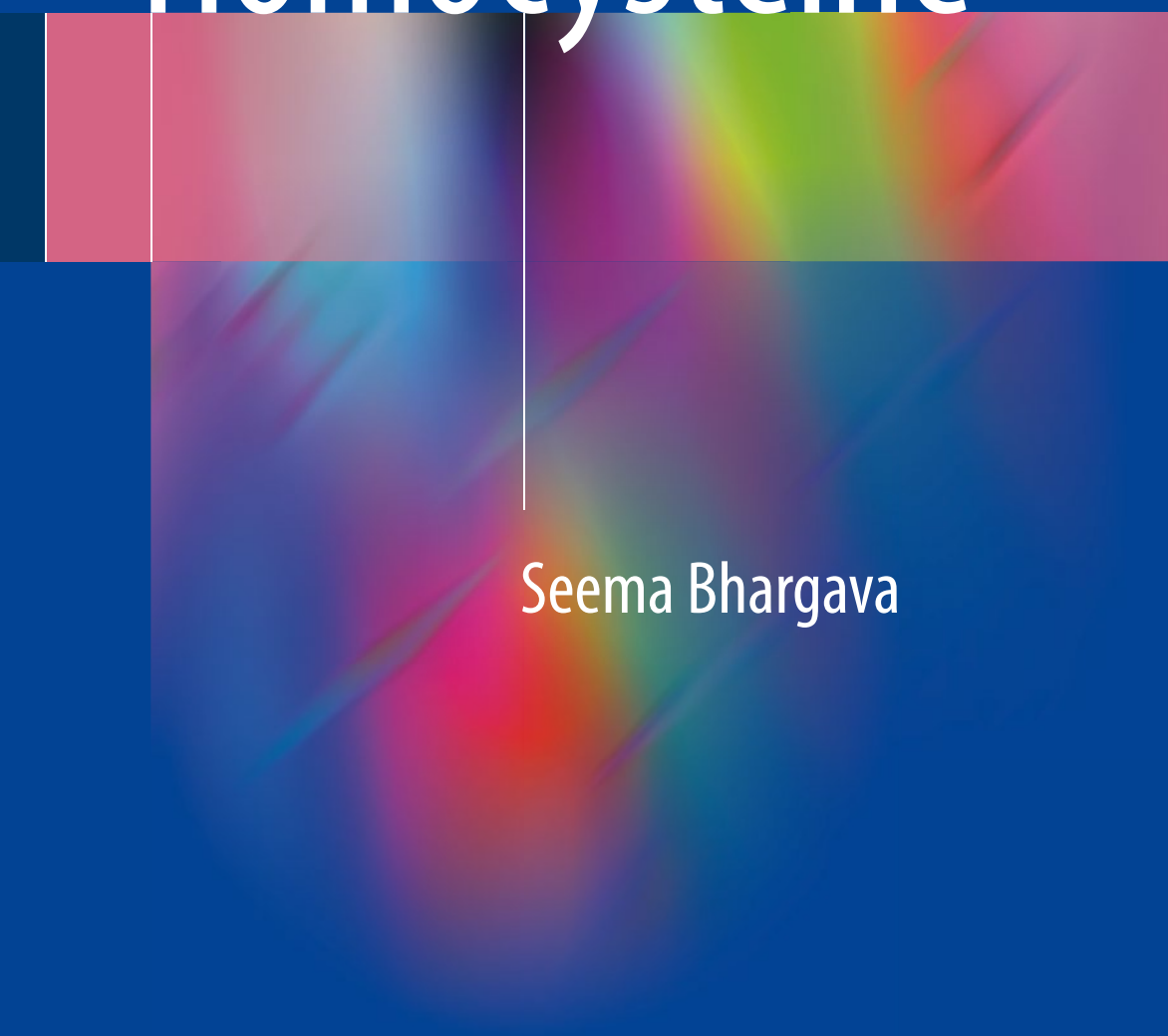
The Clinical Application of Homocysteine



Seema Bhargava

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Dedicated to
My two sets of parents

Mrs. Kanta & Dr. H. Razdan

Dr. Shanta & Late Dr. K.P. Bhargava
Who have encouraged and supported me
In all my endeavours

Foreword

In the field of medicine, where we clinicians are constantly trying to reduce morbidity and mortality for patients, a good working knowledge of all possible risk factors and risk markers is essential. This has become difficult due to the increased frequency of discovery of new biomarkers. The laboratory has, therefore, become indispensable in daily practice. Dr. Seema Bhargava is a senior consultant and chairperson of the Department of Biochemistry in our hospital and is a nationally and internationally esteemed biochemist. Her compilation on the clinical application of homocysteinemia, which has a clinical message at the end of each chapter for quick reference, would be very handy for clinicians, especially the general medical practitioner. I commend her on her efforts to compile this handy book and wish her the best.

D.S. Rana
Sir Ganga Ram Hospital Marg
New Delhi, India

Preface

Laboratory investigations are gaining importance in the diagnosis and prognosis of patients. Hence, we (laboratorians and scientists) are constantly attempting to identify new markers that would help in earlier diagnosis and better prognostication of conditions with a high morbidity and mortality, as well as a high prevalence. As per WHO[1], developing countries are home to 80% of the coronary artery disease (CAD) and stroke patients, associated with a mortality second only to cancer in the world. Intensive research in these areas has resulted in the identification of several new markers for diagnosis and prognosis of CAD and stroke. One of them is homocysteine, whose association with vascular disease was first brought forth in 1967. Since then, its implication in various disease processes emerged, emphasizing its involvement in the pathogenesis of many disease processes (apart from vascular disease). Hence, it was declared the "Marker of the Millennium."

Therefore, it is important that the clinician be aware of the pathophysiology and clinical implication of homocysteinemia[2].

It is my endeavour that this compilation assists the clinician to maximally and appropriately utilize homocysteine levels in diagnosis, prognosis, management and research.

New Delhi, India — Seema Bhargava

[1] WHO – World Health Organisation.

[2] Elevated circulating homocysteine levels.

Acknowledgements

At the outset, I would like to state that I am humbly obliged to Sir Ganga Ram Hospital, New Delhi (where I have served for the last 25 years), for the opportunity to be directly associated with patient care as well as research in the field of clinical medicine; in fact, my academic career has also been enhanced by this institute. My humble gratitude to Late Dr. K.P. Jain for introducing me to this institute.

I owe my initiation into academic writing to Prof. L. M. Srivastava who prodded me into publishing my research work.

I am also indebted to Prof. Arif Ali who guided me through my studies on various aspects of homocystenemia.

I am highly indebted to the late Dr. K.C. Mahajan who facilitated and promoted my academic career, and inspired and enabled me always.

I am also indebted to Dr. P.S. Gupta, who has always encouraged me. My warm thanks to Dr. D.S. Rana for his consistent confidence and support. I am grateful to Dr. S.P. Byotra for supporting me always.

I am thankful to Dr. N.K. Ganguly for his advice on the content of this compilation.

I am indebted to Dr. Rajiv Parakh and Dr. C.S. Agarwal who collaborated with me during my initial years of research on homocysteine.

It would be amiss of me not to mention the support and diligence of the staff and consultants of my department[1], especially Dr. Anjali Manocha, Dr. Mamta Kankra, Dr. Sabari Das, Dr. Parul Singla, Dr. Dimple Anand, Mr. Annsh Bhandari, Dr. Khageshwar Mahato, Dr. Anisha Sharma and Dr. Ashok Ahirwar.

Thanks are due to Dr. Rekha Wazir and Dr. Ashwini Saith who introduced me to homocysteine; it has been my research avenue ever since. Prof. Suresh Tyagi and his team (especially Dr. Kumar and Dr. Srikanth) exposed me to the varied aspects of homocysteinemia and its pathological mechanisms. It is during my tenure in his department[2] that I envisaged the dream of bringing forth the clinical nuances of homocysteinemia for the betterment of patient care.

[1] Department of Biochemistry, Sir Ganga Ram Hospital, New Delhi, India.

[2] Department of Physiology and Biophysics, University of Louisville School of Medicine, Louisville, Kentucky, USA.

At the helm of all my achievements is the love and motivation of my late husband, Dr. Nalin Kumar Bhargava, and my children, Dr. Eishaan Kamta Bhargava, Dr. Meghaa Shanta Bhargava and Ms. Kriti Bhandari Bhargava, who not only continue to support me unconditionally but also advise me on varied aspects of writing and publishing. They have also contributed immensely towards the final shaping of this compilation.

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About the Author

Seema Bhargava obtained her DNB from Lady Hardinge Medical College, New Delhi, and PhD from Sir Ganga Ram Hospital and Jamia Millia Islamia, New Delhi. She completed her postdoctoral studies at the Department of Physiology and Biophysics, University of Louisville School of Medicine, Louisville, Kentucky, USA.

Dr. Bhargava is senior consultant and chair of the Department of Biochemistry at Sir Ganga Ram Hospital and a professor at Ganga Ram Institute of Postgraduate Medical Education and Research (GRIPMER) and Guru Gobind Singh Indraprastha University. She was also an elected member of the National Academy of Medical Sciences. She is a lead assessor and technical assessor for the National Accreditation Board for Testing and Calibrating Laboratories (NABL) and also a member of its accreditation committee.

Her research focuses on identification of clinically relevant biochemical markers and their applications in various areas of medicine, such as neurology, Alzheimer's disease, cardiovascular disease, and sepsis. She has published numerous papers in national and international indexed journals. She has guided several PhD and DNB scholars and has been actively involved in training of MSc, BSc, nursing, and DMLT students. She is on the editorial board of two indexed international journals and is a reviewer for several international journals.

Part I

Homocysteine and Homocysteinemia

Introduction

1

In the last century, there have been dramatic improvements in health care all over the world, reflected by an increase in life expectancy from 46 years in the 1950s to 70 years in 2011 and, consequently, a rise in the percentage of geriatric population (Sen and Bonita 2000; World Health Statistics 2013).

Industrialization, economic development and social organization of human societies have moulded their health status and disease profile, resulting in an "epidemiological transition" (Yusuf et al. 2001). Thus, nutritional deficiencies and infectious diseases have given way to chronic degenerative diseases like vascular disease, cancer and diabetes.

The global burden of vascular disease, especially cardiovascular and cerebrovascular disease (CVD), is on the increase, despite the identification (and aggressive attempts at prevention) of a multitude of risk factors. Worldwide data, in 1990, revealed mortality rate due to vascular disease to be approximately 25%; today it is about 30%, and the projected figure for 2020 is >36% (World Health Statistics 2013; Murray and Lopez 1997; Braunwald 1997; Deaton et al. 2011). It is also now recognized that the epidemic of coronary artery disease (CAD) has shifted from the Western countries to the developing (middle-income and low-income) countries, so much so that 80% of the current global burden occurs in these countries (Yusuf et al. 2001). This emphasizes the requirement of continuous and unslackened efforts towards identifying various risk factors for vascular disease and taking appropriate action to minimize their pathogenicity and morbidity.

The lesions of vascular disease represent the result of a complex, multicellular, inflammatory-healing response in the vessel wall. The pathogenesis, therefore, is multifactorial. Many risk factors have traditionally been associated with atherosclerosis and atherothrombosis (Yang et al. 2008a, b). Apart from disease states like hypertension, diabetes mellitus and chronic renal failure, the cholesterols and triglycerides have been the major biochemical factors implicated and have been coined "conventional risk factors". In fact, all measures directed at reducing the burden of vascular disease have been directed at lowering the cholesterols and triglycerides by lifestyle modifications as well as medication. But recently, in many cases of

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vascular disease, the levels of these "conventional risk factors" have been within the biological reference interval (BRI). Moreover, targeting these markers for prevention and management addresses only the perpetuation of atheromatous plaques, not the actual critical initiating event of atherosclerosis and thrombosis which is injury to the vascular endothelium. Any substance contributing to this initial event would become a risk factor for occlusive vascular disease and must be tackled. *Hence, it has become necessary to identify new risk factors which will actually put an end to the beginning, i.e. prevent the loss of integrity of the vascular endothelium*.

