resulting from iron deficiency have been demonstrated in the absence of anemia. The recent NHANES III in the United States where 20% of dietary iron intake is derived from the fortification of wheat flour indicates that iron-deficiency anemia has been essentially eliminated except in toddlers (3%) and women of the child-bearing age (5%). The extent to which several decades of iron fortification have contributed to this success is uncertain, but many other factors such as the widespread use of iron supplements and improved bioavailabity of dietary iron undoubtedly have played an equally important role. It is unlikely that any further increase in the level of iron fortification will influence the prevalence of iron-deficiency anemia in segments of the population where it persists. Any further reduction in iron-deficiency anemia in toddlers is better achieved by modifying and/or enhancing the fortification of infant foods. The residual iron-deficiency anemia in menstruating women is due primarily due to excessive menstrual blood loss and could be controlled more effectively by iron supplementation or targeting fortified foods to susceptible individuals.

The rationale for continuing universal fortification in countries like the United States where there is a high frequency of the homozygous state for hemochromatosis must be seriously questioned. With the use of more bioavailable iron sources for wheat fortification in recent years, it is likely that most of the added iron is fully available for absorption. Consequently, the accelerated rate of iron-loading in homozygous individuals is directly proportional to the increase of dietary iron intake resulting from fortification. Absorption studies in hemochromatosis patients with varying degrees of iron excess indicate that the onset of clinical manifestations in a patient who presents with the disease at 50 years of age would be delayed by at least a decade if wheat fortification with iron were discontinued. This would not only result in a meaningful extension of lifespan in homozygous individuals, but also reduce morbidity and health care costs significantly. An even more important potential benefit would be a reduction of the proportion of homozygous individuals who develop disease manifestations. Continued iron fortification can be justified only if there is an early implementation of a screening program of the entire population to detect individuals with hemochromatosis prior to the development of irreversible tissue damage.

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Evidence for population-based testing for hemochromatosis[a]

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Introduction

As many as 1 million persons in the United States are affected by hemochromatosis, a genetic condition characterized by excess iron absorption and pathologic iron deposition in tissue[1]. If undetected and untreated, hemochromatosis can result in illness (such as cirrhosis, hepatoma, diabetes, cardiomyopathy, arthritis, arthropathy, and hypopituitarism with hypogonadism) and death. The identification and treatment of asymptomatic persons in whom iron measures are elevated but hemochromatosis is not clinically apparent have been recommended as a potentially cost-effective strategy for preventing hemochromatosis-associated illness and death[1–10]. Nonetheless, some experts argue that before universal screening can be recommended, the clinical expression and natural history of hemochromatosis must be clarified and the infrastructure necessary to support a universal screening program (including laboratory standardization and physician education) must be established[11]. The recent discovery of a gene[12, 13] associated with hemochromatosis has made it possible to use DNA testing along with, or instead of, iron measures in screening. Although this discovery has increased interest in hemochromatosis, it has also raised new questions about screening for and diagnosis of the disease. One objective of the meeting on Iron Overload, Public Health, and Genetics, sponsored by the Centers for Disease Control and Prevention and the National Institutes of Health in March 1997[14], was to review the scientific information available on population screening for hemochromatosis. Our assessment of the evidence and recommendations for action are presented here.

[a] This work originally appeared in the *Annals of Internal Medicine* 1998;129:971–9, and is reproduced here by permission.

Methods for evaluating the evidence for population screening for hemochromatosis

US Preventive Services Task Force criteria were used[15] to evaluate evidence related to population screening for hemochromatosis that was presented at the meeting on Iron Overload, Public Health and Genetics or was published before August 1997. In this chapter, six assumptions are examined that have been used to support the case for population screening for hemochromatosis in the United States.

(i) Prevalence: hemochromatosis is relatively common.
(ii) Burden of suffering: hemochromatosis is associated with significant morbidity and mortality.
(iii) Efficacy of treatment: phlebotomy can prevent complications by reducing the iron burden in persons with hemochromatosis.
(iv) Accuracy of screening tests: the available screening tests detect hemochromatosis earlier than no screening and do so with sufficient accuracy.
(v) Effectiveness of screening: screening and early treatment of persons with hemochromatosis improves health compared with treatment of patients after the development of clinical signs and symptoms.
(vi) Safety and adverse effects: the potential benefits of screening and treatment outweigh the adverse effects.

The studies of effectiveness that we reviewed were each assigned one of five grades of evidence on the basis of study design (Table 53.1).

Prevalence

Determination of the prevalence of hemochromatosis is complicated by variation in case definitions of the disease and by uncertainty about the progression from genetic

Table 53.1. US Preventive services task force criteria for judging quality of evidence[a]

Grade	Study design
I	Randomized, controlled trials
II-1	Controlled trials without randomization
II-2	Cohort and case-control studies (usually more than one)
II-3	Multiple time series and dramatic uncontrolled experiments
III	Opinions of respected authorities, descriptive studies, case series, and case reports

Note:
[a] Adapted from US Preventive Services Task Force [15].

susceptibility through iron overload to clinical disease. Case definitions of hemochromatosis may include one or more of the following: genetic mutations, abnormal iron measures, and clinical signs and symptoms[16]. It is difficult to reach agreement on a standard because each case definition has strengths and weaknesses.

Clinical case definitions have two chief weaknesses. First, such definitions (for example, bronze diabetes and cirrhosis) often represent the end stages of disease and have limited usefulness for prevention. Second, many clinical signs and symptoms that occur early in the course of hemochromatosis are nonspecific (for example, fatigue, abdominal pain, joint pain, and elevated liver enzyme concentrations) and may be attributed to other causes. Case definitions more specific to hemochromatosis, such as persistently elevated serum transferrin saturation or liver iron deposition without cirrhosis, allow for the detection of hemochromatosis before clinical signs and symptoms occur, but the rate and degree of progression from abnormal iron measures to clinical symptoms are uncertain. Even more uncertainty exists about progression from genetic susceptibility to clinical disease.

Although the autosomal recessive nature of hemochromatosis and its link to the HLA region on chromosome 6 have been recognized for 20 years[17], two mutations associated with hemochromatosis – C282Y and H63D – were found only in 1996[12]. Several investigators have estimated the frequency of the mutations in small groups of persons without clinical evidence of hemochromatosis (45 to 381 persons)[12, 18–24]. In the largest study published to date[25], the prevalence of homozygosity for C282Y was 1 in 1000, the prevalence of heterozygosity for both C282Y and H63D (compound heterozygosity) was 16 in 1000, and the prevalence of homozygosity for H63D was 20 in 1000 among 1450 persons from northern Europe. None of the study participants from other regions (Africa, Asia, and Australia) carried two C282Y mutations. These studies[12, 19–25] were drawn from convenience samples, and none was designed to represent the general population. Therefore, these studies may overestimate or underestimate the prevalence of genetic susceptibility to hemochromatosis.

On the basis of case definitions that use elevated iron measures (such as body iron stores) in screening studies, the prevalence of hemochromatosis is 2 to 5 per 1000 persons in white populations[11, 26–30]. The estimated prevalence of hemochromatosis in black populations is lower, less than 1 in 1000[1, 26, 31, 32]. These estimates are higher than the estimated prevalence of homozygosity for the major mutation, C282Y, but they are lower than the prevalence of compound heterozygosity or homozygosity for H63D. This discrepancy suggests either the presence of as yet undiscovered mutations for hemochromatosis or reduced penetrance of compound heterozygosity and homozygosity for H63D.

Burden of suffering

The prevalence of clinical disease due to hemochromatosis is uncertain. Hemochromatosis can lead to cirrhosis and other liver diseases, hepatocellular carcinoma, diabetes, cardiomyopathy, arthritis, hypopituitary hypogonadism, fatigue, joint pain, skin bronzing or graying, abdominal pain, impotence, amenorrhea, and cardiac arrhythmias[1]. The most common early symptom is weakness or fatigue[1]. Although diabetes and heart disease occur more often in cirrhotic patients, they are also seen in patients with hemochromatosis who do not have liver disease[33, 34]. The classic triad of liver disease (cirrhotic or noncirrhotic), diabetes, and skin bronzing occurs in a minority of patients (for example, 17% of patients in one case series[33] and 3% of patients identified through screening studies[11]. Deaths in persons with hemochromatosis are most often associated with liver disease, hepatocellular carcinoma, diabetes, or cardiomyopathy.

Morbidity

Estimates of morbidity have usually been derived from case series of patients with known hemochromatosis[33–35]. The proportion of patients with hemochromatosis who have associated illness is probably greater in case series than in the general population because a disproportionate number of patients in case series may be detected because of their symptoms (selection bias). A review of family based screening studies was done to address this limitation[36]. In

that review, 52% of 146 family members 15 to 72 years of age in whom hemochromatosis had been diagnosed by HLA haplotyping were asymptomatic. The other 48% had at least one clinical manifestation of disease, such as cirrhosis, other liver disease, diabetes, cardiomyopathy, arthropathy, skin bronzing, fatigue, weight loss, abdominal pain, or impotence. In addition, the risk for symptoms associated with hemochromatosis increased with age – 73% of men and 44% of women older than 40 years of age had at least one clinical finding.

Siblings and other family members identified through HLA testing may have a different risk for disease expression than do persons with hemochromatosis in the general population Among persons with hemochromatosis (defined by elevated iron measures[16]) in population screening studies, 45% of men and 43% of women older than 40 years of age had at least one clinical finding[11]. Screening studies have not compared the prevalence of clinical findings in persons with hemochromatosis and persons without hemochromatosis. Many clinical findings associated with hemochromatosis (such as abdominal pain, fatigue, arthritis, and diabetes) are also common in persons without hemochromatosis. Therefore, some of the illness attributed to hemochromatosis may be due to other causes. The studies with the least biased methods to date[37–51] have estimated the proportion of persons with selected clinical conditions who have underlying hemochromatosis (Table 53.2). The prevalence of hemochromatosis ranges from 11.0% to 15.0% in patients with hepatocellular carcinoma to 0.0% to 1.5% in patients with diabetes. If the population prevalence is assumed to be 0.2% to 0.5%[11, 26, 27], these studies suggest that the risk for hemochromatosis may be elevated in persons with hepatocellular carcinoma, liver disease, hepatitis, cardiac arrhythmias, arthropathy, and diabetes. The risk for hemochromatosis may be further elevated in persons with combinations of these diseases (for example, diabetes and liver disease), as was suggested by an analysis of death certificates[52].

In the studies listed in Table 53.2, with the exception of one recent case-control study[44], lack of appropriate comparison samples (for example, a sample matched for age and sex) hampers estimation of the proportion of clinical disease resulting from hemochromatosis. If it is assumed that the odds ratio is a close approximation of the risk for diabetes in persons with hemochromatosis compared with persons without hemochromatosis, then persons with hemochromatosis are 7.3 times (95% CI, 1.3 to 41.9 times) more likely than persons without hemochromatosis to develop diabetes. If the prevalence of hemochromatosis in the general population is 0.3%, the estimated proportion of the general population in which type 2 diabetes is due to hemochromatosis is 1.9%. These compelling results deserve further study in other populations. In general, heterogeneity in sample selection, population characteristics (such as age, sex, and ethnicity), and case definitions of hemochromatosis hamper comparison across studies of morbidity (Table 53.2). Precise estimation of the actual elevation in risk for chronic disease from hemochromatosis will require further study that addresses the limitations described.

Mortality

Compared with the estimated prevalence of hemochromatosis (2 to 5 per 1000) in the United States, the rate of 1.8 deaths from hemochromatosis per 1 million persons in the United States in 1992[52], obtained from death certificates, suggests that hemochromatosis is underdiagnosed or underreported or that the risk for death from hemochromatosis is low. Until recently, case detection of hemochromatosis relied on a strict clinical case definition[1, 11, 53], possibly biasing mortality estimates[52, 54]. Results of screening studies (Table 53.2) indicate that hemochromatosis is unlikely to be recognized in patients who have diabetes or liver disease alone; most patients in these studies received new diagnoses of hemochromatosis. Further studies of disease burden should estimate the risk for chronic disabling conditions and death resulting from hemochromatosis.

Efficacy of treatment

Iron overload is treated by removing excess body iron through therapeutic phlebotomy[55, 56]. The efficacy of therapeutic phlebotomy in reducing clinical disease both before and after the onset of clinical signs and symptoms is of interest. For ethical reasons (removing excess iron is the accepted treatment for hemochromatosis), no randomized, controlled trials comparing therapeutic phlebotomy with no treatment have been done[33, 35]. Until recently, no animal models for hemochromatosis were available, but a new mouse model of hereditary hemochromatosis[57] may provide opportunities to evaluate therapeutic strategies for the prevention or correction of iron overload. In this review, evidence is given from human studies only.

Results from one retrospective cohort study and a series of descriptive studies indicate that therapeutic phlebotomy decreases body iron stores and improves survival among patients with hemochromatosis who have clinical disease. In the retrospective cohort study[58], the average

Table 53.2. Estimated prevalence of elevated iron status and iron overload due to hemochromatosis among patients with chronic diseases

Chronic disease	Country of study [Ref]	Population characteristics	Patients	Patients with elevated iron status[a]	Patients with iron overload[b]
			n	*n* (%)	*n* (%)
Hepatocellular carcinoma	France[37]	In-patients who had surgical resection of hepatocarcinoma	60	—	9 (15.0)
	United States[38]	Inpatients with hepatocarcinoma identified through a hospital tumor registry	314	—	33 (10.5)
Cirrhosis	Italy[39]	Population-based cohort study	78	—	1 (1.3)[c]
Chronic liver disease	Norway[40]	Consecutively admitted inpatients with liver diseases	312	37 (11.9)[d]	18 (5.8)
Hepatitis C	Italy[41]	Patients with chronic hepatitis C (86%) or hepatitis B (14%)	98	—	9 (9.2) 4 (4.1)[e]
Peripheral arthropathy	Australia[42]	Out-patients with peripheral arthropathy, including those with previously diagnosed HH[f]	339	8 (2.4)	5 (1.5)
		Out-patients with peripheral arthropathy, excluding those with previously diagnosed HH	338	7 (2.1)	4 (1.2)
Bradyarrhythmias	Sweden[43]	Male patients with pacemakers and sinus node disease or symptomatic atrioventricular block	232	6 (2.6)[g]	3 (1.3)[e]
		Male patients with pacemakers and symptomatic atrioventricular block	149	—	3 (2.0)[e]
Diabetes	Italy[44]	Out-patients with diabetes	894	15 (1.7)[h]	12 (1.3)
		Out-patients with type 2 diabetes	777		12 (1.5)
		Out-patients with dyspepsia or irritable bowel syndrome, matched by sex and age to diabetes outpatients	467	2 (0.4)[h]	1 (0.2)
	Scotland[45]	Out-patients with diabetes, including those with previous diagnosis of HH	1194	10 (0.08)	5 (0.4)
		Out-patients with diabetes, excluding those with previous diagnosis of HH	1192	8 (0.7)	3 (0.3)
	Italy[46]	Out-patients with diabetes (86% with type 2 diabetes)	530	—	7 (1.3)
	United States[47]	Consecutive outpatients with type 2 diabetes, excluding those with other possible causes of elevated serum ferritin level	102	0 (0)[g]	—
	England[48]	Consecutive white out-patients with diabetes who were older than 18 years of age	406	4 (0.9) 2 (0.5)[i]	—
	Hungary[49]	Out-patients with diabetes who were older than 30 years of age	268		2 (0.7)[j]
		Healthy blood donors	95	—	0 (0)

Table 53.2. (*cont.*)

Chronic disease	Country of study [Ref]	Population characteristics	Patients	Patients with elevated iron status[a]	Patients with iron overload[b]
	Ireland[50]	Out-patients who receive a diagnosis of diabetes after 30 years of age	572	3 (0.5)	3 (0.5)
	Australia[51]	Non-pregnant outpatients with diabetes who were older than 20 years of age (67% with type 2 diabetes)	418	5 (1.2)	4 (1.0)

Notes:

[a] Persistent elevation in transferrin saturation (threshold values varied from 55% to 62%) unless otherwise noted.

[b] Diagnosed by iron grade of 3 or more, hepatic iron index greater than 2, or more than 4 g of iron removed by phlebotomy.

[c] Diagnosed by liver biopsy; criteria not specified.

[d] Based on one random transferrin saturation test.

[e] Patients with elevated liver iron concentration and positivity for HLA-A.