Homocysteine, a sulphur-containing non-protein-forming amino acid, is one such molecule that has been shown to cause multipronged injury to the vascular endothelium and initiate plaque formation in all types of blood vessels. Since it affects all blood vessels and several matrices, homocysteine has been found to affect virtually all organ systems.

Homocysteine: Discovery and Metabolism

2

2.1 Introduction

It is estimated that there are more than 50,000 human proteins and that the number of distinct proteins in each cell is 3000–5000. Amino acids are the basic structural units of proteins. Of the several amino acids present in nature, about 22 are required for the synthesis of human proteins. Of these 22 amino acids, 10 amino acids cannot be synthesized in the human body, and, hence, they must be derived from the diet. These amino acids have been coined "essential amino acids." Cysteine, a semi-essential amino acid, is an integral constituent of several human proteins and is derived from the essential amino acid methionine. Methionine serves as a source for available sulphur for the synthesis of cysteine and taurine, and, as *S*-adenosyl methionine, it is the most important methyl group donor in cellular metabolism. Homocysteine is an intermediate product in methionine metabolism.

During his experiments on intermediary metabolism, Vincent de Vigneaud[1] discovered homocysteine as an intermediate product of methionine metabolism (de Vigneaud 1952). Almost four decades later, McCully first associated homocysteine with atherothrombosis, and then several reports described mental retardation and specific vascular lesions (including atherosclerosis, tunica media thickness, tunica intima focal sclerosis, narrowing of arteries of all sizes and thromboembolism) in patients with homocystinuria, leading to its association with these phenomena (Carson and Neill 1962; Gerritsen and Waisman 1964; McCully and Ragsdale 1970).

To understand its pathology, it is imperative to know its physiology and metabolism.

Homocysteine is derived primarily from the breakdown of dietary methionine in the activated methylation cycle. It is not, per se, incorporated into proteins but is converted back to methionine (remethylation cycle) and further broken down by

[1] Vincent du Vigneaud was awarded the Nobel Prize in 1955 for his work on sulphur-containing compounds.

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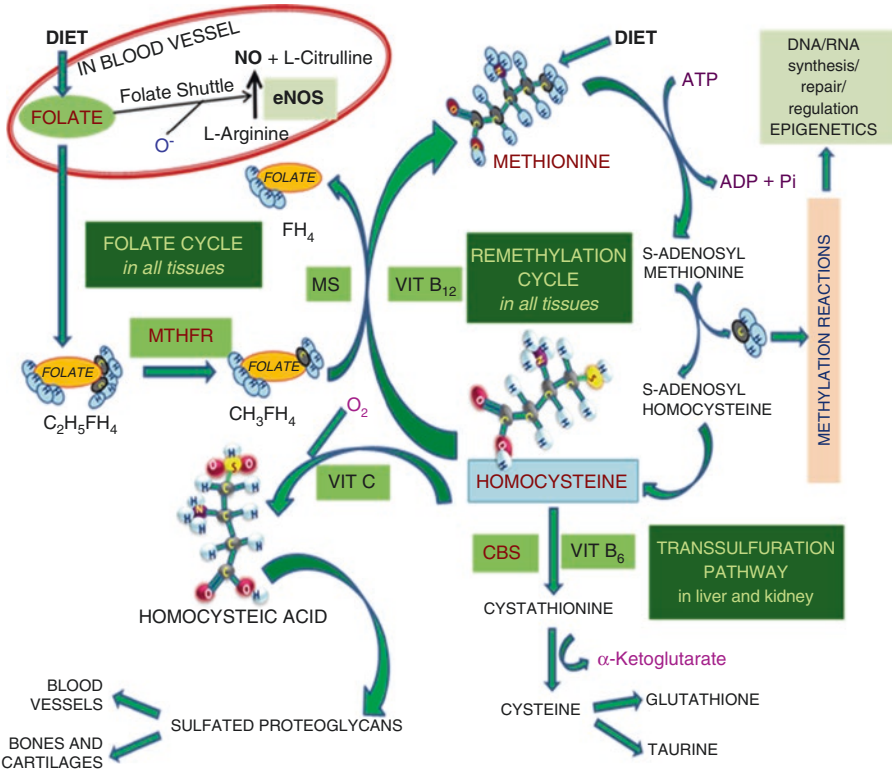

Fig. 2.1 Metabolism of homocysteine. The metabolism of homocysteine and its association with folate cycle, remethylation cycle and the transsulphuration pathway. *NO* Nitric oxide, *eNOS* endothelial nitric oxide synthase, $C_2H_5FH_4$ methylene tetrahydrofolate, CH_3FH_4 methyl tetrahydrofolate, FH_4 tetrahydrofolate, *MS* methionine synthase, O_2 oxygen, *MTHFR* methylene tetrahydrofolate reductase, *CBS* cystathionine beta synthase (Modified from Bhargava S, Srivastava LM. Hyperhomocysteinemia and its clinical implications—A short review. Current Medicine Research and Practice 2014; 4(3):112–118)

enzymes into cysteine and α-ketoglutarate (transsulphuration pathway), all of which are involved in specific protein syntheses.

The remethylation cycle is integrally related to the folate cycle (Fig. 2.1). In the folate cycle, circulating folate is converted to tetrahydrofolate which, on methylation, yields 5,10-methylene tetrahydrofolate (5,10-MTHF) first and then 5-methyltetrahydrofolate (5-MTHF) by the reducing action of the enzyme methylene tetrahydrofolate reductase (MTHFR).

The name "remethylation" cycle comes from the chemical alteration of homocysteine which gets "remethylated" to methionine by acquiring a methyl group from 5-MTHF from the folate cycle through a reaction catalysed by the enzyme methionine synthase (MS) which is cobalamin dependent. Homocysteine actually acquires a methyl group from methyl cobalamine, which is then remethylated by a methyl group from MTHF.

Thus the cobalamin cofactor serves as both donor and acceptor of the methyl group. Hence, 5-MTHF and cobalamin are necessary for the remethylation of homocysteine, and MTHFR is a rate-limiting enzyme for the remethylation cycle. Sometimes in the liver, homocysteine acquires a methyl group from betaine through a reversible reaction. All these reactions are vitamin B_{12} dependent. Approximately every 2000 catalytic cycles, the cobalamin is oxidized and reactivated.

On activation by adenosine triphosphate (ATP), most of the methionine goes into the formation of *S*-adenosyl methionine (SAM). SAM serves primarily as a universal methyl donor to a variety of acceptors involved in protein and nucleotide syntheses. *S*-adenosyl homocysteine (SAH) is the by-product of these methylation reactions, and its formation is governed by the SAM/SAH ratio.

It is subsequently hydrolysed by SAH hydrolase, in a reversible reaction, to yield homocysteine. Homocysteine, thus regenerated, becomes available to start a new cycle of methyl transfer. Here, it would be of utmost importance to note that this hydrolysis is a reversible reaction that favours the synthesis of SAH and that elevated cellular concentrations of this metabolite are likely to precede and accompany all forms of homocysteinemia (Selhub 1999; Gellekink et al. 2005).

The transsulphuration pathway, on the other hand, is not a circular pathway leading to the regeneration of homocysteine. It is a one-way condensation of homocysteine with serine to form cystathionine in an irreversible reaction catalysed by the pyridoxal-5-phosphate (PLP)-containing enzyme, cystathionine-β-synthase (CBS), the rate-limiting enzyme of this pathway. Another PLP-containing enzyme, γ-cystathionase, catalyses cystathionine to form cysteine and α-ketobutyrate. Thus, this pathway performs a dual function—firstly, catabolizing excess homocysteine (which is not required for methyl transfer) and secondly, synthesis of cysteine which gets further degraded to release hydrogen sulphide (H_2S). Vitamin B_6 is the cofactor for these reactions.

In addition, in the presence of vitamin C, homocysteine is also oxidized to homocysteic acid which is required for the synthesis of sulphated proteoglycans of the vessel walls, bones and cartilages.

Thus, homocysteine is located at a critical metabolic crossroad and, both directly and indirectly, impacts all methyl and sulphur group metabolisms occurring in the body. Decreased metabolism of homocysteine leading to homocysteinemia, therefore, causes decreased methylation reactions as well as decreased synthesis of sulphated proteoglycans, which though found in all tissues are highest in concentration in the cartilages, tendons, ligaments, synovial fluid, skin, finger- and toenails, heart valves and the basement membrane of all blood vessels (Bhargava et al. 2012a).

It is pertinent here to note that folate is required for the synthesis of nitric oxide (NO) by the vascular endothelium. In the presence of oxidative stress, the requirement for NO increases, and more folate is shunted towards its synthesis, and, consequently, decreased folate is available for remethylation, thus leading to homocysteinemia. Conversely, when there is homocysteinemia, folate utilization shifts from synthesis of NO to the remethylation cycle, causing a decreased synthesis of NO and, thereby, increased oxidative stress. This would explain why some scientists say that homocysteinemia causes oxidative stress, and yet others hold that

oxidative stress leads to homocysteinemia. This "folate shuttle" model was proposed by Hayden and Tyagi (2004).

2.2 Biological Reference Interval

Reports on biological reference interval (BRI) for concentrations of homocysteine in plasma differ greatly from one laboratory to another. In 1989, Malinow et al. (1989) had recommended keeping homocysteine below 10.0 μmol/L. This was further emphasized, by Boushey et al. (1995), when they elucidated in their meta-analysis that homocysteine does not have a threshold beyond which it is pathogenic; its pathogenicity increases with its concentration even within the biological reference interval which was confirmed to be 5–15 μmol/L. A homocysteine >15 μmol/L was termed homocysteinemia. Malinow et al. (1996) graded homocysteinemia as mild (homocysteine = 16–30 μmol/L), moderate (homocysteine = 31–100 μmol/L) and severe (homocysteine >100 μmol/L).

2.3 Hyperhomocysteinemia (or Homocysteinemia): Causes and Modulation

2.3.1 Causes

The methylation cycle has three limiting factors—the enzyme, methylene tetrahydrofolate reductase (MTHFR) and vitamins folic acid and B_{12}—whereas the transsulphuration reaction is dependent on the enzyme, cystathionine-β-synthase (CBS) and the vitamin, pyridoxine (B_6). Deficiency of any of these vitamins or polymorphisms in the genes of either of these enzymes will reduce the rate of metabolism of homocysteine and cause its accumulation in plasma, i.e. homocysteinemia.

A scrutiny of the metabolism of homocysteine, as detailed above, reveals that homocysteinemia can be a result of either:

A. *Genetic defects in one of the enzymes of homocysteine metabolism*. The MTHFR gene is present on the short arm of chromosome 1 at position 36.3 (Goyette et al. 1998), and the CBS gene is present on the long arm of chromosome 21 at position 22.3 (Münke et al. 1988). The most common defects in either of these enzymes are single-nucleotide polymorphisms (SNPs), which result in either synthesis of a defective enzyme or synthesis of less quantity of enzyme.

   MTHFR is encoded by a 20,328 base pair gene comprised of 11 exons. There are 18 known polymorphisms of this gene, but the most common are the C677T and the A1298C. The former occurs on exon 4, and the resultant thermolabile enzyme shows decreased activity due to dissociation of a dimer into monomers and decrease in its FAD-biding capacity. The latter occurs on exon 7 and apparently does not affect the functioning of the enzyme, with consequently no effect on circulating homocysteine levels (Radha Rama Devi et al. 2004).

The CBS gene is the most common site for mutations resulting in homocystinuria. It is a 23,678 base pair located on chromosome 21q22.3. More than 150 mutations causing homocystinuria have been identified in this gene, but the most common are the T833C, G919A and G1330A.

Interestingly, Alessio et al. (2008), evaluated CBS gene polymorphisms (T833C, G919A and 844ins68) in the samples of 220 children whose samples had already been analysed for MTHFR (C677T, A1298C) and MSR (A66G) polymorphisms. They observed that the insertion of 68 base pairs at position 844 (844ins68) was always associated with T833C (prevalence 19.5%); on the other hand, the G919A polymorphism was not observed, all children exhibiting only GG genotype.

In addition, there are several SNPs that modulate the other enzymes of homocysteine metabolism (e.g. SAH hydrolase, methionine synthase (MS), methionine synthase reductase (MSR)), but they do not culminate in overt metabolic disorders even though homocysteine is mildly raised. Most common among these are the A2756G of MS and the A66G of MSR, as mentioned above.

B. *Nutritional deficiency of one or more of the vitamins that participate in homocysteine metabolism* (Boushey et al.). Three vitamins of the B group—folic acid, vitamin B_{12} and vtamin B_6—play a pivotal role in the metabolism of homocysteine as detailed above. Hence, a deficiency of either one or more of these three leads to accumulation of homocysteine.

Sengupta and his team evaluated the dietary factors and polymorphisms associated with homocysteinemia in Indians, with the basis that due to the prevalent vitamin B_{12} deficiency in our population, the impact of polymorphisms is exaggerated. They observed that homocysteinemia was more prevalent in those on a vegetarian diet ($p = 0.019$) or those with the MTHFR A1298C polymorphism ($p = 0.006$). The minor allele frequency for MTHFR C677T and A1298C is 0.15 and 0.44, respectively, indicating a greater prevalence of the latter polymorphism, unlike in other populations where MTHFR C677T is more prevalent (Kumar et al. 2005).

2.3.2 Modulation

By SNPs or Vitamin Deficiencies By virtue of the different parts of the metabolic cycles affected, several SNPs and vitamin deficiencies affect plasma homocysteine concentrations to different extents. Sometimes these genetic and dietary variants coexist, and the resultant homocysteine concentration varies further (Fig. 2.2).

By Nutrition Utilization of homocysteine molecules by the transsulphuration or remethylation pathways is nutritionally regulated:

a. When a basal *methionine*-containing diet is administered, homocysteine moieties are found to go through the remethylation pathway approximately 1.5–2.0 times before being catabolized through the transsulphuration pathway (Mudd and Poole 1975).

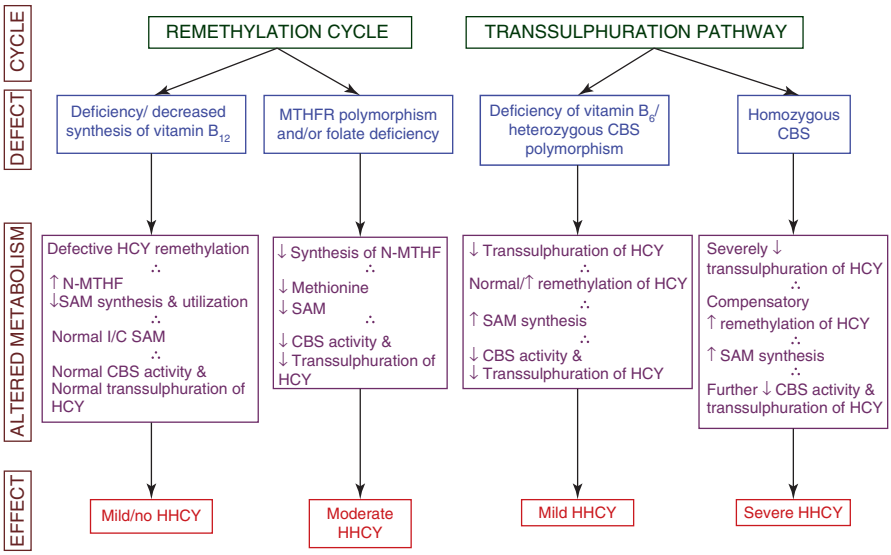

Fig. 2.2 Modulations of plasma homocysteine concentration by SNPs and vitamin deficiencies. *SNP* Single-nucleotide polymorphisms, *MTHFR* methylene tetrahydrofolate reductase, *CBS* cystathionine β synthase, *HCY* homocysteine, *SAM* *S*-adenosyl methionine, *HHCY* hyperhomocysteinemia

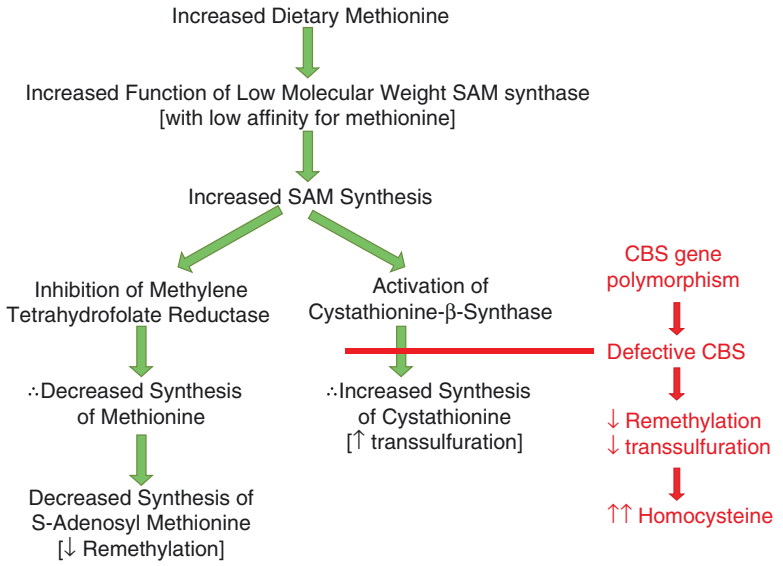

Fig. 2.3 Effects of increased dietary methionine on homocysteine metabolism

b. When dietary methionine is enhanced, this homocysteine cycling falls below basal level.
c. This capacity of the body to discriminate between remethylation and transsulphuration pathways in response to varying amounts of dietary methionine implies the existence of a coordinated regulation between these two pathways (Fig. 2.3).

   Increased dietary methionine leads to a decreased rate of remethylation and consequently an increased homocysteine concentration to be dealt with by the transsulphuration pathway. When there is excess of methionine, despite the feedback mechanism, all the homocysteine is not metabolised by the transsulphuration pathway and, hence, accumulates with resultant homocysteinemia.

   Similarly, in conditions of CBS deficiency, increased dietary methionine leads to severe homocysteinemia. Also as mentioned earlier, decreased SAM synthesis results in an inhibition of methylation reactions as well as a decrease in the formation of sulphated proteoglycans, which impact the integrity of the target organs individually or even in combination. An example of the double-pronged effect is the impairment of the vascular endothelial proteins (due to methylation) as well as impaired formation of collagen of the blood vessels (due to decreased synthesis of sulphated proteoglycans). Both these events initiate and promote the process of atherothrombosis (Gellekink et al. 2005).
d. McCully also established the correlation between the oxidative and reductive properties of *ascorbic acid* and the metabolic and pathologic abnormalities of the connective tissues in scurvy, i.e. vitamin C deficiency (McCully 1971). He demonstrated that lower levels of dihydroascorbic acid led to slower oxidation of homocysteine to homocysteic acid and a consequent accumulation of homocysteine (Fig. 2.4).

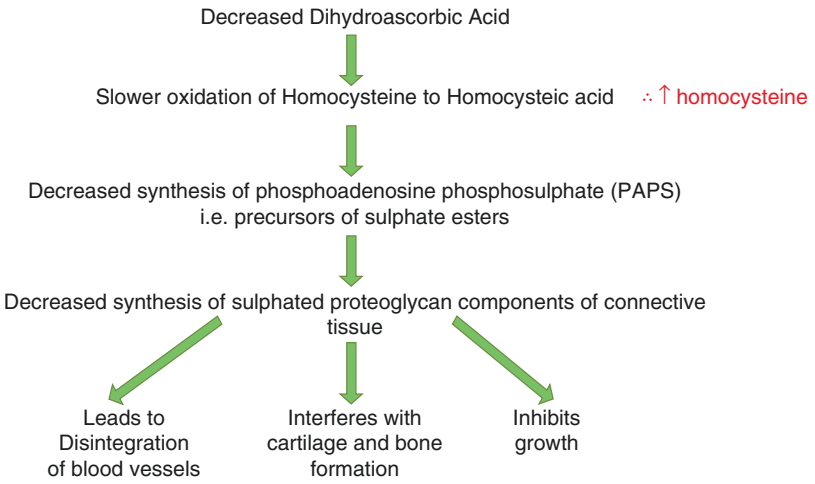

Fig. 2.4 Interplay between vitamin C (ascorbic acid) and homocysteine

Through its effect on homocysteine metabolism, deficiency of ascorbic acid leads to disintegration of walls of the blood vessels, interference in cartilage and bone formation, and consequent inhibition of growth.

To perpetuate its pathological effects on the endothelium, homocysteine has to enter these cells. In 2001, Sengupta et al. (2001) demonstrated experimentally that when in circulation, homocysteine is bound to albumin. This albumin is known to bind to several endothelial cell membrane proteins through sites other than that by which homocysteine binds to it. Thus, albumin enables homocysteine to be transcytosed via the plasmalemmal vesicles (gp 60) or endocytosed via receptor-mediated endocytosis (gp 30, 18). Once it enters these cells, homocysteine is degraded by the lysosomes and released into the cytosol where it modifies several proteins and alters the redox potential resulting in oxidative stress and endothelial cell dysfunction.

Part II

Clinical Implications of Homocysteinemia

Homocysteine in Occlusive Vascular Disease

3

3.1 Pathophysiology of Homocysteinemia-Induced Occlusive Vascular Disease

The primary pathology with which homocysteinemia has been associated is vascular atherothrombosis. The mechanisms of pathology in the vascular system can also cause pathology in other systems. Vascular endothelial injury is the critical initiating event of atherosclerosis and thrombosis, as mentioned before. This can be of three types: type I is recognized by the absence of denudation and the presence of only functional injury to the endothelium (which leads to lipid accumulation, monocyte and platelet adhesion, smooth muscle cell proliferation and plaque formation). Endothelial injury is classified as type II when there is denudation of the endothelium but no disruption of the intima. The third type of endothelial injury, called type III, includes denudation of the endothelium as well as intimal disruption. Type II and type III both lead to atherogenesis and plaque formation as a response to injury (Ross 1986).

As is evident from Fig. 3.1, the starting point of any occlusion in a vessel is a loss of integrity of the vessel wall, be it an artery or a vein. Although there are several known causes for deposition of an atherothrombotic plaque, increased plasma homocysteine concentrations actually initiate this process in arteries as well as veins (Fig. 3.1). In fact, blood vessels are more prone to deleterious effects of homocysteinemia as the vascular endothelium is inherently deficient in the enzyme CBS. As a result of their experiments and earlier observations, McCully and Wilson described the "Homocysteine Theory of Atherosclerosis" in 1974 (McCully and Wilson 1975).

Homocysteinemia (mild or severe) which increases the risk for occlusive vascular disease, thrombosis and stroke is now well-documented.