[f] HH = hereditary hemochromatosis.

[g] Elevated serum ferritin level for initial screening criteria.

[h] Persistent elevation in transferrin saturation and serum ferritin level.

[i] Elevated transferrin saturation and positivity for HLA-A3.

[j] Diagnosis includes elevated biochemistry test results, positivity for HLA-A3.

survival time was 5.25 years among patients who received phlebotomy and 1.5 years among patients identified during the same time period (1950 to 1974) who received no treatment. Reasons for lack of treatment included refusal of treatment and death before initiation of treatment. The difference in survival between the two groups was adjusted for possible differences in age and clinical presentation[58]. Other studies indicate that the average life expectancy after diagnosis of hemochromatosis increased dramatically, from 1 to 6 years[59] to 11 to 21 years[33–35, 58–63], after therapeutic phlebotomy was adopted as standard practice. Part of this trend may be related to earlier diagnosis of hemochromatosis and to improved treatment of diabetes and liver disease, but the proportion of patients with diabetes or liver disease did not change over the time period studied and all studies were done after the advent of insulin treatment for diabetes. Hence, most of the improvement in life expectancy is probably due to therapeutic phlebotomy.

Evidence from two case series (one with 251 patients and the other with 85) suggests that survival in patients who had hemochromatosis without cirrhosis (many of whom were asymptomatic) and received phlebotomy was similar to that in an age and sex-matched sample from the general population[33, 35, 60]. For ethical reasons, no data describe the natural history of untreated hemochromatosis in noncirrhotic patients; thus, the effect of treatment on life expectancy cannot be determined. Results from case series also suggest that therapeutic phlebotomy alleviates various clinical manifestations of hemochromatosis that occur in the precirrhotic or asymptomatic stages of the disease[33, 35, 58]. Niederau and colleagues[33], for example, documented an improvement after therapeutic phlebotomy in patients with hemochromatosis, as indicated by improved glucose tolerance (37% of patients), better findings on electrocardiography (34% of patients), improved liver enzyme concentrations (73% of patients), and decreased hepatic fibrosis (23% of patients). Although this study was limited by lack of an adequate comparison group, the magnitude of these improvements indicates that therapeutic phlebotomy can prevent illness in asymptomatic persons with hemochromatosis.

Accuracy of screening tests

The most common screening strategy for hemochromatosis starts with a random transferrin saturation test. If the test result is positive (that is, if the serum transferrin saturation is elevated), the test is repeated after the patient has fasted overnight[1, 5–11, 16]. The recommended cut-off value defining an elevated transferrin saturation on initial and repeated screening tests varies from greater than 45% to greater than 62%[1, 5–11, 16, 26, 27, 29]. It has been recommended

that cut-off values be lower for women than for men because the distribution of transferrin saturation is lower in women and because the transferrin saturation test has lower sensitivity in women than in men at the same threshold[64, 65]. Other tests (for example, tests for unbound iron binding capacity, serum iron concentration, and serum ferritin level) and strategies (such as measuring the serum iron concentration and then performing a transferrin saturation test under fasting conditions) have also been considered for use in screening[2–4]. However, because these alternatives are less sensitive and less specific than or have not been evaluated as extensively as the transferrin saturation test[64, 65], we focus here on the transferrin saturation test.

Evaluation of the sensitivity, specificity, and positive predictive value of screening tests for hemochromatosis is limited by lack of a standard case definition – hence, the absence of an agreed-on gold standard. Until recently, the standard for evaluating the sensitivity and specificity of hemochromatosis screening tests was HLA haplotyping of family members of clinically diagnosed index patients (as a proxy for a more specific genetic test). Regardless of clinical signs and symptoms, any family member with an HLA haplotype identical to that of an identified index case was considered to have hemochromatosis. The index case was usually identified by the clinical criterion of iron overload due to hemochromatosis[16]. This type of case definition presents three difficulties. First, a genetic predisposition does not necessarily express itself in iron overload due to hemochromatosis. Second, HLA typing is unlikely to correlate perfectly with the presence of a gene for hemochromatosis. Finally, this approach to evaluating sensitivity and specificity is not applicable to general population screening.

The sensitivity of the transferrin saturation test is equal to the proportion of persons with hemochromatosis who have a positive test result[65]. The specificity is equal to the proportion of persons without hemochromatosis who have a negative test result[65]. In one review of studies that used HLA haplotyping in family members as the standard[36], the sensitivity of the transferrin saturation test ranged from 94% (cut-off value >50%) to 86% (cut-off value >60%) in men and from 82% (cut-off value >50%) to 67% (cut-off value >60%) in women. The specificity of the test ranged from 93% (cut-off value >50%) to 98.5% (cut-off value >60%) in men and from 95% (cut-off value >50%) to 99.4% (cut-off value >60%) in women.

In family members with positive results on HLA haplotyping, a few studies have used clinical indications of iron overload (such as elevated liver iron stores) to further differentiate among cases[64, 66]. One study[66] suggested that the sensitivity of the transferrin saturation test is better in patients with iron overload due to hemochromatosis (n= 162) than in family members at earlier stages of disease (n= 12), but the number of family members included in the study was small[66]. The positive predictive value of the transferrin saturation test is the probability that a patient with a positive test result actually has hemochromatosis[65].

The positive predictive value depends on several factors, including the sensitivity and specificity of the test, the case definition or standard used, and the prevalence of hemochromatosis in the population[65]. The frequency of an elevated transferrin saturation on an initial test is greater than the estimated prevalence of hemochromatosis[63], indicating a high ratio of false-positive to true-positive cases (and, thus, a low positive predictive value). It is customary to improve the predictive value of the transferrin saturation test by repeating it in persons with initial positive results because elevated transferrin saturation can be due to factors other than hemochromatosis, such as diet or the time of day at which serum is collected[67]. In one study of blood donors, for example, the positive predictive value of an initial transferrin saturation test was as low as 3.8% when HLA typing was used as the standard[29]. Repeating the test under fasting conditions or increasing the cut-off value decreased the number of false-positive results obtained (that is, it increased the specificity) and increased the positive predictive value. The positive predictive value of repeated tests (a result >50% under nonfasting conditions followed by a result >62% under fasting conditions) was 68% when HLA typing was used as the standard and 43% when elevated liver iron stores were used as the standard[29].

The positive predictive value of the transferrin saturation test increases with the increasing prevalence of hemochromatosis. In screening studies done in patients with hemochromatosis-associated diseases (such as liver disease), the prevalence of hemochromatosis is probably elevated; thus, the positive predictive value of the transferrin saturation test may be increased. In one study done in patients with liver disease[40], the positive predictive value of a single transferrin saturation test was 41%. In patients with diabetes, the positive predictive value of repeated transferrin saturation tests was 63% to 80% when elevated liver iron stores were used as the standard[44, 45, 50]. Hence, estimates of positive predictive value in high-risk persons (such as persons with an affected family member or patients with hemochromatosis-associated illness) are much greater than estimates in blood donors or in the general population[28, 30].

Finally, the specific laboratory method used can also affect the diagnostic usefulness of the screening test because variability in iron tests causes both false-positive and false-negative results. Methods for transferrin saturation measurement vary widely among laboratories, and

within-laboratory variation for total iron-binding capacity (the denominator of transferrin saturation) is substantial. As has been learned from experience with cholesterol screening, laboratory variation, if not addressed, decreases the diagnostic usefulness of a test and can decrease the effectiveness of a screening program[68].

Since the recent discovery of the gene for hemochromatosis, DNA testing has been done on an experimental basis[18, 20, 25]. With respect to accuracy or the association of the test with the clinical expression of hemochromatosis, much less is known about the genetic test than about the transferrin saturation test. In recent clinical series of unrelated white patients with hemochromatosis (these studies included between 65 and 178 patients), 69% to 97% of affected patients carried two mutations (C282Y/C282Y, C282Y/H63D, or H63D/H63D)[12, 18–24]. Therefore, up to 31% of affected patients may carry one mutation or no mutations and would not be identified by the current DNA test. Most clinically affected patients (60% to 92%) were homozygous for C282Y. In control populations, the carrier rate of H63D is higher than the carrier rate of C282Y, suggesting that C282Y has higher penetrance than H63D. Thus, a genetic test that includes H63D might have better sensitivity but poor specificity. Case definitions varied across studies, but most of the studies were done in symptomatic patients who had progressed to iron overload disease, as determined by phlebotomy or liver biopsy[12, 18–24].

In studies in control populations (these studies included between 50 and 381 patients), 0% to 8% of participants carried two mutations (C282Y/ C282Y, C282Y/H63D, or H63D/H63D)[12, 1–24]. Population screening studies and, thus, estimates of positive predictive value are unavailable. Most important, the penetrance of these mutations has not been established.

Laboratory variability in DNA testing may also be an issue in determining the accuracy of this testing. The Task Force on Genetic Testing of the National Institutes of Health – US Department of Energy Working Group on Ethical, Legal and Social Implications of Human Genome Research[69] has stated that the technical accuracy of genetic tests is not adequately addressed by current legislation. The Task Force has called for a national accreditation program for genetic testing laboratories similar to the programs now run by the College of American Pathologists or the American College of Medical Genetics.

Effectiveness of screening

Evidence for the effectiveness of screening in the prevention of chronic disease resulting from hemochromatosis comes mainly from expert opinions, descriptive studies, and a time series analysis (grade III) (Table 53.1). No controlled trials (grade I or II-1) or observational studies (grade II-2) have compared chronic disease outcomes in populations screened for hemochromatosis using iron or DNA tests with those in unscreened populations. Because of the large number of participants and the follow-up time required, these studies would be logistically difficult and expensive. As noted, case series (grade III) suggest that survival in patients treated early in the continuum of disease expression is significantly better than that in patients with cirrhosis[33, 35, 60, 62]. Niederau and co-workers[33] indicated that the survival of patients with hemochromatosis improved over time (grade II-3), possibly in association with increases in the proportion of patients who received a diagnosis before the onset of symptoms. The proportion of asymptomatic patients with hemochromatosis in that series increased from 4.8% in the period from 1947 to 1969 to 30.1% in the period from 1982 to 1991[33].

Taken together, these data suggest that the detection and treatment of hemochromatosis in asymptomatic persons increase survival. However, data on survival from the time of diagnosis do not constitute true proof of benefit from screening. Survival analysis has two major limitations in assessing the effectiveness of population screening. First, because asymptomatic persons are likely to be identified earlier in the continuum of expression of hemochromatosis, it is unclear whether the increased length of time between diagnosis and death is the result of earlier diagnosis or of increased survival (lead-time bias)[70]. Second, neither the natural history of hemochromatosis nor the likelihood that disease progresses more rapidly in persons with hemochromatosis who present with cirrhosis or cardiomyopathy than it does in other persons with hemochromatosis is known. Increased severity of disease in persons whose cases are detected in the symptomatic phase could decrease the length of time during which persons with hemochromatosis could be detected by screening (length bias)[70]. Therefore, the proportion of illnesses and deaths averted by early detection in persons with hemochromatosis is unknown.

Safety and adverse effects

As with all screening, the benefits of screening for hemochromatosis (early detection and treatment) must be balanced against its adverse effects, which may include complications of diagnostic procedures (such as liver biopsy) and legal, social, and psychological problems (such as discrimination, loss of insurance benefits for a

person with a known genetic condition, and increased costs of health care or insurance).

Liver biopsy is often considered the final and definitive diagnostic test for hemochromatosis[16, 71]. Complications of liver biopsy can include pain, hemorrhage, bilious or infectious peritonitis, penetration of abdominal viscera, pneumothorax, and death[72]. Complications are reported in 0.06% to 0.32% of patients, and death occurs in 0.01% to 0.12%[71]. Safer diagnostic options exist, but they do not provide information on some factors (such as the presence of cirrhosis or other liver disease) that affect treatment and prognosis[16].

Individual cases of loss of insurance and employment associated with hemochromatosis have been reported[73] but, on a population level, the scale of concern about these events is unknown and must be weighed against the potential for increased health benefits from treatment. Other possible risks of screening include increased anxiety and a decreased sense of well-being caused by diagnosis and misinformation due to inaccurate results. These risks need to be balanced against the psychological benefits of finding an explanation for symptoms, alleviating symptoms with treatment, and preventing disease progression.

Population screening for hemochromatosis raises concern about diagnosis in the absence of evidence that persons can benefit from testing. Changing the case definition of hemochromatosis from iron overload to the presence of a laboratory abnormality or a mutation increases the uncertainty of eventual clinical expression. The kinds of concerns raised by the early initiation of treatment on the basis of iron status are compounded by the DNA test because we are more uncertain about the clinical expression of disease in asymptomatic persons with positive genetic test results than we are about the clinical expression of disease in asymptomatic persons with abnormal iron status. An asymptomatic person who is homozygous for a genetic abnormality may be exposed to years of unnecessary testing, follow-up, and treatment and to stigmatization and discrimination by employers, insurers, and society at large[72, 74–76] without ever developing hemochromatosis. The DNA test is more likely to offer benefits when it is used for case finding in family members of affected patients.

Economic costs

The economic costs of population screening have been estimated for both phenotypic[2–6, 8, 10] and genotypic testing[77]. Major determinants of the cost-effectiveness of screening are the prevalence and disease burden of hemochromatosis; the sensitivity and specificity of the screening tests; compliance with screening, diagnosis, and therapy; and the costs of screening, diagnosis, and therapy[2–6, 8, 10, 77]. Cost-effectiveness analyses have traditionally based the probability of developing major clinical manifestations (such as diabetes, cirrhosis, and heart failure) on data from hospital registries of patients affected by hemochromatosis[2–6, 8, 10]; this may overestimate morbidity and mortality[78]. Compliance with screening, diagnosis, and treatment is usually assumed to be greater than 80% but may be substantially lower in practice[27]. Finally, the costs associated with screening and treatment are variable and subject to change. Commercially, the cost of the genetic test (about $150; range, $75 to $250 for two mutations) is greater than that of the transferrin saturation test (about $20; range, $2 to $30). The costs of the genetic test may change as new mutations are discovered, as efficiency in laboratory procedures increases, or as patents are awarded. The costs of the phenotypic tests may decrease if the efficiency and diagnostic usefulness of laboratory procedures increases.

The cost of therapeutic phlebotomy, which varies in frequency and duration, contributes substantially to the cost of implementing population screening. The current policy of the US Food and Drug Administration dictates that blood donated by a person with hemochromatosis must be labeled as a product of therapeutic phlebotomy. Most blood banks have policies and practices that recommend against the use of such products, and they discard them[79]. Thus, patients are charged for therapeutic phlebotomy. Changing this policy (for example, allowing use of blood products that have undergone regular safety screening) could reduce the cost of treatment for hemochromatosis and could provide additional benefits to society.

Conclusions

Hemochromatosis represents a new paradigm for genetics and public health. It meets many of the US Preventive Services Task Force criteria for candidacy for population screening. Screening studies have shown that it is common in comparison with many genetic disorders; case series have demonstrated that the disease generally develops in adulthood and that illness can be prevented with treatment. The potential for preventing hemochromatosis-associated illness and death through screening and treatment may be great. Thus, timely resolution of questions about penetrance of clinical disease among persons with hemochromatosis-associated mutations or early biochemical expression of hemochromatosis is needed (Table 53.3).

Table 53.3. Recommendations for action

1. Despite the promise of prevention of hemochromatosis, this review indicates that we lack evidence to recommend hemochromatosis screening for all adults (persons >18 years of age). Gaps in the evidence include the clinical penetrance and burden of disease associated with hemochromatosis. To fill crucial gaps in the evidence, multicenter screening studies and surveillance among the general population and among persons with hemochromatosis-associated diseases (such as liver disease, arthritis, and diabetes) should be conducted[78, 80].
2. High-risk groups should be screened with the transferrin saturation test. High-risk groups include first-degree relatives of persons with hemochromatosis and persons presenting with early signs and symptoms compatible with hemochromatosis (such as elevated iron measures, arthralgia, severe weakness or fatigue, signs of chronic liver disease, chronic hepatitis C, type 2 diabetes, and bradyarrhythmias).
3. When hemochromatosis is diagnosed, periodic therapeutic phlebotomy should be initiated[55, 56, 61].
4. Laboratory standardization for the transferrin saturation test and the DNA test should be addressed at the national level[27, 69].
5. The use of DNA testing for hemochromatosis screening is not recommended at this time. This testing should be used in the context of ongoing research so that questions about its ability to predict disease and its potential adverse effects can be resolved[18, 80]. It may have a role as a confirmatory test in first-degree relatives of affected persons with two hemochromatosis mutations[16, 18].