In arterial thrombosis, the mechanisms involved are those of platelet dysfunction, and in homocysteine-induced venous thromboembolism, the mechanisms revolve around abnormalities of coagulation and/or fibrinolysis (Gellekink et al. 2005).

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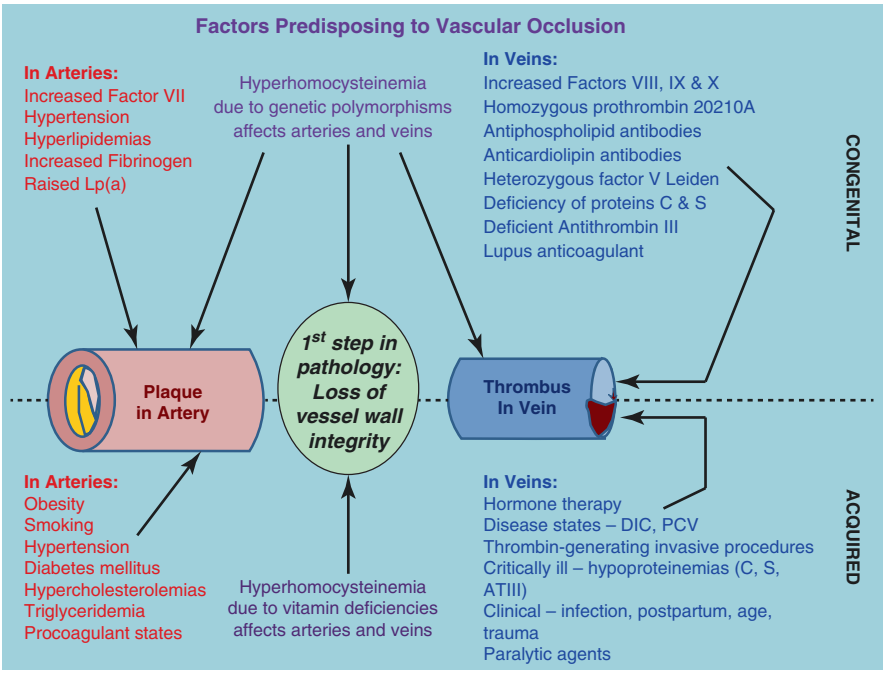

Fig. 3.1 Causes of occlusive disease of arteries and veins

The various toxic effects of homocysteine have been demonstrated over the past several decades, but it is only in the last two decades that detailed insight into the mechanisms involved has come to the fore.

Generation of Superoxides and Hydrogen Peroxide Leading to Oxidative Endothelial Damage Sen et al. demonstrated that the oxidative stress induces transforming growth factor β_1 (TGF β_1) resulting in an increased expression of α-smooth muscle actin (αSMA). In addition, homocysteine activates extracellular signal-regulated kinase (ERK) augmenting type 1 collagen deposition. These events are evidenced by vessel wall thickening, stiffness and fibrosis (Sen et al. 2006).

Increased Platelet Adhesion Due to Increased Synthesis of Thromboxane A_2 (TXA_2) Di Minno et al. (1993) demonstrated five times higher expression of TXA_2 in homocystinuric patients with homozygous CBS deficiency. This caused activation of new platelets and increased platelet aggregation, thus promoting thrombosis.

Induction of Tissue Factor Khajuria and Houston (2000) showed that increase in plasma homocysteine caused a corresponding increase in expression of tissue factor (TF) by monocytes and that this effect was not mimicked by the homocysteine analogues, homocysteine or homocysteine thiolactone (HCTL). The factor VIIa-TF complex initiates and promotes intravascular coagulation (Buetenas, 2012).

Suppression of Expression of Heparan Sulphate, Protein C and Antithrombin III (AT III) Nishinaga et al. (1993) showed that heparin sulphate from homocysteine-treated porcine aortic endothelial cells revealed less ^{125}I antithrombin III binding activity than that from control cells. No such inhibition was demonstrable in cells treated with the same concentration of methionine, alanine or valine. Hence they concluded that this reduced antithrombin III binding activity was mediated by a sulfhydryl-dependent mechanism. Harpel et al. (1996) studied the protein C enzyme system of coagulation and demonstrated that homocysteine reduces expression of thrombomodulin, causing a decrease in protein C. It also inhibits the antithrombin III binding activity of endothelial heparan sulphate proteoglycan and thereby reduces its antithrombotic activity. Further, it inhibits ADPase activity and promotes platelet aggregation and thrombosis.

Stimulation of Smooth Muscle Cell Proliferation in the Substantia Propria of the Vessel Walls It was shown by Chen et al. (2000a, b) that high homocysteine significantly stimulated both human and porcine carotid artery smooth muscle cells (mitogenic effect) in a dose-dependent fashion and inhibited endothelial cell growth (cytotoxic effect). Kartal et al. (2005) investigated the signaling molecule in the mitotic process and demonstrated that mitogen-activated protein kinase (MAPK) is involved in homocysteine-induced DNA synthesis and vascular smooth muscle cell proliferation. The resultant decreased luminal diameter leads to decreased flow in that vessel, increased turbulence of flow and, thence, predisposition to deposition of cholesterols (atheroma). Also, the imbalance between collagen and elastin caused by homocysteinemia results in decreased pulsatility of arteries and a reduction in velocity of blood flow.

Increased Expression of Metalloproteinases Results in Mucoid Matrix in Vessel Walls and Altered Dynamics of Blood Flow Steed and Tyagi (2011) demonstrated that an increase in inducible nitric oxide synthase (NOS) contributes significantly to the collagen/elastin switch which results in the decline of arterial compliance. Basu et al. (2011) demonstrated an increased expression of matrix metalloproteinases (MMPs) 9 and 12 and a decreased expression of tissue inhibitors of metalloproteinases (TIMPs) 2 and 4 in CBS$^{+/-}$ mice. This alteration in the MMP/TIMP homeostasis causes degradation of elastin and promotes the collagen/elastin switch seen in homocysteine-induced vascular remodelling. They also demonstrated the interesting concept that veins expressed arterial phenotype under the influence of homocysteinemia (Basu et al. 2011). Further, Givvimani et al. (2013) demonstrated decreased expression of connexins 37, 40 and 43 and increased expression of myostatin in CBS$^{+/-}$ mice, indicating a role of homocysteine in these expressions. This resulted in delayed conduction of vasodilation in skeletal muscle arterioles and consequent decreased tissue perfusion to contracting skeletal muscles. Increased expression of MMPs 2, 9 and 14 in the brain and cochlea of inherently hyperhomocysteinemic mice (CBS$^{+/-}$) has been demonstrated by Kundu et al. (2009), leading to an alteration of the extracellular matrix (and consequent alteration of functions) in these organs.

Impaired Regeneration of Endothelial Cells Tsai et al. (1994) showed that while in rat aortic smooth muscle cell (RASMC) homocysteinemia induced DNA synthesis, it reduced DNA synthesis in the human umbilical vein endothelial cells, inhibiting endothelial cell growth and repair and compromising the endothelial lining of blood vessels.

Impaired Regulation of Endothelium-Derived Relaxing Factor and Related Nitrogen Oxides In their experiment on cultured rat aortas, Mujumdar et al. (2001) demonstrated that homocysteine induced redox-mediated endothelial dysfunction and nitrotyrosine formation. They also demonstrated that the "length-tension relationship of homocysteine treated aortas was shifted to the left as compared to untreated aortas, indicating reduced vascular elastic compliance in homocysteine treated vessels". This is evidenced by decreased vascular capacity for dilation.

Impaired Synthesis of Proteoglycans A core protein with one or more attached side chains of glycosaminglycans (GAGs) comprises a macromolecule called a proteoglycan (Fujiwara et al. 2008). These are important constituents of extracellular matrix. As evidenced in Fig. 1.1, homocysteinemia promotes synthesis of proteoglycans, resulting in excessive accumulation of these proteoglycans in the smooth muscle cells of the blood vessels. Thus, the physiology of the blood vessels and flow mechanics are altered, promoting atherosclerosis. Similarly, extracellular matrix of other tissues is also altered by homocysteinemia. Bones are known to exhibit low density in the presence of homocysteinemia (Fratoni and Brandi 2015).

Oxidation of Low-Density Lipoprotein (LDL) Pfanzagl et al. (2003) investigated the role of sulphur-containing amino acids in LDL modification by arterial smooth muscle cells. They demonstrated that metal-catalysed LDL oxidation is observed with a mixture of homocysteine, cystine and cysteine, thus sustaining the hypothesis that homocysteine acts as a risk marker for coronary artery disease through production of oxidative stress. Physiological concentrations of homocysteine (1–5 μmol/L) inhibit the expression of the antioxidant enzyme cellular glutathione peroxidase (GPx), which results in an increase in reactive oxygen species that inactivate nitric oxide and promote endothelial dysfunction (Chen et al. 2000a, b).

Homocysteinylation of Plasma Proteins and Low-Density Lipoproteins (LDLs) by Homocysteine Thiolactone (HCTL) In the presence of normal plasma homocysteine levels, production of thiolactone is low, but it increases with the homocysteine concentration. Homocysteine thiolactone is a highly reactive molecule. It reacts with protein lysine residues and acylates their free amino groups. This results in alteration of the physicochemical properties and biological activity of these proteins. One molecule that is susceptible to its alteration is LDL. This molecule, after homocysteinylation, becomes more susceptible to oxidation, and its uptake by macrophages is accelerated, the first step of formation of an atheroma. HCTL also causes increased platelet aggregation, contributing to thrombotic phenomena. Normally HCTL is metabolized by paraoxonase 1 (PON1) which is attached to HDL and has been found to be lowered in patients of CAD (Jakubowski 2000).

Homocysteinylated LDLs Elicit Humoral Immune Response Autoantibodies have been found to the homocysteinylated proteins and LDL. These antihomocysteinyl lysine antibodies formed have been detected in higher concentrations in patients with ischemic heart disease or ischemic cerebral stroke, probably thus accounting for the accelerated atherogenesis in homocysteinemia (Beltowski 2005). LDL-homocysteine thiolactone aggregates are basically oxidized LDL particles. Like other oxidized LDL particles, they contribute to early atherosclerotic plaque formation as they are taken up by the macrophages of the artery walls to form the foam cells that are seen in these plaques. Once they migrate through the vessel walls, these foam cells degrade and release fat and cholesterol into developing plaques. In fact, these foam cells are responsible for altering the handling of oxygen by the surrounding cells of the arterial wall. It has been shown that this is a result of the release of homocysteine thiolactone by the foam cells. Consequently, there is an accumulation of highly reactive oxygen radicals within cells causing damage to the lining cells of arteries, promoting formation of blood clots and stimulating the growth of arterial smooth muscle cells.

Homocysteine Is a *N*-Methyl D-Aspartate (NMDA) Receptor Agonist NMDA receptors, when activated, increase intracellular calcium and, thereby, lead to increased cell excitability. These receptors are known to be present in cardiac tissue. Maldonado et al. (2010) described that in addition to causing oxidative stress in the cardiac cell and activating MMPs that degrade cell membranes and proteins, homocysteinemia is also an NMDA receptor agonist, whereby it induces arrhythmogenesis.

Thus, homocysteine may interact with a variety of systems and induce a cascade of events that ultimately results in plaque formation and vascular occlusion, as shown in Fig. 3.2.

3.2 Homocysteine and Occlusive Vascular Disease

A modifiable risk factor, homocysteine has been implicated as a precipitant in many disorders, but its association with vascular disease has been the longest. It has come to the fore mainly due to its established correlation with CAD (Arnesen et al. 1995; Nygard et al. 1997; Wald et al. 1998) even though it affects almost every system in the body. Since then, several studies have demonstrated an increase in mortality from myocardial infarction (Wald et al. 1998) and stroke (Perry et al. 1995) in patients with elevated homocysteine levels.

It was in 1969 that Dr. Kilmer S. McCully, while attending a conference on human genetics at Massachusetts General Hospital, Boston, first observed a similarity in the reports of vascular lesions of two postmortem cases of homocysteinuria—one an 8-year-old retarded male with cystathionine-β-synthase deficiency, reported in 1933, and the other a 7-week-old retarded male with an abnormality of vitamin B_{12} metabolism, reported in 1969. In the former, there was no gross evidence of disease in the heart, aorta, pulmonary artery, venae cavae or other major vessels, except for the carotid arteries. However, there were widespread focal alterations in the

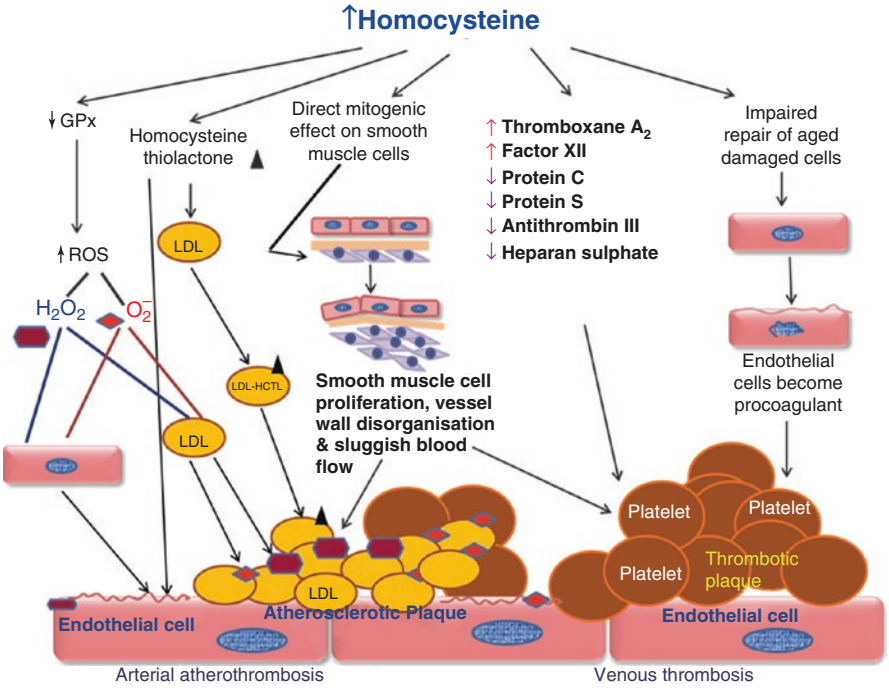

Fig. 3.2 Several mechanisms by which homocysteinemia causes atherothrombosis. *GPx* Glutathione peroxidase, *ROS* reactive oxygen species, H_2O_2 hydrogen peroxide, O_2^- nascent oxygen, *LDL* low-density lipoprotein cholesterol, *LDL-HCTL* homocysteinylated LDL, ↑ increased, ↓ decreased

medium-sized and small arteries in the thymus, adrenals, kidneys, heart and lymph nodes. In the latter, again, no gross lesions or occlusions were found in the cardiovascular system, but extensive focal microscopic alterations were found involving the large, medium-sized and small arteries in many organs (Mudd et al. 1969), which were similar to those found in the above-mentioned case. The only metabolic feature common to both these cases was homocysteinuria and homocysteinemia.

The stained tissue sections were compared with suitable age-matched controls and selected sections from three patients with CBS deficiency reported in literature (Carson and Neill 1962; Schimke et al. 1965). The arterial changes found, involving both large and small arteries, were very similar to those present in the three patients with proven homocysteinuria, irrespective of the cause of this homocysteinuria.

In 1970, McCully's (McCully and Ragsdale 1970) work on cultured cells from normal skin and that of individuals with CBS deficiency suggested that homocysteinemia produced accelerated arteriosclerosis in these children by altering the normal fibrillar structure of the arterial wall proteoglycan molecules and that a similar process may occur in individuals without enzyme deficiencies. To prove this hypothesis, McCully and his colleague conducted experiments on rabbits, which, in

addition, suggested that lipid accumulation is a secondary complication of the primary vascular alteration. Again, animal proteins are relatively abundant in methionine compared to plant proteins, and many experimental atherogenic diets contain high concentrations of methionine as well as cholesterol and other lipids (McCully and Ragsdale 1970). This interpretation of the dietary origin of arteriosclerosis correlates very well with the data on consumption of methionine-rich foods by various socio-economic groups with a high incidence of cardiovascular disease, and significant atherosclerosis is less common in people whose diet over the life span is predominantly vegetarian (Katz et al. 1985).

In 1975, once again, the pioneer of homocysteine, McKully, along with Wilson, gave the "Homocysteine Theory of Arteriosclerosis", which stated that arteriosclerotic plaques were a result of accumulation of closely related sulphur amino acids, including methionine, homocysteine thiolactone and homocysteic acid. A possible mechanism of action is the production of homocysteine from methionine and homocysteine thiolactone. This homocysteine causes endothelial injury and decreased platelet survival with resultant arterial thrombosis and fibrous arteriosclerotic plaques (Harker et al. 1974). Another action of homocysteine is the activation of Hageman factor (F XII), which results in kinin-like activity (Ratnoff 1968) and increases platelet adhesiveness (though platelet aggregation and other coagulation parameters are normal) (McDonald et al. 1964).

In 1988, Israelsson et al. (1988) found abnormally high homocysteine levels in fasting states in patients of myocardial infarction (MI) when investigated within 1–7 years after their first MI. This group comprised of patients who suffered their first MI before the age of 55 years and who had a low risk profile vis-à-vis conventional risk factors like hypertension, smoking and raised serum cholesterol. Thus, the vascular morbidity found in these patients was attributable to homocysteine.

In the early 1990s, several cross-sectional and retrospective studies have linked premature vascular disorders with homocysteinemia (Kang et al. 1992; Verhoef et al. 1994). Homocysteine was implicated even in the cases with milder homocysteinemia and without enzyme defects or deficiencies (Ueland and Refsum 1989; Clarke et al. 1991).

So far, studies on homocysteine dealt with its association with vascular morbidity, but there were no studies on its association with mortality. So cardiologist Ottar Nygard et al. (1997) and his colleagues at Haukeland University Hospital in Bergen, Norway, measured homocysteine concentration in 587 patients who were admitted to the hospital for an angioplasty to reopen a clogged heart artery. These patients were followed up for several years and continuously assessed for any recurrence or mortality. This follow-up (for a median of 4.6 years) revealed a strong, graded dose-response relation between the total homocysteine level and overall mortality. The Kaplan-Meier estimates plotted at 4 years showed that in patients with the high plasma homocysteine (≥15 μmol/L), the mortality rate was 24.7%, with 80% of these deaths caused by cardiovascular disease. On the other hand, only 8.6% of patients with homocysteine levels in the higher range of the biological reference

interval (9–15 μmol/L) had died, while the death rate for those with lowest homocysteine levels (<9 μmol/L) was 3.8%. Thus, plasma total homocysteine levels were demonstrated to be a strong predictor of mortality.

Osganian et al. (1999) showed that the distribution of homocysteine levels in children is substantially lower than that observed for adults, though a small percentage of children are still potentially at elevated risk for future cardiovascular disease.

Elevated plasma homocysteine having been established as an independent risk factor for atherosclerotic vascular disease affecting coronary, cerebral and peripheral arteries, its dose-response effect was further emphasized by Boushey et al. (1995). They found that the risk of coronary heart disease conferred by a 5 μmol/L increase in plasma homocysteine is equivalent to the risk conferred by an increase in serum cholesterol of 20 mg/dL. They also observed that an increment of 5 μmol/L in homocysteine was associated with an odds ratio of 1.6 (men) and 1.8 (women) for the development of CAD, 1.5 for CVD and 6.8 for PVD. Their data also suggested that the increment of risk is linear without threshold effect and even a small increase in plasma homocysteine leads to increased risk. Their meta-analysis of 27 studies provides considerable evidence that elevated homocysteine levels are not only associated with atherosclerotic vascular disease but also that the association of total homocysteine and coronary artery disease meets the criteria of causality for a risk factor (Hill 1965) –consistency, strength, temporality and biological plausibility. Wald et al. (2002), also elucidated that lowering homocysteine by 3 μmol/L from the current levels (which may be achieved by increasing intake of folic acid and vitamin B_{12}) would reduce the risk of ischemic heart disease by 16%, deep vein thrombosis by 25% and stroke by 24%.

A year later, Malinow et al. (1996) showed that the association of total homocysteine with vascular disease is graded significantly over homocysteine concentration, even more so after adjustment for age, body mass index, alcohol intake, cigarette smoking and lipid, lipoprotein and apolipoprotein parameters.

Selhub et al. (1996) showed the relationship between plasma homocysteine, vitamin status and extracranial carotid artery stenosis. Their main conclusions were:

- Mild elevation of plasma homocysteine occurs in about 30% of elderly population.
- Much of the high homocysteine is caused by deficient dietary intake of the following vitamins—folic acid, vitamin B_6 and vitamin B_{12}.
- High homocysteine is linked to the thickening of the walls of the carotid arteries.

Nappo et al. (1999), collected data on healthy subjects. They demonstrated that a mild to moderate elevation of plasma homocysteine levels in these subjects resulted in the activation of coagulation by modifying the adhesive properties of the

endothelium and impairment of vascular response to arginine. When these subjects were pretreated with the antioxidants vitamin E and ascorbic acid, the effects of homocysteinemia were attenuated, suggesting an oxidative mechanism for the action of homocysteinemia.

The Rotterdam study conducted by Bots et al. (1999) established that the risk of stroke and myocardial infarction increased directly with total homocysteine, and this association was more pronounced among those with hypertension. They demonstrated that an increase in plasma homocysteine of 1 μmol/L increases risk by 6–7%. The odds ratio due to plasma total homocysteine above 18.6 μmol/L was 2.43 for myocardial infarction and 2.53 for stroke. Giles et al. (2000) corroborated these findings, elucidating that there was an almost twofold increased likelihood of myocardial infarction among persons with a total homocysteine >15 μmol/L, an association unaffected by race and ethnicity.

Plasma homocysteine raises the risk associated with increasing age, hypertension and smoking, in addition to being an independent risk factor for CAD (Gupta et al. 2005). Also, Rasouli et al. (2005) elucidated that presence of elevated homocysteine >12 μmol/L strongly and independently predicts progression of coronary plaque burden.

Studies conducted in our laboratory have elucidated that the mean plasma homocysteine levels in the healthy North Indian urban population is significantly higher than that established in worldwide populations ($p < 0.01$). These studies also suggest that patients with vascular disease have higher homocysteine levels, which is even higher in cases of deep vein thrombosis as compared to those of arterial occlusion (Bhargava et al. 2003, 2004, 2006).

Other aspects of vascular disease have also been studied with relation to homocysteine levels. Tanriverdi et al. (2006a, b) observed that homocysteine levels were significantly positively correlated to intima-media thickness in patients of coronary slow flow. Plasma homocysteine was also found to be associated with quantity of coronary artery calcification independent of other CAD risk factors (Kullo et al. 2006).

Thus, it emerged that the pathogenicity of homocysteine is primarily due to its various effects on the vasculature, and over two decades after the discovery of its association with atherosclerosis, homocysteine attained a place parallel to cholesterol and triglycerides as an independent risk factor in atherothrombotic vascular disease.