Collaboration among primary care providers, scientists, and policy-makers is crucial to the gathering of the additional information required to determine the most effective preventive strategy for decreasing morbidity from hemochromatosis. The need for population data to answer questions about penetrance among asymptomatic persons should not impede increased case detection in high-risk groups or efforts to increase laboratory standardization of screening tests. We hope that our paper and others in this issue will increase knowledge and awareness about iron overload due to hemochromatosis and will motivate physicians to examine and establish strategies to prevent the morbidity and mortality associated with this disorder.

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Ethical issues and hemochromatosis

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Introduction

Hemochromatosis is the most common genetic disorder among Caucasians of Western European descent. Increasing recognition of its frequency and the recent development of a DNA-based genetic test have increased concerns about the methods used to diagnose and treat hemochromatosis at the level of individuals, families, and the general population, and the possible legal and ethical ramifications of widespread testing for and diagnosis of a heritable disorder. Therefore, we review the general legal rights of all patients, with attention to how they apply to the situation of patients with hemochromatosis and the special legal rights of some patients conferred by certain contracts. In addition, ethical rights, responsibilities, and unresolved questions are explored concerning hemochromatosis, including those of patients, physicians, and public policy.

Rights and responsibilities of patients

Privacy and confidentiality of medical information

Persons with hemochromatosis have moral and legal rights to privacy. The notion of a right conveys that respectful treatment is an entitlement, not a privilege or a benefit bestowed at the discretion of another. Each patient is entitled to be treated with dignity and in a way that avoids unnecessary exposure of the body or the disclosure of embarrassing information to others. The right to confidentiality is the right to control the communication of sensitive personal information, and the assurance that there will be no unnecessary communication of personal medical facts. However, the term 'right' does not necessarily convey the idea of a specific list of actions or omissions. Maintaining patient privacy and confidentiality varies with the individual and the situation, and there is no substitute for the physician's judgment and sensitivity in this regard.

Relevant information

Patients have a right to receive relevant information so that they can make informed choices about issues that arise in the course of their diagnosis and treatment. This includes the question of whether or how to inform relatives at risk for genetic disorders. Physicians have an obligation to provide patients with this information (see discussion below).

Prevention of harm

Most ethical views recognize an obligation not to cause harm to innocent persons – one of the most weighty and clear moral obligations. Most also recognize a general obligation to prevent serious harm. The obligation to prevent harm that one does not cause is understood to be more restricted than that not to cause harm[1]. Unlike the obligation not to cause harm, the obligation to prevent harm, unless suitably restricted, would be burdensome. Because there is no limit to preventing harm to the human population at large, an unlimited obligation to prevent harm would, in effect, make the conscientious slaves of those who engage in irresponsibly risky behavior. The most important restriction on this obligation is that persons are not morally required to prevent a harm they do not cause, if doing so would impose extreme costs or risks on themselves. Moreover, there is often more moral blame associated with causing than not preventing harm. Therefore, the obligation to prevent harm is generally a limited or conditional one.

Individuals have special obligations to prevent harm. These arise from voluntary undertakings, such as contracts

and promises, and from special relationships, such as those among family members and between friends and lovers. Special obligations are often more demanding. For example, the special obligation to prevent harm to a member of one's family or to a close friend usually carries more moral weight and requires greater costs for fulfilment than the general obligation to prevent harm to a stranger. This duty is strongest in circumstances in which there is a contractual undertaking, or in which one person has taken responsibility for the welfare of a vulnerable persons, e.g., parents' obligations to their children. Consequently, the broad moral consensus that parents' obligations are special is reflected in the existence of laws defining child neglect as a criminal offense. The obligation to prevent harm to other relatives is less well-delineated legally, and it is not so clear morally. Because marriage vows include pledges of mutual support and fidelity, a special duty to one's spouse is compelling. It is less clear whether special obligations to siblings are as unambiguous and weighty as those of parents to minor children or spouses to one another. The nature and extent of special obligations also varies among cultures. In some, relationships among relatives beyond the nuclear family are very close. Members of the extended family are connected by a dense web of cooperative interactions that can give rise to special obligations that would not exist, or would not be so demanding, in a different 'family culture.'

Informing relatives at risk

Suppose that an individual 'John' has been diagnosed to have hemochromatosis. At present, John has no legal obligation to inform relatives at genetic risk. Does John have an ethical responsibility to inform relatives that they are at risk for hemochromatosis? If so, which relatives, and what counts as fulfilling it? John has two brothers, each over 40 years of age. Because hemochromatosis is an autosomal recessive genetic disorder, each sibling typically has a 25% chance of having hemochromatosis. If either brother has hemochromatosis and his diagnosis is not timely, there is a high probability that he will sustain serious injury to his liver, heart, and endocrine system, and have increased risk to develop cancer of the liver and arthropathy. Because hemochromatosis often goes undiagnosed in routine health care delivery, even long after characteristic symptoms appear, John's siblings may not be diagnosed until serious and irreversible damage occurs – unless they learn that a close relative has hemochromatosis. If siblings have hemochromatosis and are diagnosed sufficiently early to avoid significant organ injury, they can undergo iron depletion therapy by phlebotomy. This is relatively inexpensive and imposes a very low risk of complications or serious burdens to the patient.

With respect to the diagnosis of hemochromatosis, the harm to be prevented is great. The action that will prevent the harm (therapeutic phlebotomy) usually produces no harms of comparable weight to offset the harm prevented, and it is unlikely that the harm will be prevented by some other means if John does not inform his at-risk siblings. It seems clear that John has a prima facie ethical obligation to inform his brothers that he has hemochromatosis, and that they are at risk. Whether this is an obligation all things considered depends, however, upon whether informing them would impose unreasonable costs on John. Three possible costs to be borne by John if he informs his brothers of his diagnosis are: (a) stigmatization by family members or others who thus learn that he has hemochromatosis; (b) the risk of insurance or employment discrimination; and (c) the cost of the measures needed to inform his brothers.

Although genetic stigmatization does occur, all genetic disorders do not carry the same risk of stigmatization. A genetic disorder like Alzheimer disease or inheritance of a BRCA1 gene associated with increased risk to develop breast cancer carries a greater risk of stigma or other psychological harm than does hemochromatosis. Because there are no reliable data to indicate that persons with hemochromatosis have reported or are likely to suffer significant stigmatization, this risk would appear to be very low. John might be stigmatized by other family members for being 'genetically defective.' This risk, if any, can be minimized if John convinces his brothers that persons with hemochromatosis can live normal, healthy lives. Alternatively, he might convince his family members that they should not tell others about his hemochromatosis. His siblings need not even inform their own physicians that John has hemochromatosis. Instead, they can tell their physicians that there is hemochromatosis 'in the family,' and they are therefore at higher than normal risk and wish to be tested.

Whether the risk of insurance discrimination due to the diagnosis of hemochromatosis constitutes an unreasonable cost and hence undercuts John's prima facie obligation to inform his siblings is doubtful. The question is whether any risk of insurance discrimination John has already incurred by virtue of being diagnosed with hemochromatosis will be augmented if he informs his siblings of his diagnosis. The evidence that insurance discrimination occurs against those with hemochromatosis consists almost exclusively of studies in which persons with genetic disorders are asked if they have suffered insurance discrimination as a result of their condition[2]. The chief

difficulty with such surveys is that some affected persons are refused insurance on other grounds, yet assume that it was their genetic disorder that was the basis of exclusion. Contrariwise, anecdotes suggest that some insurance companies reverse initial decisions to exclude hemochromatosis patients when they receive documentation that the patient is undergoing successful treatment and that serious injury due to iron overload has been avoided. Persons with hemochromatosis often incur increased health care expenditure risks (i.e., for diagnostic evaluation and lifetime therapeutic phlebotomy), but may qualify for life or disability income insurance (e.g., if they do not have hepatic cirrhosis or diabetes mellitus). Whether there is a risk of insurance discrimination also depends partly upon applicable statutes. Several states now have laws prohibiting insurers from seeking and utilizing information about genetic disorders, and current proposals for federal legislation would make insurance discrimination on genetic grounds illegal[3].

John's costs to inform his siblings of his diagnosis and their risk are trivial, and not comparable to the harm possibly avoided by early detection of hemochromatosis in his brothers. If there is a general obligation to prevent harm, and a special obligation to prevent harm to those with whom we have special relationships, a strong case is made for the conclusion that John should inform his siblings. There are unlikely to be 'unreasonable costs' that extinguish this obligation. One must also assume that John's siblings have not instructed him that they do not wish to be informed, and that he has no other valid reason to believe that they wish to remain uninformed. In the case of some genetic disorders, e.g., Huntington's disease, some persons do not wish to be informed of their relatives' test results and refuse to be tested themselves[4]. It is much less likely that a person would feel similarly about hemochromatosis, because hemochromatosis, unlike Huntington's disease, can be treated successfully.

John's other first-degree relatives are also at increased risk to have hemochromatosis. If these are women, they are at lower risk to develop iron overload than men. Menstruation is a natural 'de-ironing' mechanism, and women have lesser risk to develop iron overload for this and other reasons. However, iron absorption in women with hemochromatosis increases after menopause to levels similar to those in men, and the complications of iron overload become increasingly likely with each decade of life. Therefore, the fact that women are at somewhat lower risk than men does not justify that John ought not to inform them. John also has other, more distant relatives. Because he may feel less 'close' to them, both logistically and psychologically, the task of informing them may be more onerous and less successful. However, the more biologically distant the relative, the lower the risk that they will have hemochromatosis. This also decreases the probability that informing them will prevent a serious harm. If John attempts to inform a distant relative, he incurs a greater risk of invading that person's privacy than if he informs a relative with whom he has a closer, more significant relationship. The weight of John's obligation to inform more distant relatives may differ, depending upon his 'family culture.' In cases in which there are long-standing patterns of reciprocal aid among members of an extended family, a person with hemochromatosis may have more substantial obligations to inform more relatives. However, different 'family cultures' may have different attitudes about privacy, and this may influence the nature of the obligation to inform relatives about genetic risk.

The nature and extent of our obligations to prevent harm depend in part upon the nature of our institutions. The current health care system does not diagnose most persons who have hemochromatosis, and the general level of education of physicians, other healthcare personnel, and the non-medical public about hemochromatosis is not great. Therefore, John's informing his brothers is likely to be more consequential than if testing for hemochromatosis were a routine part of healthcare delivery. The duty to disclose a risk of serious harm is exemplified by the famous Tarasoff case, in which a university student (Mr Proddar) informed his therapist that he planned to kill his estranged girlfriend, Ms Tarasoff[5]. The therapist, feeling he had a duty to breach the confidentiality of the therapist–client relationship, informed the campus police, but Ms Tarasoff was not living in the USA at the time. The police lowered their guard. When she returned later, Mr Proddar killed her. The California Supreme Court held that the therapist did not do enough to prevent harm to Ms Tarasoff, and that he had a duty to inform the potential victim (in addition to the police). There are three important differences between the Tarasoff case and that of John, our hypothetical hemochromatosis patient. First, Mr Proddar was about to engage in a criminal act. Second, Proddar was himself the cause of the harm to Ms Tarasoff, and thereby lost his claim to confidentiality. In contrast, John would not have caused his siblings to be damaged by hemochromatosis and iron overload. Third, the probability of harm to Ms Tarasoff was close to certainty. Contrariwise, the probability of John's siblings having hemochromatosis is only 25%, and all affected siblings do not develop life-threatening clinical manifestations of iron overload (according to some estimates, $<80\%$ of homozygotes are symptomatic).

The main elements of our analysis of John's ethical responsibilities can now be assembled. The chief moral

ground for an obligation to inform is the obligation to prevent harm. If a patient has an obligation all things considered (rather than merely prima facie), the extent and weight of the obligation depend upon these factors: (a) the severity of the harm to be prevented (the more severe the harm, the weightier the obligation); (b) the probability that the harm will be averted if the patient informs relatives at risk (the higher the probability, the stronger the case for attributing an obligation to inform); (c) the probability that the harm will occur if the individual is not informed (the higher the probability, the weightier the obligation); (d) whether there is a 'special' relationship between the individual who can inform and the one who would benefit from being informed (special obligations generally carry more weight than general ones, at least so far as that which falls within the scope of 'reasonable costs'); (e) the costs of preventing harm to the individual who is at risk for the harm (e.g., a regimen of phlebotomies in the case of hemochromatosis vs. surgery to treat colorectal cancer); and (f) the costs of informing incurred by the person who does the informing, including the additional risk of insurance or employment discrimination (i.e., the risk beyond that of the initial diagnosis); and (g) the risk of stigma or other negative psychological effects for those who informed.

Physicians' ethical obligations

Obligations regarding testing and test results

The threshold ethical question is when a physician ought to discuss the option of testing for hemochromatosis. However, there is no general answer. Whether testing is a worthy option for a particular patient depends on many factors. In this context, the physician's ethical responsibilities are also professional responsibilities, the most fundamental of which is to practice medicine according to professional standards of competence. However, an appropriate standard of care regarding the diagnosis of hemochromatosis has not been effectively disseminated. Sources of professional standards include recommendations by expert individuals or groups, statutes, regulations or court decisions, and the actions taken by most physicians. An expert panel recently concluded that general population screening at this time would be premature, and no professional recommendations currently suggest that routine screening for hemochromatosis is the standard of care[6]. However, the present standard of care may be incorrect. Courts sometimes create so-called objective standards of care, arguing that a doctor has a duty to offer a test or treatment when a reasonable person might want it, regardless of what other professionals say or do[7]. Consequently, physicians are not always the sole arbiters in questions of the information or services patients should have.

Many physicians are not well informed about hemochromatosis, and therefore most affected persons remain undiagnosed and untreated. Much needs to be done to educate many physicians (and the public) about the prevalence of hemochromatosis, the means for detecting it, and the success of therapy. This collective professional responsibility may be increased in certain medical specialties, e.g., gastroenterology, in which there should be greater sensitivity to the prevalence of hemochromatosis due to the effects of iron overload on the liver. The medical profession and various medical specialty groups have an ethical obligation to develop and enforce appropriate standards of care. Individual physicians also have an ethical obligation to practice medicine in accordance with accepted standards of care. Recommending diagnostic testing to patients depends upon a number of factors, none of which are peculiar to hemochromatosis. Because there is some risk of insurance or employment discrimination to an affected patient, the physician should inform the patient of this possibility when he presents options and recommendations. Once a diagnosis of hemochromatosis has been made, physicians have more specific obligations, including the provision of quality care and respect for their patients' right to accept or refuse treatment under conditions of informed consent. It is less clear whether the physician should suggest informing relatives at risk.

Many genetic counselors and some medical geneticists believe that they should be 'non-directive' toward patients who have a genetic disorder or who are at risk for producing children with a genetic disorder. The first interpretation of non-directiveness is that a physician should never tell a patient that he has an ethical obligation to inform any relatives that he has a genetic condition. According to the second interpretation, it may be appropriate or ethically obligatory for the physician to tell the patient that he ought to inform relatives at highest risk (e.g., siblings), although the physician should avoid coercing or pressuring the patient. Consequently, one might assume that the norm of non-directiveness applies to hemochromatosis, as it is thought to apply to reproductive counseling for other genetic conditions such as Down's syndrome or cystic fibrosis. At present, the dominant view in the USA is that the first interpretation of non-directiveness is the correct one[8]. The justification for non-directiveness is that the physician should refrain from 'imposing values' on the patient. However, it does not follow that 'strong' non-

directiveness is required to avoid the imposition of values; weak non-directiveness may suffice.