The excitement of a new marker for vascular disease was followed by a period of scepticism. Brattstrom and Wilcken (2000) suggested that though reducing markedly elevated levels of homocysteine, as seen in inborn errors of metabolism (CBS deficiency), reduced the cardiovascular risk, lowering mildly elevated levels of plasma homocysteine was of undetermined value so far as risk reduction was concerned. Similarly, Abdu et al. (2001) demonstrated a lack of significance of homocysteine levels in cardiovascular disease in patients with growth hormone deficiency.

At the same time, reports correlating homocysteine positively with varied aspects of vascular disease continued. Rasouli et al. (2005), in an American population, elucidated that presence of elevated homocysteine >12 μmol/L strongly and independently predicts progression of coronary plaque burden. Tanirvedi et al. compared plasma homocysteine levels to carotid artery intima-media thickness in a Turkish population with coronary slow flow and elucidated that plasma homocysteine significantly correlated positively with the carotid intima-media thickness and mean thrombolysis in myocardial infarction (Tanriverdi et al. 2006a, b). In a Korean population, Yoon et al. (2012) demonstrated that endothelial dysfunction preceded carotid artery intima-media thickening and that both correlated to plasma homocysteine in patients with slow coronary flow. Plasma homocysteine was also found to be associated with quantity of coronary artery calcification independent of other CAD risk factors (Kullo et al. 2006).

3.3 Homocysteine and Occlusive Vascular Disease in Indians

In several experiments conducted outside the Indian subcontinent, scientists reported that Asian Indians had higher plasma homocysteine levels. In their parallel case-control studies on Europeans (n = 801; 294 cases and 507 controls) and Indians (n = 775; 257 cases and 518 controls), Chambers et al. (2000) reported that though plasma homocysteine levels were 8% higher in cases as compared to controls in both ethnic groups, fasting plasma homocysteine concentrations in controls were 6% higher in Indians as compared to Europeans. They also concluded that elevated homocysteine levels may contribute to twice as many CAD deaths in Asian Indians as compared to Europeans. Chandalia et al. (2003), in their study on Asian Indians living in the United States, reported an elevated homocysteine in this population as compared to the Caucasians with a low vitamin B_{12} and a significant negative correlation between the two parameters.

In their case-control study on 565 subjects (221 controls and 344 patients of coronary artery disease), Sastry et al. (2000) concluded that homocysteine was not significantly different in cases and controls, the mean plasma homocysteine levels in their control subjects being 18.04 ± 10.69 μmol/L and in angiographically proven coronary artery disease being 18.49 ± 10.04 μmol/L. It is interesting to note, however, that these homocysteine levels are higher than the universally accepted BRI of 5–15 μmol/L. This would indicate that the Indian population is prone to homocysteinemia.

Studies conducted in the biochemistry laboratory of Sir Ganga Ram Hospital, New Delhi (n = 788; 252 controls and 536 patients), have elucidated that mean plasma homocysteine levels in North Indian patients of vascular disease are significantly higher than that in healthy controls ($p < 0.001$). During our review of literature, we had noticed that previous studies correlating homocysteinemia to vascular disease included only patients with arterial occlusion. One of the few studies

conducted on deep vein thrombosis (DVT) elucidated a lack of correlation between plasma homocysteine levels and DVT (Amundsen et al. 1995). This seemed at variance with the postulated mechanisms of vascular pathology due to homocysteinemia. Therefore, we conducted a study to elucidate the correlation, if any, between plasma homocysteine levels and DVT in Indian patients. We were the first in India to demonstrate that plasma homocysteine concentrations as well as prevalence of homocysteinemia are similar in deep vein thrombosis (DVT) and arterial occlusion. The highest mean homocysteine concentrations in patients of PVD were seen in DVT, especially if it were complicated by pulmonary embolism (PE). This indicated that homocysteine could be used as a prognostic marker in DVT and that reducing homocysteine could help prevent PE, a potentially fatal condition (Bhargava et al. 2003, 2004, 2007). Gupta et al. (2005) elucidated that plasma homocysteine raises the risk associated with increasing age, hypertension, serum cholesterol levels and smoking, in addition to being an independent risk factor for CAD in Indians.

Having established that homocysteinemia is a significant risk factor for occlusive vascular disease in Indians more than in other populations, we felt that elucidating the risk it conferred for vascular disease as compared to that due to conventional biochemical risk factors [cholesterols, triglycerides, Lp (a)] would help in better prognostication of these patients and establish probable therapeutic measures required in Indian patients of occlusive vascular disease. This would enable us to formulate an integrative approach towards reducing the incidence and morbidity of vascular disease.

In our study in the Indian population, normal controls exhibited a highly atherogenic milieu in their blood with mean cholesterols and triglycerides near the upper limit of the biological reference interval as depicted in Fig. 3.3.

The patients, too, had similar mean blood concentrations of these parameters, i.e. near the upper limit (or lower limit in case of HDL cholesterol) of the BRI. Mean homocysteine, on the other hand, was normal in the controls and more than double of that in every category of vascular disease patients (2.1 times normal in CAD, 2.2 times in CVD and 2.7 times in PVD). Maximum ratios (mean homocysteine in patients as compared to controls) reported in earlier literature are 1.8 for CAD (Baby et al. 2009), 1.5 for CVD (Araki et al. 1989) and 2.1 for PVD (Marcucci et al. 2001), which were much less than the corresponding ratios in the Indian population as mentioned above. These results indicated that in addition to lifestyle modifications and lipid-lowering measures, homocysteine levels in Indian patients of vascular disease need to be modified.

Several studies from different parts of the globe have reported the prevalence of homocysteinemia in occlusive vascular disease as 30–50%. Our study demonstrated a higher prevalence of almost 60% and higher in these patients as shown in Fig. 3.4. Multivariate analysis of our data also revealed that the only parameter that was significantly different in all three vascular disease categories was homocysteine; in CVD, triglycerides also showed significance, and in CAD HDL cholesterol, triglycerides and lipoprotein (a) were also significant.

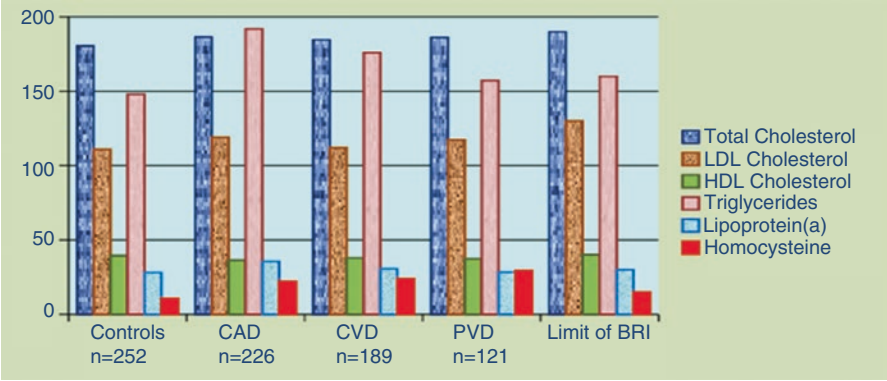

Fig. 3.3 The mean blood concentrations of cholesterols (mg/dL), triglycerides (mg/dL), lipoprotein(a) (mg/dL) and homocysteine (μmol/L) in Indian controls and patients of vascular disease. *CAD* Coronary artery disease, *CVD* cerebrovascular disease, *PVD* peripheral vascular disease, *BRI* biological reference interval

To further exemplify the importance of homocysteine in Indians, we elucidated the odds ratio of vascular disease due to blood concentrations above the population mean of all these parameters (Table 3.1).

The odds ratio conferred by the cholesterols, triglycerides and Lp (a) ranged between 1.034 and 1.855 for all categories of vascular disease, whereas that conferred by homocysteine was 4.153 for CAD, 3.336 for CVD and 3.170 for PVD, almost three times as much risk as conferred by any of the other "conventional risk factors". Also, when the odds ratio was calculated for homocysteine >15 μmol/L (the upper limit of the biological reference interval), the risk almost trebled, becoming 11.792 for CAD, 9.607 for CVD and 9.859 for PVD. This indicated that pathology due to increasing homocysteine was enhanced even within the biological reference range, corroborating the findings of the meta-analysis done by Boushey et al. (1995), who concluded that the less the circulating homocysteine, the better. Hence, measures to reduce homocysteine are imperative, especially in this population.

This brought forth the query as to whether or not Indians are genetically or nutritionally prone to homocysteinemia.

MTHFR C677T is the most common polymorphism causing homocysteinemia. Table 3.2 summarizes data on global prevalence of MTHFR C677T polymorphism.

It shows that the prevalence of the T allele is much lower in the Indian and Sinhalese population (0–16%) than in many other global populations, being highest in the Costa Rican Indians (59–70%). The frequency of the T allele in North Indian patients of vascular disease was about 20% (Table 3.2). Thus, though the mean homocysteine was twice as high in vascular disease patients as compared to controls, the frequency of the T allele in the MTHFR gene could not account for it.

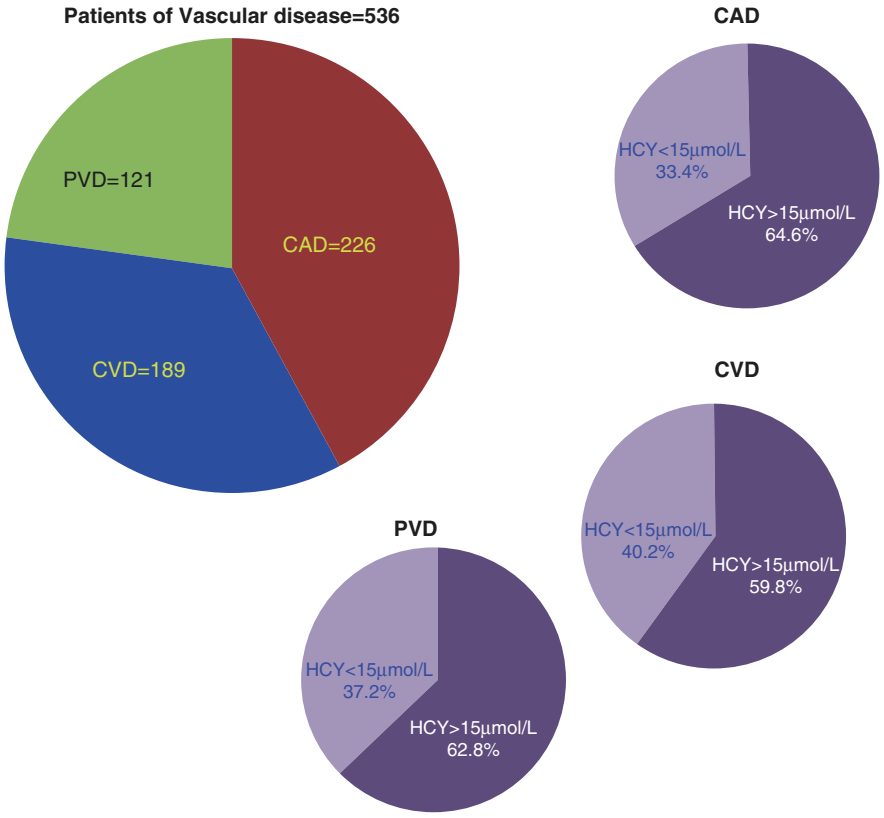

Fig. 3.4 Distribution of homocysteinemia in Indian patients of vascular disease, coronary artery disease, cerebrovascular disease and peripheral vascular disease. Prevalence of homocysteinemia in Indian patients of CAD, CVD and PVD was 64.6%, 59.8% and 62.8%, respectively (Bhargava et al. Current Medicine Research and Practice 2014;4(3):112–118)

Table 3.1 Odds ratio for vascular disease due to biochemical parameters above the population mean in Indian patients of vascular disease