Consider the physician's options in John's case: (a) The physician has a duty to take a family history to learn if other relatives are at risk for hemochromatosis. (b) The physician has a responsibility to inform John that his siblings (and possibly other blood relatives) are at risk for hemochromatosis, because most patients feel affection and concern for their relatives and want to alert them to preventable harm. John's relatives may want to consider their choices about reproduction, because their offspring will be at higher risk for hemochromatosis than members of the general population. (c) It is appropriate for the physician to ask John whether he planned to inform his siblings, and whether he wants the physician's assistance in providing written information or contacting the siblings directly. If the physician believes that John has a moral responsibility to ensure that his siblings are informed, this suggests that the physician should participate in the process. John's awareness of his illness does not qualify him as a genetic counselor, and there is considerable risk that he may inform his relatives incorrectly, leading them to have false beliefs about their own risks. If John were to assume complete responsibility for the education of his siblings, the physician has no way to know whether John has completed the task. Whether the physician has a responsibility to ensure that John's siblings are informed is separate from John's responsibility. Although we argue that John has a responsibility to inform his siblings, the physician does not necessarily have a similar duty. (d) If John refuses to inform his siblings, or acts in a way that makes the physician unsure that he will do so, the physician must consider whether he has an obligation to identify this as an ethical issue, a question of moral responsibility. The obligation to prevent harm favors the physician's ascertaining that John understands that he has an ethical obligation to inform his siblings, because it is improbable that they will learn of their risk otherwise. (e) If John's responses leave the physician unsure about John's plans, the physician must then decide whether he has a responsibility to try to persuade John to discharge his duty, or even (f) to insist that the siblings are informed. This implies that the physician will ensure that disclosure of the risk occurs. Does the physician's need to avoid 'imposing values' on John preclude telling John that he ought to inform his siblings? If a physician does this in an intimidating or moralistic way, he might be 'imposing values.' However, it is demeaning and disrespectful to assume that stating an opinion about a person's obligations is intimidating or infringes on one's autonomy. Thus, ironically, 'strong' non-directiveness may seem paternalistic. However, physicians can appeal to a patient's moral sense and acknowledge patient autonomy by pointing out such obligations.

Physicians, like other persons, have an obligation to prevent harm, and can fulfil this obligation by informing patients of their obligations (as the physician sees them). The physician could insist that John's relatives be informed – a coercive position. Although John has a moral duty to inform his relatives, the physician does not necessarily have a comparable duty if John refuses. First, the physician has no special relationship to John's siblings. Second, the physician's duty to John obliges him to put John's interests above those of others, unless those other interests are of very great weight. The physician's special relationship with John generates an implied promise of confidentiality. Although there can be no assurance of absolute confidentiality, the conditions under which confidentiality can be breached include a high probability of serious harm to others, or a definite benefit to be achieved from the breach. A breach of confidentiality must be a last resort – the only means of averting serious harm. The risk of harm to John's siblings is no greater than 25%, and most persons like John want to inform their siblings. Threats of coercion or non-consensual disclosure by physicians are usually unnecessary. However, physicians who believe that they have an obligation to inform relatives at risk ought to inform their patients as soon as feasible.

The historical context of the norm of non-directiveness

Non-directiveness is a social product of a certain historical experience in the area of genetic testing concerning reproductive choices, not a fundamental principle of morality or medical ethics. The paradigm in this area of medical practice is the disclosure to prospective parents that there is a risk that their child will have a serious genetic disorder. This situation has distinctive features that lend credibility to the norm of non-directiveness according to the 'strong' interpretation. Among the most important are these: (a) the disclosure of genetic information touches intimate human relationships; (b) the only option for responding to the revelation of genetic risk is to avoid conception or terminate pregnancy; and (c) medical professionals are aware of the need to dissociate themselves from such decisions. These factors support the conclusion that physicians should avoid imposing their values or the presumed values of society on patients regarding reproductive choice. However, none of these factors is directly relevant to the different context of whether to inform relatives that they are at risk for hemochromatosis. As in most other medical situations in the USA, patients typically expect

their doctors to express opinions and make recommendations.

Observance of 'strong' non-directiveness may encourage patients to believe that they have no ethical obligations. When physicians refrain from informing patients of these obligations or fail to acknowledge that there are issues of responsibility, they may convey the message that the patient's choice is a matter of 'personal values' – that the idea of ethical responsibility is not applicable. Ethical responsibilities are not usually reducible to acting consistently on whatever values or preference one happens to have, but are subject to moral appraisal. In the case of the diagnosis of hemochromatosis, little can be said in favor of 'strong' non-directiveness. To fail to see this is to be mesmerized by the fact that hemochromatosis is a genetic disorder, and to commit 'the fallacy of genetic exceptionalism.' At issue is whether there are plausible grounds for concluding that patients have ethical responsibilities to their relatives at risk, and whether physicians can advise them of these responsibilities without infringing their autonomy – not whether hemochromatosis is a genetic disorder.

Insurance-related risk

This issue of physician responsibility arises in the clinical management of hemochromatosis and many other conditions, genetic or otherwise. Although the extent of insurance discrimination against persons diagnosed with hemochromatosis is unknown, there is some risk. Therefore, physicians ought to inform patients with hemochromatosis that they are at risk for insurance discrimination without conveying the impression that this is certain. If the patient is refused insurance, the physician should supply documentation to the underwriter explaining the complications of iron overload (if any) that have occurred, that hemochromatosis is treatable, and that this patient is cooperating with the treatment program. In some cases, patients may ask the physician to aid them in preventing insurers from gaining access to the fact that they have hemochromatosis (with or without complications). Fearful of insurance discrimination, the patient may ask the physician not to record the diagnosis in the official medical record (but instead to keep a 'shadow' record for purposes of their clinical management). It may seem extremely unfair that persons with hemochromatosis should be excluded from insurance coverage. However, 'risk-rating' is 'actuarially fair,' in that it treats individuals at the same risk equally; insurers themselves are at excessive financial risk when patients hide information about their health risks. Nonetheless, many believe that adequate health care is a moral right, and that a system in which individuals are vulnerable to insurance discrimination is unjust. Accordingly, some physicians believe that aiding patients to hide the diagnosis of hemochromatosis is analogous to a justified act of civil disobedience. It can be argued that a necessary condition of justified civil disobedience is that it be done openly, so that the legitimacy of the disobedience can be evaluated and the law in question can be subjected to public scrutiny. Without this, it is abusive to break laws simply because one thinks they are unfair.

Physicians have many legal responsibilities within the health care system. Among these are contractual obligations to insurers and health care organizations for which they provide patient services to keep accurate and honest records. Adherence to these obligations is not a matter of slavish adherence to arbitrary rules; there are sound medical and moral reasons for having a system in which the integrity of medical records is preserved, especially by physicians. Accordingly, physicians are not obligated to help patients 'game the system.' If physicians refuse patients' requests to hide the diagnosis of hemochromatosis, they do not default on obligations to their patients (or those to healthcare insurers). Further, patients have no right to demand that physicians distort their medical records.

Public policy issues

Population screening

A minority of physicians have advocated general population screening for hemochromatosis for several years. In 1997, there was a sharp increase in the volume and intensity of the discussion of this proposal[6]. Despite the fact that hemochromatosis is much more prevalent and can be treated much more effectively and cheaply than many other genetic disorders, more attention was given in the past to the positive and negative aspects of screening for other disorders (especially cystic fibrosis)[9,10]. However, these issues are not exactly parallel. Proposals for carrier detection for cystic fibrosis are directed toward reproductive choices, whereas proposals for hemochromatosis are directed toward pre-symptomatic detection of a disorder in individuals who already exist. This would seem to favor giving priority to hemochromatosis screening, on the grounds that our obligations to prevent harm to existing persons are weightier than those to future possible individuals. Other differences include the different cost–benefit

ratios of screening for the two disorders (that strongly favor hemochromatosis), and the fact that screening for hemochromatosis does not involve significant debate over human reproduction.

Escalation of the discussion concerning hemochromatosis screening was triggered by the discovery in 1996 of two mutations in a gene on the short arm of chromosome 6 that account for the majority of Caucasians with hemochromatosis[11]. This discovery permits DNA-based genetic testing for hemochromatosis. However, biochemical tests to detect hemochromatosis and iron overload (transferrin saturation and serum ferritin concentration) are very reliable, are less expensive to perform, and have been available for decades. A consensus at a recent conference on the prospects of performing general population screening for hemochromatosis was that biochemical tests may be more appropriate than DNA-based genetic tests for use in a screening program, at least for the present[6].

Important ethical issues are raised by proposals to perform mass screening programs for genetic disorders, whether diagnosis is achieved by DNA-based genetic testing or more conventional clinical and biochemical criteria. These are: (a) equity in the distribution of health care resources in funding general population screening for a particular disorder; and (b) the manner in which the screening program is to be conducted. Ideally, the decision to undertake a screening program for a particular disorder should be based on a systematic account of distributive justice in health care, and reflect a consistent and morally defensible ordering of priorities. Unfortunately, many screening programs in the USA have been political responses to advocacy group pressures and have incurred much criticism. For example, national screening of newborns for phenylketonuria (PKU) was implemented without adequate studies to assess the accuracy of the screening test, or the toxicity of the diet designed to prevent damage from the disorder. This was largely the result of a powerful 'PKU lobby,' including President John F. Kennedy, whose interest was spurred by his personal experience with a retarded sister who had phenylketonuria. Similarly, mass screening to detect carriers of the sickle-cell gene was triggered by Congressional funding supported by President Richard M. Nixon as part of a political strategy to garner African American votes in the 1972 presidential election. Thus, the results of preliminary cost-effectiveness studies suggest that the case for screening for hemochromatosis is at least as compelling, if not more so, than for other screening programs for genetic and non-genetic disorders[12].

The voluminous literature on the ethics of population screening for cystic fibrosis, sickle-cell disease, and Tay–Sachs disease provides answers about how population screening for hemochromatosis should be conducted. For over 20 years, there has been a broad consensus that responsible screening programs should include evidence that: (a) the incidence of the disorder in the population(s) being tested is sufficiently great to warrant screening; (b) the diagnostic test for the disorder is accurate and affordable; (c) a safe and effective intervention can be provided for persons who test positive; (d) education and counseling can be delivered in such a way that patients can make free, informed choices; and (e) safety and efficacy of the program as a whole can be evaluated, based on the assessment of the screening and intervention in well-designed pilot studies[13–15].

A distinction is sometimes made between the terms screening, that connotes programs implemented under state auspices, and testing, that is understood as an interaction between individual patients and physicians. Advocates of screening programs involving adults generally support the same high standards of informed consent required for interactions between individual patients and physicians. From an ethics standpoint, the distinction between screening and testing may be minor, at least in the case of hemochromatosis. Thus, widespread testing for hemochromatosis by physicians in their offices can become indistinguishable from a screening program with high standards of informed consent.

Hemochromatosis blood policy

It is the usual practice of blood banks in the USA not to accept blood donations from hemochromatosis patients. This practice has arisen largely from statutes in the USA that require that blood and blood products obtained from persons with known genetic disorders be so labeled. This, in effect, severely limits or prohibits the use of such blood products by physicians and blood banks. In some other countries, such labeling requirements do not prejudice the use of blood from persons with hemochromatosis who are otherwise qualified as donors. Patients with hemochromatosis and certain advocacy groups have recommended for some time that these policies be rescinded. Blood procurement organizations have resisted this recommendation, because it is their general policy and statutory obligation to accept blood donated only 'for altruistic reasons,' and collecting non-transfusable blood is costly. If general population screening were undertaken, some one million new cases of hemochromatosis could be diagnosed. Thus, the costs to society of adherence to this policy

(at least in the case of hemochromatosis) may be great.

Many undiagnosed persons with hemochromatosis donate blood on a regular basis in the USA. However, most patients who are truthful about their diagnosis of hemochromatosis are unable to get phlebotomy therapy by 'donating' their blood, and much potentially transfusable blood is discarded when the blood is removed in a physician's office. However, blood donated for reasons of 'self-interest' is more likely to be contaminated by transmissible microbes or by unwanted drugs. Some believe that blood donation by hemochromatosis patients undergoing therapeutic phlebotomy may represent an exception to the general policy rule about volunteerism among blood donors. Not all blood donated to blood banks is given with altruistic motives. For example, college sororities and fraternities, corporations, and military units sometimes exert considerable pressure on their members to contribute to blood drives, and often reward them with time off from work, parties, or other prizes when they do so. Some believe hemochromatosis patients, as a group, are at lower risk to have diseases transmissible by blood than members of the general population, although this is unproven. Some persons with hemochromatosis are older and hence are not in younger groups that have the highest incidence of human immunodeficiency and hepatitis virus infections. It could be argued that an individual who is complying with a regime of therapeutic phlebotomy, including the intensive initial 'de-ironing' process to induce iron depletion, is thereby demonstrating that he or she takes future consequences seriously and has a rational attitude toward risk-avoidance. Some persons with hemochromatosis do not have insurance coverage for therapeutic phlebotomy, and might receive treatment more readily if blood procurement organizations provided therapeutic phlebotomy without charge.

However, there are many problems with the notion that blood procurement organizations should be required to treat persons with hemochromatosis using therapeutic phlebotomy. These patients often need medical care for complications of iron overload, genetic counseling, and other services not available at blood procurement organizations, many of which are not geographically convenient for routine patient use. Screening programs would be expected to identify many young persons with hemochromatosis whose risk of transmitting microbial disease to others by blood transfusion could be increased. Further, most young persons with hemochromatosis do not have severe iron overload (if any), thereby decreasing the volume of blood that might be harvested. Importantly, the acceptance of widespread use of hemochromatosis patients as donors by the general physician and patient public who deal with blood transfusion has not been evaluated. It seems morally unjust that any person with hemochromatosis (or hemochromatosis advocacy groups) has the right to demand that patients in need of blood transfusion must, in effect, receive blood products derived from persons with hemochromatosis. Should persons with other genetic disorders also be allowed to donate their blood for transfusion? The percentage of patients with hemochromatosis and the fraction of their units of blood removed for treatment that would be acceptable for transfusion in accordance with other existing guidelines have not been estimated. There has been no objective comparison of projected costs involved in the present manner of providing phlebotomy therapy, and those that would be provided at blood procurement organizations. Without doubt, a re-examination of current blood bank policy in the context of using hemochromatosis blood is forthcoming, and data (not opinion and speculation) are needed to support future debate on these issues.

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Hemochromatosis patients as blood and tissue donors

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Introduction

Hemochromatosis, the prevalence of which is estimated to be 1 in 250 persons, is the most common inherited disorder of Caucasians. The prognosis for persons with hemochromatosis depends on the amount of iron they accumulate and the duration of their iron overload. The most effective treatment of this genetic disorder is therapeutic phlebotomy which removes approximately 200 mg of iron with every unit (500 ml) of blood. Many persons with hemochromatosis might qualify as volunteer blood donors[1, 2]. Because these presumably healthy patients undergo repeated, lifelong therapeutic phlebotomy, blood collected from them has the potential to enhance the blood supply in the United States (US).

The use of blood from individuals with hemochromatosis for transfusion in the US is impeded by a provision in the Food and Drug Administration's Code of Federal Regulation (Title 21 640.3(d)) that states:

> Blood withdrawn in order to promote the health of the donor otherwise qualified . . . , shall not be used as a source of Whole Blood unless the container label conspicuously indicates the donor's disease that necessitates the withdrawal of blood.

Thus, the practice of transfusing blood that has been drawn from patients with hemochromatosis with therapeutic intent is allowed, but the source of the blood must be specified on the label. Accordingly, the clinician is alerted that the particular unit of blood has a 'disease association' and is therefore different from the usual unit drawn from a volunteer donor. It is left to the clinician to approve the use of the blood and to explain the significance of this labeling difference to the intended recipient as part of the informed consent process. The American Association of Blood Banks, a nonprofit organization that sets professional standards for blood banks and transfusion services, assumes an even stronger position by suggesting that blood drawn for therapeutic phlebotomy should not be used for allogeneic transfusion[3]. The practical effect of the labeling regulation, and the transfusion standard is to preclude allogeneic transfusion of blood units drawn from persons known to have hemochromatosis. In the US, the need for blood for transfusion, in addition to the need for organs and tissues for transplantation, exceeds the supply. The shortfall is a major public health problem. Individuals with hemochromatosis constitute a large group of potential donors. This chapter explores the rationale for the blood and tissue labeling practices, the appropriateness of applying these practices to people with hemochromatosis, and the legal, ethical, and practical issues that surround the use of blood, tissues, and organs from persons with hemochromatosis.

Blood donor voluntarism and labeling requirements

Almost all blood collected in the US comes from volunteer donors. Before 1970, many donors in the US received monetary payment for their blood. Studies conducted during the 1970s concluded that paid blood donors are more likely to carry a hepatitis virus than those who receive no money[4]. Thus, since 1978, any blood used for transfusion in the US must be labeled with the source of the blood, i.e., 'volunteer' or 'paid.' The Food and Drug Administration's Code of Federal Regulation (Title 21 600–799, 1991 paragraph 606.21 (c)[5] defines these terms:

> A paid donor is a person who receives monetary payment for a blood donation; a volunteer donor is a person who does

not receive monetary payment for a blood donation. Benefits . . . that are not readily convertible to cash do not constitute monetary payment . . .'