Biochemical parameter	Odds ratio in CAD	Odds ratio in CVD	Odds ratio in PVD
Total cholesterol >180.44 mg/dL	1.134	1.051	1.034
HDL cholesterol <39.53 mg/dL	1.855	1.590	1.389
LDL cholesterol >110.90 mg/dL	1.425	1.201	1.235
Triglycerides >147.90 mg/dL	1.817	1.275	0.898
Lipoprotein(a) >28.5 mg/dL	1.316	1.108	0.873
Homocysteine >10.78 µmol/L	4.153	3.336	3.170
Homocysteine >15 µmol/L	11.792	9.607	9.859

Odds ratio for each category of vascular disease due to each biochemical parameter above its mean serum concentration in controls (below its mean for HDL cholesterol) revealed the high odds ratio for vascular disease due to homocysteine. Odds ratio of vascular disease due to homocysteine >15 µmol/L (the upper limit of the biological reference interval) (Modified from Bhargava et al. 2012b)

Table 3.2 Prevalence of T allele of the MTHFR C677T polymorphism

Author (year)	Population	No. of subjects	% frequency of T allele
Malik et al. (1998)	United Kingdom	233	7.3
Herrmann et al. (2001)	Costa Rican Blacks	95	16.3
	Indians (Punjab)	150	16.6
	NE-Germans	170	29.1
	Costa Rican blood donors	194	39.7
	Costa Rican Chorotega Indians	76	59.9
	Costa Rican Bribri Indians	77	70.1
Sadewa et al. (2002)	Indonesian Japanese	68	6
	Japanese	174	37
Wilcken et al. (2003)	Mexican Americans	500	57
	Italians	1343	41–46
	North Chinese	643	44.2
	Atlantan Hispanics	62	41.1
	French	178	35.7
	South Chinese	430	34.7
	Spanish Whites	601	33.9
	Hungarian	378	33.7
	Atlantan Whites	300	31.7
	Australians	288	28.6
	Netherland	188	27.4
	Russians	587	26.9
	Israelis	210	25.7
	Finnish	545	25.1
	Asians in the United States	26	21.2
	American Blacks	298	12.6
Krajinovic et al. (2004)	French Canadian	174	63.2
Chiusolo et al. (2004)	Italian Caucasian	110	43.2
Gemmati et al. (2004)	Italian Caucasian	257	44.7
Ferrazi et al. (2005)	Northern Italian	50	32.4
De Oliveira et al. (2008)	Brazilian	209	30.6
Dissanayake et al. (2009)	Sinhalese	80	13
	Tamils	80	9
	Moors	80	9
Cyril et al. (2009)	South Indian	120	0
Tripathi et al. (2010)	North Indian	331	7.3
Bhargava et al. (2012c)	North Indian	70	16

The prevalence of T allele of the MTHFR polymorphism ranged from 0% to 16.6% in Indians. The highest prevalence was observed in Costa Rican Indians (Modified from Bhargava et al., Vascular 2012; 20(2): 88–95)

Indians could, therefore, be nutritionally deficient in the B vitamins leading to higher homocysteine concentrations in Indian patients of vascular disease than in other populations.

Nutritional deficiencies as a cause of homocysteinemia had received a lot of attention from scientists. Kang et al. (1987) demonstrated an inverse correlation between plasma homocysteine levels and serum folate concentrations in an American population. Scientists corroborated these findings in subsequent studies in several different populations (American and Swedish), adding that plasma homocysteine levels also inversely correlated to serum levels of the vitamins B_{12} and B_6 (Moller and Rasmussen 1995; Brattstrom et al. 1992; Robinson et al. 1995).

Studies in Western and European populations demonstrated that increasing dietary intake of folate reduced homocysteine levels even in absence of overt folate deficiency, whereas dietary supplements of vitamin B_{12} were effective in lowering homocysteine only in the presence of overt deficiency of this vitamin (Wilcken et al. 1988; Brattstrom et al. 1990; Ubbink 1994).

In their meta-analysis, Boushey et al. (1995) demonstrated that plasma homocysteine levels could be lowered by administration of folate supplements and that reducing homocysteine by 3–4 μmol/L reduces the risk of vascular disease by 30–40%. In 2002, Wald et al. (2002) also did a meta-analysis in which they elucidated that lowering plasma homocysteine by 3 μmol/L from their current levels (by increasing folic acid intake) would reduce the risk of ischemic heart disease by 16%, deep vein thrombosis by 25% and stroke by 24%.

Selhub et al. (1996) showed the relationship between plasma homocysteine, vitamin status and extracranial carotid artery stenosis. They reported that approximately 30% of the elderly population has mild elevation of plasma homocysteine, mostly due to a dietary deficiency of folate, vitamin B_6 and vitamin B_{12}. In addition, high levels of homocysteine were associated with thickening of the walls of the carotid arteries.

These studies included several populations from around the world, but not Indians living in the Indian subcontinent. So our laboratory conducted a study in Indians and revealed that Indian patients of vascular disease had significantly ($p < 0.001$) lower blood concentration of folate than controls, but B_{12} levels were within the BRI and not significantly different from B_{12} in controls (Bhargava et al. 2012a). Despite the normal B_{12} levels, plasma homocysteine bore a significant inverse correlation with these B_{12} levels in the patients, manifest in CVD ($p < 0.05$) and PVD ($p < 0.01$) but not in CAD ($p > 0.5$). Plasma homocysteine correlated inversely with serum folate levels in controls ($p < 0.05$) as well as patients ($p < 0.005$), which was manifest in CAD ($p < 0.05$) and CVD ($p < 0.05$), but not in PVD. This indicated that homocysteinemia is caused by folate deficiency in CAD, vitamin B_{12} deficiency in PVD and deficiency of both vitamins in CVD. Also, folate levels determined homocysteine levels in controls as well. Interestingly, in our study, irrespective of the pretreatment blood concentrations of folate and B_{12}, the response to therapy in terms of percent reduction of homocysteine was similar (over first 6 months of treatment) with a single daily dose of 5 mg of folate or a daily combination therapy with 1.5 mg folate

and 500 mg B_{12} (Bhargava et al. 2012a). This could be accounted for by the dual role played by folate in attenuating homocysteine and its deleterious effects, as described by Hayden and Tyagi (2004). They postulated that not only does an increased folate promote the remethylation of homocysteine to methionine, but also it floods the folate shunt, whereby it acts as cofactor for the nitric oxide synthase (NOS) enzyme and negates the oxidative stress of a high homocysteine.

Hence, to prevent the morbidity of vascular disease in Indians, it would be advisable to employ large-scale measures to reduce homocysteine in this population, possibly by food fortification with these vitamins.

In the early 1980s, the developed countries instituted food fortification with folate to prevent neural tube defects caused by homocysteinemia. Boushey et al. (1995) had predicted that this would prevent 50,000 deaths annually due to CAD alone. This turned out to be an overrated figure. As per the Centers for Disease Control and Prevention who studied the outcome of food fortification in the United States, the overall stroke-associated mortality rate annually declined by about 1% in 1995–1997 period, and this decline increased to 5.4% in the 1998–2001 period after food fortification, accounting for 16,700 fewer annual deaths due to stroke alone (Yang et al. 2006). This decline spanned both sexes and all ethnic races in America, being consistent in the whole population.

Several European countries too have started food fortification (e.g. Germany). The dose used by the FDA was 350 μg per 100 g of flour. Hanky and Eikelboom brought to the fore that fortification with folate alone leads to the masking of early vitamin B_{12} deficiency. By the time these patients were identified, they had already progressed to neurological manifestations. Hence, food fortification now incorporates both folate and B_{12}.

The predominance of non-vegetarian diet, which has high methionine content, could be a contributing factor for homocysteinemia in European and Western populations. Therefore, food fortification in such countries should be more therapeutic than it would be in the predominantly vegetarian countries. But the dietary deficiency of folate and vitamin B_{12} prevalent in Indians seems to put us at a greater disadvantage in terms of homocysteinemia than does a high methionine diet.

3.4 Homocysteine, Lipid Peroxidation and Antioxidant Status

As discussed earlier and as shown in Fig. 3.1, it has been postulated that homocysteine promotes enzymatic as well as non-enzymatic peroxidation of lipids, thereby aiding the process of atherosclerosis. Studies to demonstrate this postulate have been few and far between. There were several that supported lipid peroxidation as a mechanism for homocysteine vascular pathology and many that did not.

In support of this postulate, Jones et al. (1994) examined the toxicity of homocysteine, alone and along with copper, in cultured human umbilical vein endothelial cells. They found that these toxic effects could be prevented by antioxidant catalase and desferal. They also demonstrated that though lipid peroxidation accompanied the

toxicity, inhibiting lipid peroxidation did not affect cell viability. As mentioned earlier, Nappo et al. (1999) showed that mild to moderate elevation of plasma homocysteine levels in healthy subjects leads to the activation of coagulation, modification of adhesive properties of the endothelium and impairment of vascular response to arginine. They also demonstrated that pretreatment with antioxidant vitamin E and ascorbic acid blocks these effects of homocysteinemia, suggesting an oxidative mechanism. In the same year, a study in Finland provided the first conclusive evidence of a role for elevated fasting plasma homocysteine in lipid peroxidation in vivo. They measured F2-isoprostane as a marker of lipid peroxidation and demonstrated a significant simple correlation with plasma homocysteine (Voutilainen et al. 1999). In their attempt to evaluate the role of homocysteine in inducing oxidative stress in coronary artery disease, Cavalca et al. (2001) measured homocysteine and malondialdehyde (MDA) in their subjects. They found that though both homocysteine and MDA levels were significantly higher in the CAD patients as compared to controls, homocysteine at the detected values could not be considered completely responsible for the oxidative stress. Rahbani-Nobar et al. (2004) studied the correlation of homocysteine, total cholesterol and LDL cholesterol with total antioxidant status (TAS) in Iranian patients of hypothyroidism and concluded that enhanced production of free radicals may contribute to the abnormalities seen in homocysteine and cholesterol metabolism. In an Italian population, Caruso et al. (2006) studied the redox status consequent to homocysteine lowering by 5-methyltetrahydrofolate (5-MTHF). 5-MTHF showed a favourable interaction with glutathione (GSH) metabolism. They showed that high doses of MTHF ensured marked lowering of homocysteine indicating an inverse relationship between GSH metabolism and homocysteine.

At the same time, there were several reports that did not support the postulate that homocysteine acts by increasing lipid peroxidation. In 2003, Bayes et al. (2003) in their study on homocysteine as a risk factor for CAD in Spanish haemodialysis patients, found no correlation between homocysteine and oxidized LDL antibody titre. A South American study on oxidative stress of hyperhomocysteinemia was conducted in 2004 by Hirsch et al. (2004), in which they correlated several parameters of oxidative stress (F2-isoprostane, TBARS) and total antioxidant status with plasma homocysteine. They deduced that homocysteinemia was not associated with oxidative stress in presence of normal serum folate.

Evidently, all these studies are equivocal in establishing the presence or absence of a correlation between homocysteine and antioxidant status and lipid peroxidation of an individual. No such studies had been conducted in India, and to the best of our knowledge, our study was the first to demonstrate a definite link between lipid peroxidation as a mechanism in the development of vascular pathology due to homocysteinemia.

We measured homocysteine, MDA and TAS in 170 consecutive patients of vascular disease. The data indicated that homocysteine correlated directly with lipid peroxides (LPOs) in CAD and CVD, but not in PVD (Table 3.3). This could be explained by the fact that among our 42 PVD patients, 38 were diagnosed with DVT, which is purely a thrombotic process without lipid peroxidation (Bhargava et al. 2014).