Today, most blood used for transfusion is donated by volunteers who provide blood altruistically for unknown recipients. This blood comprises the bulk of the allogeneic blood supply and is specifically labeled as derived from a 'volunteer donor.' Donors who are motivated to give blood for other reasons such as money (paid donors), or who give blood for themselves in advance of planned surgery (autologous donors), must have their blood labeled as such. There are no specific labeling requirements for donors who give blood for family or friends (directed donors), though for purposes of appropriate tracking and final disposition of these units, special labeling must be part of a written operating procedure for their handling.

The policy of labeling blood according to donor source virtually eliminated paid-donor blood and associated components for use in direct transfusions. Paid donors continue to contribute to plasma pools collected largely by commercial pharmaceutical firms for preparation of fractionated blood products such as albumin and anti-hemophilic factor. Demographically, paid plasma donors differ from volunteer donors: they are younger, more often male, and socioeconomically disadvantaged. Though not directly comparable with volunteer whole-blood donors, paid plasma donors from California are reported to have a higher frequency of positive tests for infectious disease markers than do blood donors who volunteer[5]. Plasma used to manufacture blood products such as clotting factor concentrates or immune globulins is subject to viral inactivation processes. In contrast, cellular blood components cannot be processed with current viral inactivation methods. Those who collect plasma from paid donors justify payment as necessary to provide the volumes of plasma required for plasma fractionators, and point to the absence of direct evidence of any increased risk of viral transmission. Maintaining the supply of fractionated blood components may be another rationale for acceptability of paid donations for use in manufacture of components, although it is an issue that is not well documented.

Potential blood donors with a diagnosis of hemochromatosis may be altruistic, but they may be motivated to donate blood for reasons other than altruism. There are several potential benefits that could accrue to hemochromatosis patients undergoing therapeutic phlebotomy. Although no money changes hands at the time of blood donation, hemochromatosis patients are donating blood units for personal advantage, i.e., to benefit their own health. Furthermore, sparing hemochromatosis patients or their insurance companies the cost of the therapeutic phlebotomy could be perceived as a direct (or indirect) financial incentive for donation. Even token, non-monetary incentives (such as T-shirts given to blood donors) are accompanied by increased rates of serologic markers for viral disease, medical deferrals, and self-deferral of donors at blood drives[6]. Thus, concern arises over whether blood donated by people with hemochromatosis would be as 'safe' for the allogeneic blood supply as blood given by persons motivated only by the spirit of voluntarism. National blood policy mandates that blood for transfusion be as safe as possible. Therefore, the burden of proof is not to demonstrate that blood from a given source is less safe, but to demonstrate that it is as safe as the current supply.

It is unclear why volunteer blood appears to be safer than 'paid' blood. The act of accepting compensation should not in itself render blood any less safe than does the presence of a particular genetic polymorphism. Among other reasons, it has been proposed that payment makes donors less candid about risky practices, but this has never been proved. The frequency of viral markers has always been 3–10 times greater in paid donors than in volunteers. The US blood supply is extraordinarily safe, and the overall risk of acquiring a bloodborne virus in the US is <1 per 100 000 units transfused[7]. However, a short but measurable period of time exists during which bloodborne viral infections are serologically 'silent' and undetectable by present-day blood testing. No data suggest that patients with hemochromatosis would deliberately mislead blood banks about their risk factors for infectious diseases, as could be the case for paid commercial blood donors. A study to prove that hemochromatosis patients as allogeneic donors would be equally safe or less safe as volunteer donors would be lengthy, difficult to perform, and prohibitively expensive. Even among volunteers who qualify to donate after pre-donation screening, a small but significant percentage (0.4%) have engaged in a behavior considered high-risk for acquiring a transfusion-transmissible infection within three months before blood donation, yet they chose not to reveal this history before giving blood[8]. This topic has not been studied among donors who are given some incentive, such as hemochromatosis patients who might serve as regular blood donors. The US public demands that donors of blood for direct allogeneic transfusions be proven individually and as a group to be universally 'safe.'

Several problems can arise when blood is labeled with a 'disease association' in accordance with federal regulations, in addition to the concerns over donor motivation and infection risks of blood transfusion. First, though the labeling is not to imply that hemochromatosis patients are

ill or that their blood is unsafe as a result of the genetic polymorphism, clinicians and their patients may perceive such blood as 'unacceptable' because it is labeled with a 'disease association.' Certainly, if hemochromatosis blood is transfused, the transfusing physician should be aware of its known and unknown advantages and disadvantages. A decision to use hemochromatosis blood for a hospital's allogeneic transfusion supply is made by the hospital's blood bank director, but he/she must justify its use to clinicians who know that 'volunteer' blood is ordinarily transfused and is generally available. Blood collection centers that provide blood to hospitals recognize this lack of universal acceptability to practitioners. Because specially labeled blood may be returned and even expire due to poor acceptance, many blood centers do not deem the collection and labeling of hemochromatosis units to be worthwhile. Second, the special handling required for units from individuals with hemochromatosis presents operational problems for large blood centers that collect hundreds or thousands of units per day. The segregation and labeling creates an additional 'control point' at which there is possibility for clerical error. Any such exception to standard operating procedures or addition of alternative processing systems makes room for error. With regulatory emphasis on Good Manufacturing Practices for licensed facilities and increased scrutiny by regulatory and accrediting agencies of issues that concern blood quality and safety, the correct labeling of blood is an issue of utmost importance.

Hemochromatosis patients as allogeneic blood donors

It is useful to consider the blood collection process (including pre-donation screening and blood testing) in the US to better understand problems that could be encountered by a patient with hemochromatosis who is contributing to the allogeneic blood supply. Numerous units of blood from persons with hemochromatosis are already collected and transfused in the US each year. Certainly, many of the annual 8 million volunteer blood donors have undiagnosed hemochromatosis and receive unrecognized treatment of their disorder with each blood donation. If, as estimated, 5 people per 1000 are calculated to be homozygous for the hemochromatosis gene[9], then the disorder is vastly under diagnosed. Furthermore, use of a serum iron assay to screen Caucasian blood donors at risk for developing hemochromatosis is preferred to waiting for evidence of disease complications, and is also a cost-effective practice[10]. Consider the hemochromatosis patient who presents with the intent of therapeutic phlebotomy for iron overload. To donate his or her blood for the allogeneic supply, patients with hemochromatosis must meet all the same requirements as volunteer blood donors. Potential blood donors must undergo a pre-donation screening process, including a focused medical history and check of vital signs to qualify for blood donation. In the medical history, donors are asked about their general health. Donors may be disqualified from donation to protect their own health or the health of the potential blood recipient. Mobile blood collection units dispatched from a large center are not staffed by a physician and are not well-equipped to handle a life-threatening medical emergency for a donor on-site. The transient blood volume depletion associated with whole-blood phlebotomy could be risky for hemochromatosis patients with advanced iron overload and end-organ damage if they are unable to compensate for a rapid change in blood volume, e.g., by increasing their cardiac output. Questions regarding donor acceptability or problems with a donation are generally referred to medical staff by telephone. Approximately 27% of patients with hemochromatosis are asymptomatic at the time of diagnosis[11]. Fatigue is reported commonly by patients with hemochromatosis (38%), but is not ordinarily cause for disqualification from donation. Medical disorders that could affect the safety of donation for the donor and/or the safety and quality of the donated blood commonly occur in older persons (>70 years) with hemochromatosis. Diabetes mellitus and hepatic cirrhosis occur in 14% and 22%, respectively, of hemochromatosis patients diagnosed in routine medical care delivery, and these medical problems disqualify potential donors for the allogeneic supply. Likewise, blood from a hemochromatosis patient who reported recent jaundice or a history of hepatitis could not be used. It is well accepted that hemochromatosis patients, in particular those who are older and those with hepatic cirrhosis, have an increased risk to develop hepatocellular carcinoma. Individuals with a history of malignancy, other than localized cancers that have not required chemotherapy, have traditionally been excluded as donors for the allogeneic blood supply. Because primary hepatic cancer accounts for a high proportion of deaths among persons with hemochromatosis, should individuals with known increased risk be encouraged to donate? The risk to the recipient of a transfusion from a hemochromatosis patient with defined or occult cancer is unknown. Other individuals who undergo phlebotomy therapy, who are predisposed to malignant conditions, such as those with polycythemia vera, are not considered acceptable donors due to concern over transmission and engraftment of a malignant clone of cells. Generally, screening of volunteers for blood donations is

performed by trained nurses or phlebotomy staff. From a medical and legal standpoint, if blood from patients with hemochromatosis is to be used for a hospital's supply, initial medical scrutiny of hemochromatosis patients by a qualified physician would be prudent to insure discovery of details not immediately obvious to the patient or donor interviewer that would compromise the safety or quality of the donated blood.

Suitability of hemochromatosis blood for allogeneic transfusion

Screening for bloodborne disease

All volunteer blood donations are screened for bloodborne viruses using sensitive antibody screening assays for the human immunodeficiency virus (HIV), hepatitis C virus (HCV), hepatitis B virus (HBV), human T-cell lymphotropic viruses I and II, and syphilis. Alanine aminotransferase (ALT) testing of blood was used for a decade in the US as a surrogate marker for what was previously called non-A, non-B hepatitis. This marker is no longer required but is still widely used (see below). Though the use of donor incentives may increase the rate of infectious disease markers in blood, has the blood drawn therapeutically from a patient with hemochromatosis been shown to be less safe for transmitting retroviruses or hepatitis viruses than blood drawn from a volunteer donor population? Are donors with hemochromatosis at any greater risk than healthy volunteers for acquiring bloodborne transmissible agents? There are few data on this topic in this patient group in the medical literature, and that which exists yields conflicting information.

In a retrospective study, Adams et al. found there were no hemochromatosis patients among more than 200 persons who had evidence of chronic hepatitis or positive serology for HBV or HCV[11]. However, among 272 French hemochromatosis patients in a liver disease clinic[12], the prevalence of HBV infection markers, especially antibodies to hepatitis B core antigen (anti-HBc), was significantly increased in patients with hemochromatosis (complicated or not by primary hepatic cancer) compared to a blood donor population (Table 55.1). The reason for low rates of HBV markers in blood donor controls may be that many are repeat donors who have been previously screened and found negative. As noted earlier, blood donors undergo an intensive medical selection process before their blood is used for the allogeneic supply. In contrast, the incidental discovery of higher rates of HBV markers in hemochromatosis patients may represent the frequency in an unselected, unscreened population. Although the units testing positive for past or present HBV infection would be discarded, the rates are still of some concern because they suggest that this group of subjects is at higher risk of having other infectious agents. These rates also suggest that, as a group, the proportion of hemochromatosis donors who harbor hepatitis infections but test negative by screening assays could be higher than among volunteer blood donors. That is, their rates of low-level viremia and window-period infections might exceed those expected among volunteer blood donors.

Table 55.1. Markers associated with hepatitis B virus infection among French blood donors and patients with hemochromatosis

	n	HbsAg, %[a]	anti-HBc, %[b]
Blood donor controls	255 029	0.07	3.7
Hemochromatosis without HCC[a]	239	1.1	13 3
Hemochromatosis with HCC[a]	33	6.2	16.6

Notes:

[a] Frequency of hepatitis B surface antigen.

[b] Frequency of antibodies to hepatitis core antigen.

Data regarding other markers of infectious disease are similarly sparse. A high prevalence (5.8%) of HCV infection confirmed by recombinant immunoblot assay (RIBA), was reported in a cohort of 150 patients with hemochromatosis in France. However, it was learned upon questioning that all these individuals had received transfusions during prior orthopedic procedures[13]. No direct comparison of the prevalence of HIV in hemochromatosis patients and in blood donors has been performed. In general, most hemochromatosis patients in these studies have symptoms attributable to iron overload disease. Asymptomatic individuals with hemochromatosis who would be more suitable as blood donors and the frequency of infectious markers in this group might be different.

ALT testing

Because sensitive and specific hepatitis screening assays are available, the US Food and Drug Administration discontinued the requirement for screening all blood units by 'non-specific' testing for the hepatic enzyme ALT in 1995. ALT and anti-HBc testing to screen and exclude blood donors were adopted as a 'surrogate' markers in the 1980s in the US in an attempt to identify donors at high risk for hepatitis viruses, in particular the non-A, non-B (NANB) agent. Implementation of these surrogate tests for screening the blood supply was expected to decrease hepatitis

transmission by approximately one-third. In fact, the estimated rate of transfusion-transmitted hepatitis in the US fell to 2%. However, because antibody testing was developed in 1990 and was implemented rapidly for screening the blood supply for NANB hepatitis virus (now known as HCV), and because HCV accounts for more than 90% of the transfusion-transmissible hepatitis virus infections, the value of the ALT test for donors was deemed to be no longer worthwhile. Nevertheless, many blood collection centers continue to apply the ALT test to the blood units they collect in order to continue international exportation of blood components (particularly plasma) to European manufacturers, who still require ALT testing. Elevations in serum ALT concentrations in potential blood donors with hemochromatosis are expected, given that hepatic disease is the most common abnormality in this group of individuals[14]. Plasma from such persons would not be suitable for exportation. Yet, when increased ALT concentrations occur in patients with hemochromatosis, they are generally less than twice the upper limit of normal[15]. Therefore, although ALT elevation would not be a major factor in preventing the transfusion of hemochromatosis blood in the US, hemochromatosis patients with elevated concentrations of hepatic enzymes might need to seek out those donor centers that no longer measure ALT concentrations.

Bacterial contamination

Individuals with hemochromatosis sometimes have increased susceptibility to bacterial infection with organisms such as *Yersinia enterocolitica* and *Vibrio vulnificus*. However, episodes of bacteremia and sepsis are uncommon in persons with hemochromatosis, and a causal association between these two disorders remains difficult to establish[16]. Because *Y. enterocolitica* infections can cause no symptoms or only minimal gastrointestinal disturbances, it would be difficult to ascertain whether, or to what degree, bacteremia is more common in potential hemochromatosis donors than in volunteers. No standardized, sensitive, and rapid screening procedure exists to detect bacterial infection in blood units, but *Y. enterocolitica* thrives in stored red blood cell units. This causes increasing concern for quality and safety of blood units, because approximately two transfusion-associated deaths per year are attributable to this organism and the risk is considered to be greatly underestimated[17]. If there is substantial bacterial growth in a red blood cell unit, the visual appearance of the unit after storage can be abnormal; it may appear darker than usual or contain clots. Routine testing for bacterial contamination is not performed prior to release of the blood. Though careful assessment and prudence is warranted for all units issued to patients, a blood unit that is slightly abnormal in appearance may not be detected by the technical staff issuing blood, even when a high index of suspicion exists.

Quality

Would blood drawn from hemochromatosis patients have any properties considered advantageous or disadvantageous, compared to blood from normal healthy donors? Red blood cell phenotypes among persons with hemochromatosis are expected to be largely those characteristic of Caucasians of Northern European descent; an unusually high proportion of rare red blood cell types would not be expected. Because weekly or biweekly therapeutic phlebotomy is performed during the first year of therapy in many patients, the proportion of 'neocytes' (young red blood cells) in each bag of blood would be expected to increase. In theory, 'neocytes' may be a desirable cell type for transfusion, particularly in patients with chronic hemolytic anemia, to prolong the interval between transfusions and thus decrease iron accumulation. However, there are limited data support this hypothesis[18].

Excessive iron storage in persons with hemochromatosis has been implicated in the pathogenesis of hepatic fibrosis and cirrhosis[19]. Iron-induced peroxidative injury to phospholipids in cellular and subcellular membranes is thought to lead to cell injury in iron-overload states. Theoretically, impaired erythrocyte membrane integrity could result in impaired survival or function of transfused hemochromatosis red blood cells, but this has not been studied. Presently, there is no evidence of any quantitative or qualitative defects in blood cells from hemochromatosis patients. In addition, there are no facts available regarding the use or quality of hemochromatosis plasma for fractionation. Blood drawn from hemochromatosis donors contains 15–20 times more free iron than that of normal subjects[20, 21]. Hypothetically, a greater load of transfused, non-protein-bound iron might be advantageous for recipients who need supplemental iron. Conversely, the extra iron could be construed as deleterious due to its prooxidant effects. Although no studies in humans have been performed to demonstrate the latter, transfusion of iron has caused nucleic acid oxidative damage in the rat[22, 23]. The total protein-bound and free iron remaining in concentrated red blood cell units derived from a hemochromatosis patient would be lower than that reported in fresh whole blood, because red blood cell units are stored in anticoagulant-preservative solutions and often contain only small amounts of residual plasma. Any beneficial or adverse effects of extra free iron in the blood supply are

largely academic. It is very unlikely that any individual blood recipient would receive enough transfusions of hemochromatosis blood containing sufficient free iron to produce any significant detriment or benefit.

Informed consent for transfusion

Mentally competent transfusion recipients should be advised and give written informed consent before receiving transfusion of any blood in non-emergent situations. This policy has been recommended by the American Association of Blood Banks since 1986. The advent of acquired immunodeficiency syndrome (AIDS) generated much public anxiety over the source of blood for transfusion. If hemochromatosis blood is incorporated into a hospital's supply, should the patient give consent before receipt of hemochromatosis blood, just as they do for directed or autologous donations? Because hemochromatosis blood is not, strictly speaking, derived from a 'volunteer' donor, it would be legally prudent to inform patients that they might receive hemochromatosis units or the alternative volunteer allogeneic units.

Augmentation of the blood supply

If blood from patients with hemochromatosis were considered to be 'volunteer' blood, might these extra contributions produce a substantial increase in the US blood supply? Approximately 14 million units of blood are donated annually by approximately 8 million volunteer donors in the US. This supply is used by 4 million patients who receive more than 23 million blood components every year. There are an estimated 1 million Americans with hemochromatosis. After diagnosis of hemochromatosis, a 'routine' therapeutic phlebotomy schedule is usually started. For patients with a very heavy iron body burden ($>$30 g), it may take more than two years of weekly phlebotomies to achieve iron depletion. In a study by Edwards and colleagues[24], 20 men required an average of 68 units of therapeutic phlebotomy to achieve depletion of iron stores; 10 women required an average of 25 units of phlebotomy. After total mobilization of iron, three or four units of phlebotomy are usually needed each year to maintain iron depletion.

Among these estimated 1 million patients with hemochromatosis, approximately 750 000 would be older than 17 years, making them age-eligible as blood donors. As noted above, 27% of patients in a recent study of hemochromatosis patients were asymptomatic at diagnosis. However, this percentage would likely increase if screening for hemochromatosis with serum iron testing were implemented widely. To make a conservative estimate, this would introduce 202 500 donors into the blood donor population, if the asymptomatic 27% of 750 000 age-eligible hemochromatosis patients qualified as 'volunteer' donors after pre-donation screening. If only 1% of this population were phlebotomized 26 times (i.e., in the first year of biweekly therapeutic phlebotomy), after phasing in all who were diagnosed with hemochromatosis, this would add 52 650 blood units to the annual blood supply. This would represent an increase of nearly 0.4% over the current 14 million donations in the US. Although these estimates could be criticized for originating from the prevailing rate of hemochromatosis among Americans, routine diagnostic screening for hemochromatosis in at-risk populations would inflate the number of potentially usable blood units because asymptomatic persons with hemochromatosis would be diagnosed incidentally.

Is the extra blood that could be generated by the use of hemochromatosis blood units necessary? A nationwide survey of blood collection and utilization (National Blood Data Resource Center, American Association of Blood Banks, Bethesda, MD) has projected that, if current trends continue, transfusion demand will exceed the available supply in the year 2000. Although blood shortages arise intermittently, annual red blood cell usage in the US has been stable over the last several years. Only about 5–7% of eligible blood donors give blood each year. Although this estimate seems low, blood shortages may reflect a maldistribution of various blood groups rather than an absolute shortage of donors. New biological drugs, e.g., erythropoietin, provide attractive alternatives to allogeneic transfusion for certain patients. Research involving extended storage of red blood cells to prolong shelf-life and to cushion periods of relative shortage is being performed. Military and some civilian hospitals keep depots of frozen red cells, an effective, albeit expensive strategy in anticipation of shortages. With recent scrutiny of the blood supply and vigilance concerning potential transfusion-transmissible agents (e.g., new variant Creutzfeldt–Jacob disease), recalls of manufactured blood products have reduced supplies and increased expense of such blood-derived products as albumin. Thus, plasma derived from hemochromatosis donors could be more desirable than their red blood cells.

Use of hemochromatosis blood in the volunteer blood supply would represent cost savings to the hemochromatosis patient and a donor incentive. Presently, therapeutic phlebotomy costs an average of approximately $100 US dollars per phlebotomy, and the estimated cost to the individual hemochromatosis patient or the patient's insurance company amounts to approximately $2600US for the first

Table 55.2. Results of a questionnaire from countries with high prevalence of hemochromatosis regarding the use of blood from individuals with hemochromatosis in their allogeneic blood supply[a]

	Canada	UK	Australia	US
Permitted in blood supply	Y	Y	Y	Y[b]
Patients considered 'volunteers'	Y	N	Y	N
National policy to permit transfusion of hemochromatosis blood?	Y	N	Y	Y[c]
How long has it been policy?	5 years	NA	3–4 years	NA
Hemochromatosis patients pay for phlebotomy?	N	N	N	Y[d]
Is/Was transfusion advanced by hemochromatosis advocacy groups?	Y	Y	N	Y
Differentiates those with and without symptoms? [e,f]	N	NA	N	N
Labeled with disease association?	N	NA	N	Y
Labeling creates a problem	N	NA	N	Y
ALT test required?	N	N	N	N[g]
Adverse events reported with transfusion of hemochromatosis blood	N	NA	N	N

Notes:

[a] Y = yes, N = no, NA = not applicable.

[b] Blood may be used in the allogeneic supply, but must be labeled with 'disease association.'

[c] Individual hospitals set their own policies. The American Association of Blood Banks, a professional standard-setting organization recommends against the use of hemochromatosis blood for the allogeneic supply.

[d] Patient or health insurance/third-party reimbursement unless blood-collection center does not charge the hemochromatosis patient.

[e] Allogeneic blood donors qualify after undergoing a health history questionnaire prior to each donation.

[f] Patients with hemochromatosis who have symptoms may not qualify to donate blood for the allogeneic supply in the US (see text).

[g] Alanine aminotransferase (ALT) testing not required by law, but often performed.

year of therapy. If one were to use the estimates above, this represents a collective US cost savings of more than $5 000 000US for hemochromatosis patients and their third-party payers. The issue of using hemochromatosis donor blood to augment supply has been raised internationally also. In Wales, for instance, it has been proposed that donors undergo screening for hemochromatosis to increase the number of donations by 22%[25]. In the Brittany region of France, it was estimated that approximately 6250 liters of blood could be collected each year if hemochromatosis blood were used, increasing the blood supply by almost 30% for that region[26]. Potential contributions to the blood supply by individuals with hemochromatosis are not trivial, and augmentation of blood supplies has been an influential argument for those who advance the unrestricted use of hemochromatosis blood.

Use of hemochromatosis blood in other countries

Approaches to the collection and use of blood from hemochromatosis patients differ among countries (Table 55.2). Individuals with hemochromatosis have been well-received as donors for years in Sweden and Australia. The Canadian Red Cross (CRC) Society Blood Transfusion Service acted in 1992 to accept blood from hemochromatosis donors who are otherwise suitable. The CRC requires that hemochromatosis blood used in the allogeneic supply be drawn at a CRC blood collection center according to their guidelines. If drawn at a hospital, physician's office, or other site outside of the CRC, the blood cannot be used for transfusion. In practice, most of the hemochromatosis phlebotomies are not captured by the CRC system, and therefore these units are not entered into the allogeneic supply.

In countries such as the United Kingdom (UK) where there is no charge to the patient for therapeutic phlebotomy, there is no financial motivation on the part of the hemochromatosis patient to donate. However, this does not mean that therapeutic phlebotomies are used for the allogeneic blood supply. To the contrary, national policy in the UK stipulates that these donations cannot be used because the donors are not considered 'volunteers.' The reasoning behind this conservative medical approach in the UK is similar to that in the US: hemochromatosis patients are not completely altruistically motivated to

donate. Some individual and hospital-based blood donor centers outside the American Red Cross system do accept hemochromatosis patients as blood donors. However, because approximately 80% of the US blood supply is obtained by the American Red Cross or America's Blood Centers (large community blood centers), the required labeling of hemochromatosis blood precludes its general use for the reasons stated above.

Novel research applications for hemochromatosis blood

Blood donated by patients with hemochromatosis can be used for research if it tests negative for markers of transfusion-transmissible diseases. A novel use of the reticulocytes derived from individuals with hemochromatosis during frequent therapeutic phlebotomies is to propagate *Plasmodium vivax,* the most widespread cause of human malaria. This parasite uses the Duffy blood group antigen present on the red blood cells of most Caucasians as a specific receptor to invade the cells and subsequently cause malaria. All attempts to develop an in vitro method for culture of this parasite have failed. However, *P. vivax* thrives in reticulocyte preparations[27]. Reticulocytes harvested from blood donated by hemochromatosis patients appear to be ideal for the propagation of *P. vivax.* During the course of therapeutic phlebotomy of patients with hemochromatosis, reticulocyte counts increase. When the reticulocyte count exceeds 3%, as is usually the case after 5–6 weeks of weekly phlebotomy, an enriched layer of reticulocytes suitable for invasion by *P. vivax* can be harvested and used as a preparation for the production of many schizonts. A potential application for use of hemochromatosis donor blood is the manufacture of hemoglobin-based blood substitutes that could be treated to inactivate infections agents. Such formulations have not yet entered routine clinical use in the US, though clinical trials of red blood cell substitutes are on-going. There may be additional uses unrelated to transfusion for these units of hemochromatosis blood.

Donations of hemochromatosis organs and tissues

For decades, the medical and humanitarian value of donating and transplanting organs and tissues has been recognized. Like the professionals in blood banking, those involved with organ and tissue banking strive to maintain the availability of safe and effective donated cells and tissues. The American Association of Tissue Banks is a nonprofit organization that sets professional standards to provide guidance for individual tissue banks[28]. Because demand for organs greatly exceeds supply, patient advocacy groups and some collectors promote enlarging the donor pool by including cadaveric donors who would have been excluded previously due to advanced age, coexisting illnesses, or other factors thought to pose a risk for transplantation to the recipient. Like blood banks, organ and tissue collectors rely on peer-generated standards for donor suitability based on medical, sexual and social history and physical examinations. Living or cadaveric donors whose history reveals risk of harboring viral hepatitis or HIV are excluded. Autopsy findings and laboratory tests are also important in determining the acceptability of organ and tissue donations. Acceptance criteria include baseline laboratory tests such as viral serologies and surveillance blood cultures that are outlined by the United Network for Organ Sharing[29]. However, although organ/tissue bank technical staff perform preliminary screening of donors to evaluate their acceptability before procurement and processing, tissues and organs are not released for transplantation without final review of donor suitability by the tissue bank medical director or licensed physician designee. Both the safety and quality of the tissue are evaluated from the medical history and medical record review. The evaluation and reasons for donors acceptance or exclusion are documented in the donor record and in a release/disposition statement. Ultimately, the final decisions on the suitability of donor organs or tissues are made by the transplantation programs.

How is a subject with hemochromatosis viewed as a potential organ or tissue donor? In contrast to their status as blood donors, subjects with hemochromatosis are clearly 'volunteers' for organ and tissue donation. Unlike donating blood, there is no personal gain for an individual with hemochromatosis, living or dead, by donating organs and tissues. Thus, people with hemochromatosis are no different from other volunteer organ or tissue donors in regard to risks for transmission of viral diseases. A hemochromatosis patient with hepatocellular carcinoma would be deemed unsuitable for organ or tissue procurement. In general, however, other hemochromatosis patients would not be considered unfit for organ or tissue donation unless the tissue quality were compromised by disease. Assuming that infectious agents have been excluded, the liver of a hemochromatosis patient would receive the greatest scrutiny of any organ or tissue before its release for transplantation. The presence of iron overload, cirrhosis, or hepatocellular carcinoma would certainly affect the quality of the liver for transplantation. The heart and pancreas would warrant careful examination before release,

because these also could be affected adversely by iron overload. Medical urgency may be considered by an individual or organ procurement organization and might influence the decision to use an organ. Unlike blood, organs and tissues have no labeling requirement concerning the donor's disease association. When the potential advantages outweigh the potential risks, the organ is harvested and transplanted.

In contrast to organ and tissue donations, genetic pedigree of a donor is important for semen donation. Family and personal medical histories are integral to the donation procedure. Specific criteria for anonymous semen donation are detailed elsewhere[27]. Because hemochromatosis is a genetic defect with potential risks for the uninformed offspring of an artificial insemination, the presence of hemochromatosis or a family history of hemochromatosis ordinarily disqualifies a man with hemochromatosis as an anonymous semen donor. However, because symptoms of hemochromatosis would be generally absent in young men (usually those $<$40 years of age) who donate semen anonymously, it is unlikely that hemochromatosis would be detected in a prospective semen donor unless serum iron screening studies were performed routinely.

Conclusions

By current definition, hemochromatosis donors who undergo therapeutic phlebotomy are not considered to be volunteers. At this time, medical and scientific data are incomplete; the safety and quality of blood derived from therapeutic phlebotomies of hemochromatosis patients are not unequivocally established. In addition, federal regulations require blood drawn to promote the health of the donor be labeled by 'disease association.' Until studies of the viral safety and characterization of this blood for transfusion are performed, the most medically prudent position, despite potential economic advantages, is the recommendation against the unlabeled use of hemochromatosis donor blood in the allogeneic volunteer supply. Unrestricted use of hemochromatosis donor blood without specific informed consent of the recipient also presents potential legal liability. Organ and tissue donations by individuals with hemochromatosis are voluntary and are evaluated by the same criteria applied to all donations, albeit with special medical attention given to organs whose quality might be adversely affected by iron overload. Individuals with hemochromatosis are ordinarily excluded from anonymous semen donation, because the current semen banking system excludes individuals with genetic disorders.

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Genetic counseling for hemochromatosis

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Introduction

Hemochromatosis often eludes diagnosis, although its early detection and treatment by phlebotomy can prevent complications of iron overload and premature death[1]. Thus, screening persons over 20 years of age by measurement of serum iron concentration and transferrin saturation has been proposed[2]. The recent cloning of a gene (*HFE*), mutations of which are found in 60–100% of Caucasian patients presenting with hemochromatosis, provides the opportunity for pre-symptomatic DNA-based genetic testing[3–9]. Genetic counseling and education are strongly recommended as integral components of screening and genetic testing programs[2, 10].

Genetic counseling has been defined as 'the process by which patients or relatives at risk of a disorder that may be hereditary are advised of the consequences of the disorder, the probability of developing or transmitting it and of the ways in which this may be prevented, avoided or ameliorated'[11]. This chapter provides the basis for counseling patients diagnosed with and those at risk of developing hemochromatosis. Although we rely on basic principles of genetic counseling, recommendations for the use of genetic testing for late-onset disorders will also be considered[10]. Our recommendations will assist healthcare providers in educating and counseling patients with hemochromatosis and family members at risk. As knowledge of the genetic etiology of hemochromatosis and iron overload increases, and our ability to predict, treat, or cure hemochromatosis, these recommendations may change.

Identifying those at risk

When a person is diagnosed to have hemochromatosis, the healthcare provider's immediate concern is management of that person's illness. In addition, the provider must be aware that siblings, children, and other family members may also have this disorder. Collecting detailed family history of disease information is helpful in identifying those at risk and in assessing patients in whom the diagnosis is not clear, e.g., those with abnormal serum iron or ferritin concentrations that may be due to chronic viral hepatitis, non-alcoholic steatohepatitis, or alcoholic liver disease[12]. The rationale for obtaining family history of disease information is documented in a study of Canadian patients, in which additional persons with hemochromatosis were detected in 3.6% of the families of probands[13]. A multivariate discriminative analysis revealed that hepatic cirrhosis, diabetes mellitus, and arthritis predicted the diagnosis in 48% of these cases. Although recording a detailed family history of disease should be part of the history and physical examination routine for the evaluation of all patients, it is often not obtained or is incomplete. As more genetic tests become available to identify persons at risk for genetic disorders (including variants of hemochromatosis), it is increasingly necessary to obtain complete family history of disease information to identify those who would benefit from genetic testing.

Collecting family history of disease information

The quality and completeness of family history of disease information gathered by health care providers varies widely. The authors have found that these are increased if the history is obtained in a structured manner[14, 15]. The information can be obtained by interview or by use of self-administered forms in which diseases that occurred in each relative over four generations are checked by the patient at home. The latter method requires less time of the health care provider, and the amount and quality of the information obtained is greater than that acquired by

interview while the patient is in the physician's or counselor's office.

An accurate diagnosis is needed for all subjects in a family reported to have a disorder. Although family history of disease information obtained for many common illnesses can be quite sensitive and specific[16], the diagnosis should be confirmed by obtaining medical records or re-examining the persons believed to be affected. In hemochromatosis families, an effort should be made to evaluate for diseases that are part of the clinical phenotype of hemochromatosis and iron overload, including hepatic cirrhosis, arthritis, diabetes mellitus, hepatocellular carcinoma, and cardiomyopathy[17, 18]. Disorders the frequency of which may be increased in family members who are heterozygous for hemochromatosis include diabetes mellitus, colorectal neoplasia, hematological malignancies, and gastric cancer[19]. Other disorders in which iron overload is often seen include alcoholism[20], porphyria cutanea tarda[21–23], and sideroblastic anemia[24].

The gender of all persons for whom information is obtained should be recorded. Although hemochromatosis is not gender-specific, there may be three-fold more men presenting with hemochromatosis than females[18]. On the average, men accumulate twice as much excess iron as women, and have a higher incidence of hepatic cirrhosis and hypogonadotrophic hypogonadism. However, women have arthropathy as frequently as men, and often have a higher frequency of fatigue and hyperpigmentation[18]. Because full phenotypic expression of hemochromatosis and iron overload occurs in women, they should not be overlooked when evaluating a family in which hemochromatosis occurs.

The clinical symptoms of hemochromatosis can be present at diagnosis in adolescents (rarely) and in adults of all ages[25]. Neonatal hemochromatosis, a rare disorder due to accumulation of excess iron in utero, does not have the same genetic etiology as 'classical' hemochromatosis[26, 27], and the two disorders should not be confused. Thus, the outline for genetic counseling presented here is directed toward adult-onset hereditary hemochromatosis. Because the frequency of hepatic disease, diabetes mellitus, and arthritis at the time of diagnosis usually increases with age, it is important to record the date of birth of all persons for whom family history of disease information is obtained. This is useful in determining those who should receive screening or genetic tests.

Hemochromatosis is most prevalent in Caucasians of Northern European descent, and its origin in persons of Celtic origin has been postulated[28]. The clinical and pathological findings and genetic etiology of iron overload in Africans[29] and African Americans[30] are unlike those seen in Caucasians. The frequency of the C282Y mutation among various racial and ethnic groups is consistent with the prevalence of hemochromatosis in these groups[7, 31–33]. The highest frequency of this Caucasian mutation is found among the Irish (10%). Other groups in which the frequency of the C282Y mutation is also relatively great include the Danes (9.6%), Icelanders (6.7%), Norwegians (6.4%), British (6.4%), and Bavarians (5.6%). Like Africans and African Americans, natives of the Americas, Askenazi Jews, and Chinese rarely have the C282Y mutation. Thus, obtaining information about the racial and ethnic origin of the family being assessed for risk of hemochromatosis is also helpful.

Consanguinity

When estimating any individual's risk of developing a genetic disorder (particularly an autosomal recessive one), it is important to determine if marriage has occurred between close relatives; one needs to determine the genetic relationships of the married couples in a family. Because the carrier frequency of the gene(s) for hemochromatosis is approximately 0.1, a known carrier has a one in ten chance of marrying another carrier if that person mates with someone in the general population. If the person marries a first cousin, there is a one in eight chance that the cousin carries the same gene. The increased risk of having children who are homozygotes will depend on the family relationship of the two individuals in a consanguineous mating.

Constructing the pedigree

After obtaining family history of disease information, one should construct a pedigree using standardized human pedigree nomenclature[34]. Although the pedigree can be drawn by hand, we use a computer program developed 'in house'[35]; there are several computer programs commercially available for the same purpose. After review, a copy is sent to the person who provided the information, and the person is queried for additional information or asked to confirm certain items. When the person providing the family history of disease information and his/her relatives have a copy of their pedigree, they usually make corrections or provide additional information. Often, the diagnosis for a family member is uncertain. In such cases, responsibility for obtaining the medical records is placed on the person whose medical history is in question, or on the next- of-kin for whom a risk assessment is requested. After review of the pedigree and confirmation of diagnoses of family members, a risk assessment is generated and a

genetic counseling session is scheduled. The American College of Medical Genetics Laboratory Practice Committee recommends that a molecular genetics laboratory report include a copy of a pedigree if a family study was conducted[36]. Because a test for C282Y mutation analysis is often requested to assess the risk of hemochromatosis in the family member of a proband, a pedigree should be included with the report.

Screening for iron overload and genetic testing

Recent guidelines recommend general screening of all persons over the age of 20 years for hemochromatosis by measurement of serum iron concentration and transferrin saturation[2]. The rationale for these recommendations is that (i) hemochromatosis often is not diagnosed until organ damage due to iron overload has occurred; (ii) screening tests are inexpensive; and (iii) a relatively benign treatment (therapeutic phlebotomy) is available that can prevent iron-associated organ damage and prolong life. These recommendations were written before the C282Y mutation was discovered[3]. Although the C282Y mutation is not present in all patients presenting with hemochromatosis[3–9], the availability of a DNA-based genetic test that predicts disease before symptoms appear can be used to select those who should be evaluated further. One problem with the iron overload screening and genetic tests is that the penetrance of the gene(s) is not 100%, and many C282Y homozygotes never develop disease due to iron overload. Moreover, other gene(s) may be involved. The maximum benefit from the use of iron overload screening using biochemical and DNA-based genetic tests cannot be realized until the number of genes involved in the causation of hemochromatosis has been defined, until the penetrance of these genes is known, and until the sensitivity and specificity of the biochemical and genetic tests are validated. Thus, it may be inappropriate to begin population screening for iron overload or genetic predisposition until this information is available. However, this does not preclude the use (or necessity) of performing these tests in evaluating individuals and in family studies.

The process of genetic counseling

The manner in which the genetic counseling session is conducted is determined by the status of the patient. The information conveyed and concerns of the patient depend on whether the patient is affected with hemochromatosis, is at risk to develop clinically significant hemochromatosis with iron overload, or is not at risk. Our suggestions for conducting these sessions are illustrated with actual cases referred to our laboratory for genetic testing and counseling.

Newly diagnosed patients

The first concern of the physician who has diagnosed a person to have hemochromatosis is the institution of medical management for iron overload and its complications. Many patients (and healthcare personnel) have never heard of hemochromatosis or understand the consequences of having this disorder. Thus, an education and genetic counseling session is needed, during which the counselor explains the nature of the disease, the associated medical complications that can occur, and the need to reduce body iron stores to prevent further organ injury. If the diagnosis of hemochromatosis has been made early in the course of the disorder, the patient's initial anxiety can be reduced by emphasizing that, with early diagnosis and proper medical management, affected persons can live a normal or near-normal life without developing complications associated with iron overload[19]. For patients whose hemochromatosis and iron overload are diagnosed relatively late, the optimism about the benefits of treatment and future health must reflect their individual medical circumstances and reasonable expectations of treatment.

The next step is to determine who in the family may be at risk. Review the pedigree with the patient and discuss the risk that siblings and children have to hemochromatosis and iron overload. Each sibling has a 25% chance of also being homozygous for the gene(s) that cause hemochromatosis and a predisposition to develop iron overload. Emphasize to the patient that their siblings should undergo initial biochemical testing for hemochromatosis and iron overload (using measurement of serum iron concentration, transferrin saturation, and serum ferritin concentration) and DNA-based genetic testing (using mutation analysis to detect the C282Y mutation). For family members to learn that a sibling has been diagnosed to have a disorder, and that they may also be at risk to have the same disorder, is understandably disturbing to some. These persons will also need counseling prior to performing biochemical or DNA-based genetic testing.

A person recently diagnosed to have hemochromatosis may have children at risk to have hemochromatosis or iron overload. Each child of persons with hemochromatosis is an obligate heterozygote, although it is unclear at this time which hemochromatosis-associated mutation they may possess. Nonetheless, the risk that the child will be homozygous for the hemochromatosis gene(s) can be estimated.

Any child will have received one copy of a hemochromatosis gene from the affected parent. Because approximately 1 in 10 Americans carry a gene for hemochromatosis, the probability that a child will receive a second hemochromatosis-associated mutation from an unaffected parent is approximately 1 in 20. The patient also should be told that approximately 25% of homozygotes do not develop clinically significant iron overload, despite their inheritance of two hemochromatosis genes[37]. Several groups have recommended that children not be tested for a disorder that is not manifest until after the age of consent, and for which a cure or preventive treatment does not exist[10, 38]. Based on current knowledge about hemochromatosis and iron overload, these recommendations seem appropriate for general application to testing for hemochromatosis.

The patient should be informed that there is the possibility of discrimination with respect to obtaining health, life, and disability income insurance, and employment. Such discrimination was reported to occur in patients diagnosed with hemochromatosis before the C282Y mutation was discovered[39]. In general, health care costs for any person with hemochromatosis are increased, even among persons diagnosed before the development of iron overload. Accordingly, costs of health care insurance are sometimes increased, particularly when insurance is obtained through individual or small group policies. Persons with severe complications of iron overload, especially hepatic cirrhosis, primary hepatic cancer, diabetes mellitus, or disabling arthropathy, may be unable to obtain healthcare or disability income insurance, or only at markedly increased rates. Life insurance can usually be purchased at competitive rates by persons who have completed iron depletion therapy, whose health is otherwise good, and who do not have hepatic cirrhosis or diabetes mellitus, the two complications of hemochromatosis associated with a statistically decreased length of survival. There appears to be little practical experience with the possible prejudicial effects of the diagnosis of hemochromatosis on employability. Although there are statutes based on genetic risk of disease to limit discrimination against obtaining health insurance and employment[40–43], they are likely to increase in number and specificity in the next few years.

An important component of genetic counseling is to ensure that the patient has the support of immediate family members and that he/she is aware of other available support services. Some patients need help in coping with the psychological burdens associated with learning that they have a genetic disorder. Often, patients and their family members remember little that was discussed in the initial counseling session. Questions that arise later can be usually discussed and resolved by a telephone conversation. The genetic counselor should be available to provide support, re-enforce the physician's recommendations, and, if necessary, refer the patient to an appropriate support service. A telephone call to the patient or family members to remind them of follow-up visits or to provide additional requested information regarding the status of a family member is important and is appreciated. Using a checklist of the items to be discussed in an education and genetic counseling session assures that nothing will be omitted, and promotes uniformity of the process. The checklist should be signed and placed in the patient's chart to document the topics discussed.

The person at risk

A person at risk to develop hemochromatosis and iron overload may be identified by several means. Some learn that a family member has been diagnosed to have hemochromatosis; others are identified through a family history taken by their physician. Regardless, the process of genetic counseling for these persons is similar to that for a patient diagnosed through routine health care delivery or screening programs. This process includes confirming their family history of disease, describing the disease and its natural history, performing iron overload screening and genetic testing, estimating risk for disease, providing medical management, and referring the persons to support services.

Sometimes, persons in a family with hemochromatosis who feel healthy deny that they could be affected also, and thus may resist testing using biochemical testing or DNA-based genetic testing. Historically, much emphasis has been placed on the need to be non-directive in conducting genetic counseling[11, 44]. Others have argued that non-directive counseling is not possible[45]. Directive genetic counseling has been defined as 'a deliberate attempt – through deception, threat, or coercion – to undermine the individual's autonomy and comprise his or her ability to make an autonomous decision'[46]. With this in mind, it seems that it is appropriate to be more directive when counseling a person who is at risk for hemochromatosis, the complications of which can be prevented by relatively benign intervention.

Assessing a typical family

The process of genetic counseling can be illustrated using information taken from one of the families referred by a gastroenterologist to our fee-for-service laboratory for genetic counseling and testing (Fig. 56.1). The proband

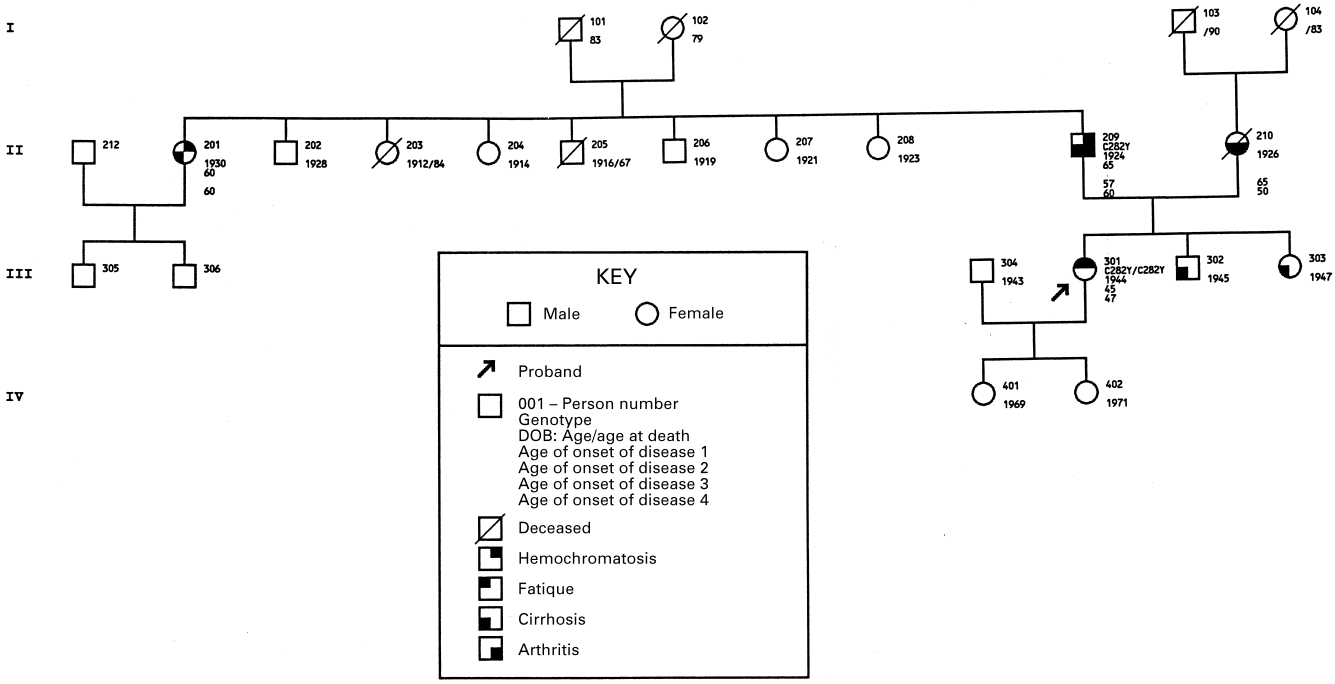

Fig. 56.1. Family history of disease assessment hemochromatosis family 053-00142.

(number 301 on pedigree) was a 52-year-old Caucasian female diagnosed with hemochromatosis and mitral valve prolapse at age 45, and with chronic fatigue at age 47. Her mother (number 210) and father (number 209) were third cousins. Her 51-year-old brother (number 302) and 49-year-old sister (number 303) had 'abnormal liver test results' at ages 44 and 49, respectively. The proband's 72-year-old father was diagnosed with hypertension at age 60, hepatic cirrhosis at age 57, and hemochromatosis at age 65. Her 71-year-old mother was diagnosed with non-insulin-dependent diabetes mellitus at age 46, arthritis at age 50, and non-alcoholic hepatic cirrhosis at age 65. A 67-year-old paternal aunt (number 201) was diagnosed with hemochromatosis and non-alcoholic hepatic cirrhosis at age 60. The two sons of this individual (no ages provided) (numbers 305 and 306) had 'abnormal liver test results.' The proband was homozygous for the C282Y mutation; her father was a C282Y heterozygote.

This family study illustrates many of the problems and opportunities for intervention that exist when evaluating a family with hemochromatosis. The information initially provided was incomplete. In analyzing this pedigree, information about children of the proband's brother, sister, and paternal uncles (her first cousins) who might be at risk for the disease is needed. More information is needed about those individuals who had 'abnormal liver test results.' All of the aforementioned individuals and the proband's two daughters are at risk. Their status could be defined further by measuring serum iron parameters and performing DNA-based genetic analysis for the C282Y and H63D mutations associated with hemochromatosis. This was explained to the proband who was especially concerned about the risk to her daughters (obligate heterozygotes), and who was eager to inform her relatives of her diagnosis and help gather additional data.

Conclusions

Collecting family history of disease information, assessing risk to develop hemochromatosis and iron overload, providing accurate information, and performing genetic counseling are important in the overall diagnosis and management of hemochromatosis. More patients and their family members will need educational and genetic counseling sessions as greater emphasis is placed on identifying those at risk for hemochromatosis and instituting treatment before organ damage occurs. Although the molecular genetics of hemochromatosis is not fully understood, the tools to identify those with a genetic predisposition for hemochromatosis and the capability to test for abnormal iron parameters are available at present.

Unresolved issues surrounding population screening for hemochromatosis should not diminish efforts to identify individuals with a positive family history of hemochromatosis who could benefit from genetic counseling and indicated medical testing. Appropriate medical evaluation of persons at risk can result in early detection of hemochromatosis and iron overload, and permits intervention before irreversible organ injury occurs.

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Part XIII

Final issues

57

Hemochromatosis: problems to be solved and directions for future investigations

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Introduction

At the conclusion of the First International Conference on Hemochromatosis in New York City in 1987, attendees discussed unsolved problems concerning hemochromatosis[1, 2]. One of the most important of these was identification of the hemochromatosis gene. Other questions that prevented an adequate understanding of hemochromatosis in 1987 included: Why is there a preponderance of males with hemochromatosis? What are the possible interactions of the hemochromatosis gene with X chromosomes? How does regulation of iron absorption differ between normal persons and those with hemochromatosis? What are the differences in iron transport in normal persons and in those with hemochromatosis? Why does iron loading of some organs occur in hemochromatosis, but there is little or no excess iron deposition in other cells and tissues, e.g., reticuloendothelial cells and organs? What are the mechanisms of iron-related tissue injury? Does hemochromatosis represent a constellation of disorders of iron absorption and metabolism?

Progress in understanding some features of hemochromatosis

A few of the questions posed in 1987 have been partially answered. Identification of the first hemochromatosis-associated gene (*HFE*), a class I-like gene linked to the major histocompatibility complex (MHC) on 6p, was published in 1996[3]. Two mutations of *HFE* were reported: nt 845G→A (Cys282Tyr; C282Y) and nt 187C→G (His63Asp; H63D). Of these, the C282Y mutation is considered to be a major cause of iron loading. However, the results of multiple studies performed in several populations indicate that the clinical phenotype associated with Caucasian hemochromatosis can only be explained in all patients by the occurrence of multiple genes that cause increased iron absorption.

Remaining problems and directions of future research

Mutations of *HFE*

Hemochromatosis is transmitted as an autosomal recessive trait. In some studies, 82–89% of individuals with hemochromatosis are homozygous for the C282Y mutation of *HFE*[4, 5]. However, 11–18% of individuals who have the hemochromatosis clinical phenotype are heterozygous for the C282Y mutation that is considered to be causative[4, 5]. Do the hemochromatosis subjects who are heterozygous for the C282Y mutation have an additional, unidentified mutation of *HFE*, or a mutation of another gene locus important for the control of iron absorption? In a study of Australian sibships with at least two individuals affected with hemochromatosis, 100% were homozygous for the C282Y mutation. Does this apparently perfect concordance of homozygosity for the C282Y mutation among affected siblings suggest that other reports of the frequency of C282Y include some iron-loaded heterozygotes? Although possible, this does not seem likely, because it is unusual for heterozygotes to develop heavy iron overload[6].

Another mutation present in some individuals who have hemochromatosis with heavy iron loading is H63D. To date, no haplotype has been identified in which the H63D and C282Y mutations exist simultaneously[4, 5]. What is the

significance of the H63D mutations in subjects who have hemochromatosis? How does H63D protein affect iron absorption? Does it augment iron absorption beyond the effect of C282Y heterozygosity? If the H63D mutation does not cause iron loading, why do some C282Y/H63D compound heterozygotes have the iron-loading phenotype? Are there other genes that interact with C282Y or H63D that cause iron overload in persons who are heterozygous for C282Y? It is probable that more than one mutation, possibly localized in more than one gene, causes the clinical disorder recognized as hemochromatosis. What are the respective genetic and clinical characteristics and relationships of these mutations and forms of hemochromatosis?

Molecular events in iron absorption

What are the molecular events associated with the *HFE* gene interaction with β_2-microglobulin that cause progressive iron accumulation? How does the *HFE* gene cause iron overload? Does it act as a signaling molecule or cause increased iron absorption directly by interacting with transferrin receptor in enterocytes[7]? Does it function abnormally to increase transport of iron in an abluminal direction in the intestine, or fail to down-regulate duodenal iron absorption? Does it bind an iron chelator moiety abnormally? What is the presumed iron chelator moiety?

Non-intestinal expression of *HFE* genes

Does an MHC-like gene code for a protein with extra-intestinal expression that directs the intestine to absorb excess iron? Could such a protein participate in the transport of iron into parenchymal or storage cells in hemochromatosis? Regardless of its localization, what are the effects of age, sex, hormone status, iron status, and other factors on expression and production of this protein?

Absorption of non-ferrous metals

Hemochromatosis homozygotes absorb and retain excess quantities of certain non-ferrous metals. What is the biochemical basis of absorption and organ deposition of these metals? What, if any, are the adverse (or beneficial) effects of increased stores of these metals on hepatocytes and other cell types? Does an MHC-like gene code for a protein that is normally involved in the absorption and metabolism of non-ferrous metals? Is there a metal chelator moiety in enterocytes that interacts with the *HFE* protein and that binds iron and non-ferrous metals in a competitive manner? It seems likely that answers to questions about the molecular mechanism of the increased iron absorption associated with the *HFE* gene will clarify the physiology of iron and non-ferrous metal absorption in normal persons.

Prevalence of hemochromatosis

The prevalence of hemochromatosis is 3–5 per 1000 in some populations, corresponding to a heterozygote frequency of 10–13%[4, 5, 8]. There are reports of higher and lower prevalences in some countries. In the past, investigations of the prevalence of hemochromatosis were based on the presence or absence of elevated transferrin saturation, or serum ferritin concentration (or both), or on the occurrence of hepatic iron loading. It was difficult to establish the diagnosis in individuals who did not have the iron loading phenotype. Now that the *HFE* gene has been identified, it will be possible to determine the frequency of heterozygosity and homozygosity for the C282Y mutation.

Frequency of C282Y mutation in different populations

The frequency of the C282Y mutation is known in a few populations. It is expected that 10–13% of Caucasian ancestry will be heterozygous for the C282Y mutation, and that the prevalence of C282Y typical of western Caucasians will be very low in populations that are not of European derivation.

Prevalence of the hemochromatosis gene among Caucasians

Because 10–13% of some Caucasian populations are heterozygous for the hemochromatosis gene, heterozygosity for the hemochromatosis gene probably conferred some advantage in the past. If a reproductive advantage were present, it either had to occur prior to the age of adolescence, or it increased a woman's likelihood of delivering a viable newborn. Recent studies suggest that fertility is normal among persons with hemochromatosis[9]. Heterozygotes often have normal serum iron parameters and infrequently develop iron overload. Thus, the advantages of heterozygosity may not have been related to iron accumulation. All cells require iron for normal function, but many bacteria also require iron for their own growth and reproduction. Does heterozygosity for a hemochromatosis allele enhance the ability of immune system cells to suppress infections? Is this effect related directly to altered body iron stores or metabolism? Is this effect related to expression of HLA or other MHC types that are somehow more favorable?

Prevalence of iron overload among hemochromatosis homozygotes

Do all subjects homozygous for the C282Y mutation develop iron overload? It is expected that most children and many women of reproductive age who are homozygous for the C282Y mutation will not be iron-loaded at the time of diagnosis[8, 10]. Further, it is likely that a large proportion of persons in whom the diagnosis of hemochromatosis is established by screening will have modest elevations of serum ferritin or of liver iron stores, rather than advanced iron overload[10].

Age after which iron loading is common

At what age does iron loading begin in hemochromatosis? By what age do most homozygotes have iron overload? It is expected that after age 40 years, 90% of homozygous men and 61% of homozygous women will have iron overload manifest by elevated serum concentrations of ferritin and/or increased quantities of iron deposited in the liver[11].

Iron overload-related morbidity

What proportion of homozygotes will develop the disease-related morbidity of hemochromatosis? Among homozygotes, it is expected that approximately 50% of men and 40% of women have symptoms attributable to morbid complications of hemochromatosis by age 40 years[11].

Arthropathy in persons with hemochromatosis

Approximately 40% of hemochromatosis homozygotes diagnosed during routine medical care delivery develop arthralgias and arthropathy. An increasing body of data suggests that the joint disease typically associated with hemochromatosis may not be entirely attributable to iron overload. Do persons with both hemochromatosis and arthropathy have an additional disorder that causes joint disease? Will homozygotes identified at a young age and treated to prevent iron loading eventually develop arthropathy characteristic of hemochromatosis? Should studies of genetic markers of heritable forms of premature osteoarthritis and other forms of arthropathy with genetic components (e.g., rheumatoid arthritis) be undertaken in patients with hemochromatosis?

Diseases other than iron overload among hemochromatosis heterozygotes

A small proportion (1–3%) of heterozygotes develop iron overload[6]. It has been suggested that heterozygotes may have an increased collective frequency of certain malignancies, arteriosclerosis, or other disorders. What is the basis, if any, of these possibilities? Can other hereditary disorders that also increase iron absorption (e.g., hereditary anemias or porphyria cutanea tarda) account for the occurrence of iron overload among heterozygotes who develop iron overload? Do some of these persons have other hemochromatosis-associated mutations for which testing is not presently available? What other disorders should be studied among hemochromatosis heterozygotes? What other genetic and environmental factors could account for current observations regarding iron overload and other diseases among heterozygotes?

The ideal age to screen for hemochromatosis

Which combination of transferrin saturation, serum ferritin, and tests of the *HFE* mutation will identify iron-loaded and non-loaded homozygotes? Will a test for the *HFE* mutation be the only population (or individual) screening test needed[11]? Should transferrin saturation and/or serum ferritin concentration be measured only after a person is found to be homozygous for the C282Y mutation? Because 11–18% of iron-loaded persons with hemochromatosis are not homozygous for the C282Y mutation, use of the test to detect C282Y only will not identify a substantial proportion of persons who are at risk to develop iron overload and its associated morbidity. Iron overload is common in clinically unselected homozygotes after age 40 years. The disease-related morbidity of hemochromatosis only occurs in heavily iron-loaded homozygotes, and hepatic cirrhosis occurs after massive hepatic iron loading is present. Based on current knowledge about hemochromatosis, it seems reasonable to screen for hemochromatosis before age 20 years. This could result in the identification of most homozygotes prior to their accumulation of massive quantities of stored iron, and should lead to the initiation of therapeutic phlebotomy of all homozygotes with an elevated serum ferritin concentration.

The potential utility of genetic population screening for hemochromatosis

The cost of DNA-based tests of hemochromatosis-associated mutations will probably decrease rapidly due to automation, demand, and market forces affecting reference and genetics laboratories. What is the potential role of testing for hemochromatosis-associated DNA mutations in population screening? How will DNA test results correlate with the serum iron parameters that are presently recommended for screening? What additional provisions

must be made to accommodate medical, social, and ethical concerns that surround increasing use of DNA testing for diagnostic purposes[4, 5]?

Cost-effectiveness of screening for hemochromatosis

There have been some estimates of the cost of screening for hemochromatosis[4, 5, 10]. However, large-scale screening studies designed to identify subjects who are homozygous for a mutation of *HFE* have not yet been performed. It is likely that the most cost-effective strategy for screening for hemochromatosis will employ some combination of an *HFE* or other DNA-based hemochromatosis-associated mutation test and measurement of serum concentration of ferritin in individuals who are homozygotes or compound heterozygotes for the causative mutation(s).

Relationship of *HFE* to iron overload in African Americans

Iron loading has been described in sub-Saharan Africans and among African Americans[12, 13]. Is the iron loading that occurs in these populations due to the presence of a non-HLA-linked African iron-loading gene alone? Is it due to admixture of Caucasian genes, resulting in the introduction of the *HFE* mutation into individuals of sub-Saharan African ancestry? Does the non-HLA-linked African iron overload gene interact with a mutation of *HFE* among African Americans? What is the prevalence of *HFE* mutations in African Americans? Current data suggest that primary iron overload among sub-Saharan Africans is not related to a mutation of the *HFE* gene. It seems reasonable to propose that the prevalence of a mutation of *HFE* among African Americans will be similar to the prevalence of other Caucasian genes that have been introduced into African Americans. However, African American populations (like Caucasian American populations) are genetically and historically diverse. There are probably significant regional differences among African Americans with respect to the frequency of African iron overload and Caucasian hemochromatosis genes.

HLA types and marrow and organ transplants

Hemochromatosis homozygotes and heterozygotes constitute 10–13% of some Caucasian populations. Collectively, these persons have relatively restricted HLA immunophenotypes due to the limited expression of HLA-A and HLA-B haplotypes caused by linkage disequilibrium. This results in a significantly increased frequency of the antigens HLA-A3 and HLA-B7 (and of the haplotypes -A3, B7 and -A3, B14) in persons who inherit hemochromatosis genes in comparison with persons in general Caucasian populations[14]. Taken together, these observations suggest that persons who inherit hemochromatosis genes who need marrow or organ transplants are more likely to find HLA-compatible unrelated (or related) donors among other persons with hemochromatosis or among hemochromatosis heterozygotes. Investigation is needed to determine whether establishment of a separate marrow and organ donor registry and specific recruitment of homozygotes and first-degree family members as potential donors would be beneficial and feasible. This could increase the availability of tissues needed for transplantation among persons who are positive for HLA-A3, B7, HLA-A3, B14, and other common hemochromatosis-associated haplotypes. Because persons who inherit hemochromatosis gene(s) represent a substantial proportion of the Caucasian population[4, 5, 10], the potential value of such a project could be relatively great. Should physicians continue to perform HLA typing of patients who have hemochromatosis and their relatives? Emerging computerized DNA automation technology is likely to provide HLA typing quickly and economically at the same time genetic testing for hemochromatosis genes is performed.

Using blood from therapeutic phlebotomy

In some countries, it is the policy to use blood obtained from persons with hemochromatosis for purposes of transfusion. In the United States, blood from known hemochromatosis patients can be used for transfusion only if the blood is labeled to identify its source. The major issue surrounding the requirement of labeling, or of rejecting individuals who have hemochromatosis as potential donors, is that these individuals are not volunteer donors. Persons with hemochromatosis have a vested therapeutic interest and a medical indication for removal of their blood. Will a change in American or other national public health policy occur as a result of identification of mutations of the *HFE* gene? Hemochromatosis homozygotes represent a potentially vast source of blood that could be used for transfusion or for the manufacture of blood derivatives, assuming the blood is otherwise safe. Accordingly, some individuals who have hemochromatosis insist that their blood is safe for transfusion, and that it should be used for this purpose. However, it seems important to emphasize that no one has a right to insist that his or her blood be transfused into someone else. Recent conference conclusions make it seem likely that public health policy in the United States will change on the basis of the discovery of the *HFE* gene and its mechanism of causing excess iron absorption and

overload. Objective information about the potential eligibility of hemochromatosis patients as blood donors and about the feasibility of treating them in blood collection centers is needed.

New iron overload disorders

In the last few years, several new iron overload disorders have been described[15–18]. Collectively, these have provided new insights into the mechanisms of normal and abnormal iron and non-ferrous metal absorption and metabolism. Further, their discovery suggests that additional related disorders are likely to be found in the next few years. Some of these disorders may be rare hereditary disorders that will provide valuable lessons at the molecular level. Others may be relatively common, and will require additional clinical and epidemiologic research to establish standards of diagnosis and care.

Conclusions

Because scientific questions usually are answered partially and in increments, many of the questions posed in 1987 at the First International Conference on Hemochromatosis remain unanswered, and many new questions are raised with each partial answer. Hopefully, many of today's questions will be answered within the next decade.

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