Table 3.3 Correlation between homocysteine, lipid peroxides and total antioxidant status

Category	Statistical analysis	Homocysteine with lipid peroxides	Lipid peroxides with total antioxidant status	Homocysteine with total antioxidant status
All patients ($n = 170$)	Correlation	0.322	−0.405	−0.006
	p value	<0.001**	<0.001**	0.939
CAD ($n = 43$)	Correlation	0.463	−0.349	−0.099
	p value	0.002**	0.022*	0.527
CVD ($n = 84$)	Correlation	0.435	−0.121	−0.119
	p value	<0.001**	0.281	0.286
PVD ($n = 43$)	Correlation	0.063	−0.206	0.282
	p value	0.690	0.185	0.067

*$p < 0.05$ significant
**$p < 0.005$ highly significant
Modified from Bhargava et al., CJPP 2014; 92:592–597

Our results also indicate that LPO levels in Indian patients with CAD and CVD and with homocysteinemia were significantly higher than in those without homocysteinemia. Total antioxidant status (TAS) was significantly lower in CVD patients with homocysteinemia as compared to those without. Interestingly, TAS levels were higher in CAD and PVD patients with homocysteinemia than in those without (Table 3.3 and Fig. 3.5).

"The natural response to oxidative stress due to homocysteinemia is a surge in the antioxidant defence system (Wilcken et al. 2000; Bea et al. 2009). It may be hypothesized that these antioxidants are consumed in countering the oxidative stress. Therefore, their levels still remain low in the serum in the initial stages of disease process because the antioxidant response is comparatively less than the oxidative stress. Hence, it would be expected that TAS would be lower in patients with homocysteinemia in the initial stages of the disease". Later, as the antioxidant response would continue to surge, a time would come when the antioxidant response balances the oxidative stress due to homocysteinemia.

But before the system recognizes this fact, antioxidants continue to be produced as part of the extended defence mechanism of our system. Hence, in later stages of homocysteinemia, TAS would be higher than in the earlier stages. This sequence of events could be compared to weighing a substance in a two-pan balance; if the substance being weighed is lighter than the weights, one keeps adding the substance till a time comes when the balance tips in the other direction before readjustment of the amounts of substances in the two pans equalizes them.

Since the pathological progression of "atherosclerosis/thrombosis in CAD and PVD is slow and continues for years, symptoms are delayed and these patients present themselves in a hospital at an advanced stage of the disease process. On the other hand, CVD becomes symptomatic at a much earlier stage as even a slight decrease in blood flow to any part of the brain can have grave consequences; hence these patients come to the hospital at a very early stage. This

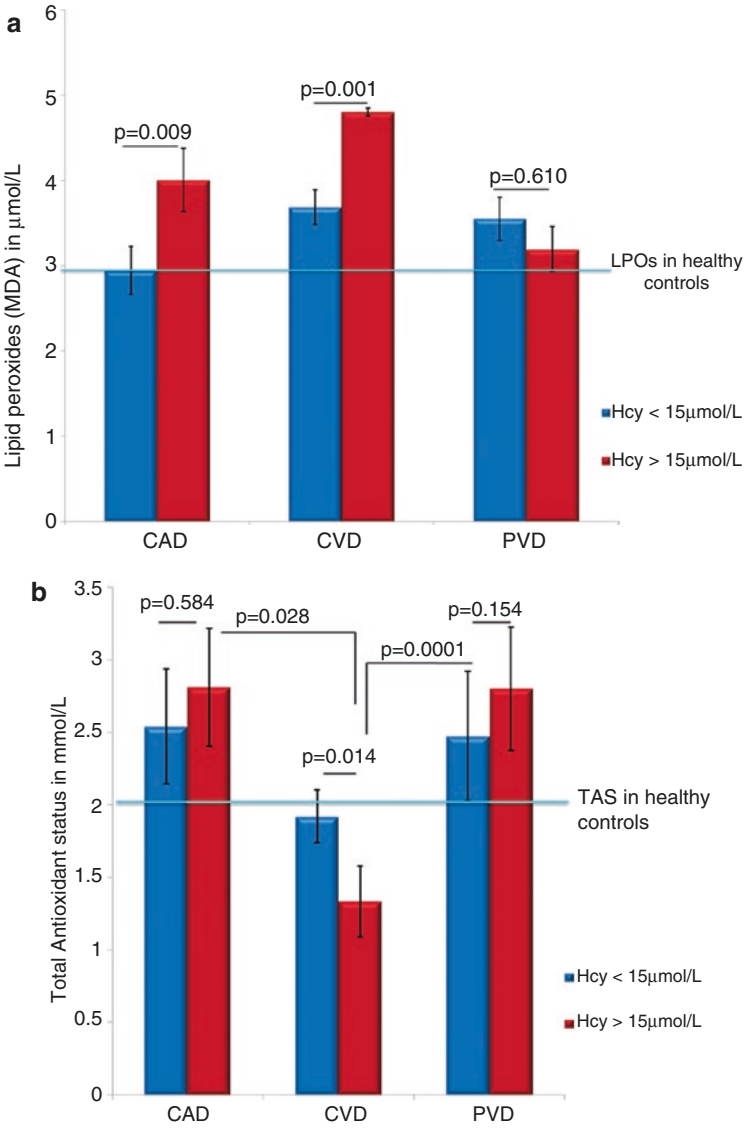

Fig. 3.5 Serum LPOs in Indian patients of occlusive vascular disease with hcy more than and less than 15 µmol/L. (**a**) Lipid peroxide levels were significantly higher in CAD and CVD patients with hcy >15 µmol/L than in those with hcy <15 µmol/L. In PVD patients, there was no significant difference in the two groups. (**b**) In CAD patients, TAS was lower in patients with hcy >15 µmol/L than in those with hcy <15 µmol/L (though not significantly), and, hence, antioxidants could be used as adjuvant therapy in these cases. Paradoxically, TAS was higher in patients of CVD and PVD with hcy >15 µmol/L (Reproduced from Bhargava et al., Can J Physiol Pharmacol 2014; 92:592–597)

could probably explain the significantly lower TAS in CVD patients with homocysteinemia than in those without homocysteinemia; whereas the opposite is the case in CAD and PVD".

Our results indicated that homocysteinemia-induced lipid peroxidation plays an important role in CAD and CVD. In peripheral vascular disease (especially deep vein thrombosis), this is not the case. Moreover, in patients of CVD, antioxidants could be an important therapeutic adjuvant towards buffering the homocysteinemia-induced oxidative stress.

Thus, morbidity of occlusive vascular disease due to homocysteine could be reduced further.

Lacunae in Knowledge

The role of homocysteine in vascular disease is the most extensively studied aspect of homocysteine. Yet, there is a requirement of studies to elucidate the common polymorphisms of the enzymes of its metabolism that are associated with homocysteinemia in the Indian population. Also needed are case-control studies on the results of vitamin supplements in each type of occlusive vascular disease in all populations.

Clinical Message

1. Homocysteinemia is emerging as a major modifiable risk factor in vasculo-occlusive disease. Although the degree of risk varies among different populations, several studies have shown a positive correlation of homocysteinemia with all types of occlusive vascular disease across populations. As a susceptible population, Indians are at a higher risk of developing homocysteinemia which confers a much higher odds ratio for vasculo-occlusive disease in this population than in many others. Hence, it would be pertinent to include measurement of homocysteine in the vascular risk assessment in this population.
2. In persons at high risk of vascular disease, especially if it is recurrent, vitamin B supplements in appropriate doses may be instituted to minimize risk.
3. In CAD and CVD patients, serum levels of lipid peroxides directly correlate with circulating homocysteine concentrations; in addition, in CVD patients, antioxidants are inversely related to homocysteine. Hence, antioxidants could be instituted as supportive therapeutics in such patients.
4. Since high level of homocysteine can be lowered by dietary modifications, developed countries have adopted food fortification with folate and vitamin B_{12}. Indeed such measures have successfully reduced deaths due to CAD and stroke, e.g. food fortification with folate and vitamin B_{12} has been shown to prevent the morbidity and mortality by normalizing homocysteine levels. It would be, therefore, beneficial to our population as a whole if such a measure were to be adopted.

3.5 Homocysteinemia and Skeletal Muscles

When we talk of homocysteinemia-induced vascular alterations, skeletal muscles deserve individual mention as the short-term mechanisms have a greater role to play. Under situations of increased muscle action, there is a demand-induced conductance vasodilatation of the arterioles to take care of the enhanced metabolic needs of the tissue; this is mediated through increased nitric oxide synthesis as well as adequate connexins required to carry the hyperpolarization signals for vasodilatation. Homocysteinemia interferes with this process by reducing the expression of inducible nitric oxide synthase, thus reducing the available nitric oxide and decreasing the vasodilatation of the arterioles. It also reduces the expression of connexins (Veeranki and Tyagi 2013). Earlier it was postulated that homocysteinemia increased production of the reactive oxygen species, causes oxidation of the key enzymes of the glycolytic and Kreb's pathways and thereby decreased energy yield from these processes. In their experiment on $CBS^{+/-}$ (hyperhomocysteinemic) and C57 (control) mice, Veeranki et al. (2016) demonstrated that the enzymes were not altered but ATP production was reduced through marginal reduction in dystrophin levels along with a decrease in mitochondrial transcription factor A (mtTFA). It was also observed that the morphology of the muscle fibres remained the same, but there was a reduction in large muscle fibres in the $CBS^{+/-}$ mice which corresponded to the fatigability. Thus, homocysteinemia induces oxidative and metabolic stress in the muscle tissue and, thereby, enhanced fatigability, mainly through mitochondrial dysfunction and epigenetic changes. This fatigability is especially evident in the elderly who exhibit homocysteinemia. Interestingly, in the mice, the molecular elevations seen due to homocysteinemia were reversed after exercise.

Clinical Message

1. In patients complaining of muscle fatigue, homocysteinemia should be ruled out.
2. While treating elderly patients, the presence of concomitant vitamin B deficiencies leading to homocysteinemia, or homocysteinemia per se, should be kept in mind and managed accordingly.

Homocysteinemia and Hypertension

4

4.1 Homocysteine and Hypertension

As has been described in the previous text, homocysteine affects the vascular endothelium as well as the smooth muscle layer of the vessel walls. In addition, if one looks at the metabolism again, one observes three more effects:

Firstly that homocysteine also gets oxidized to homocysteic acid which is involved in the synthesis of proteoglycans of the vessel wall as well as bones and cartilages. When homocysteine is in excess, the synthetic components of these proteoglycans are altered, affecting the compliance of the vascular wall.

Secondly that when homocysteine is in excess, more SAM is available for methylation reactions modulating protein synthesis with resultant alteration in the elastin/collagen ratio (Fig. 3.4).

Thirdly, increased SAM also induces the MMP/TIMP axis leading to alterations in the increased formation and accumulation of extracellular matrix protein, such as collagen, once again resulting in an altered elastin/collagen ratio (Jakubowski 2000). The end result is a stiff vascular wall and a narrowed lumen with consequent hypertension (Fig. 4.1).

The Third National Health and Nutrition Examination Survey (NHANES) studied the association of homocysteine with blood pressure and with the risk of hypertension using cross-sectional data. They found that, after adjusting for cardiovascular risk factors, homocysteine had an independent positive correlation with blood pressure (Lim and Cassano 2002).

Jain et al. (2003) observed that plasma homocysteine in patients of essential hypertension was significantly higher ($p < 0.0001$) than in controls, and, interestingly, these levels in their normotensive siblings were also significantly higher ($p < 0.0001$) than in the controls. However, the Framingham study did not find any significant causal role of plasma homocysteine in the incidence of hypertension in their large community-based sample (Sundstrom et al. 2003). Several years later, in

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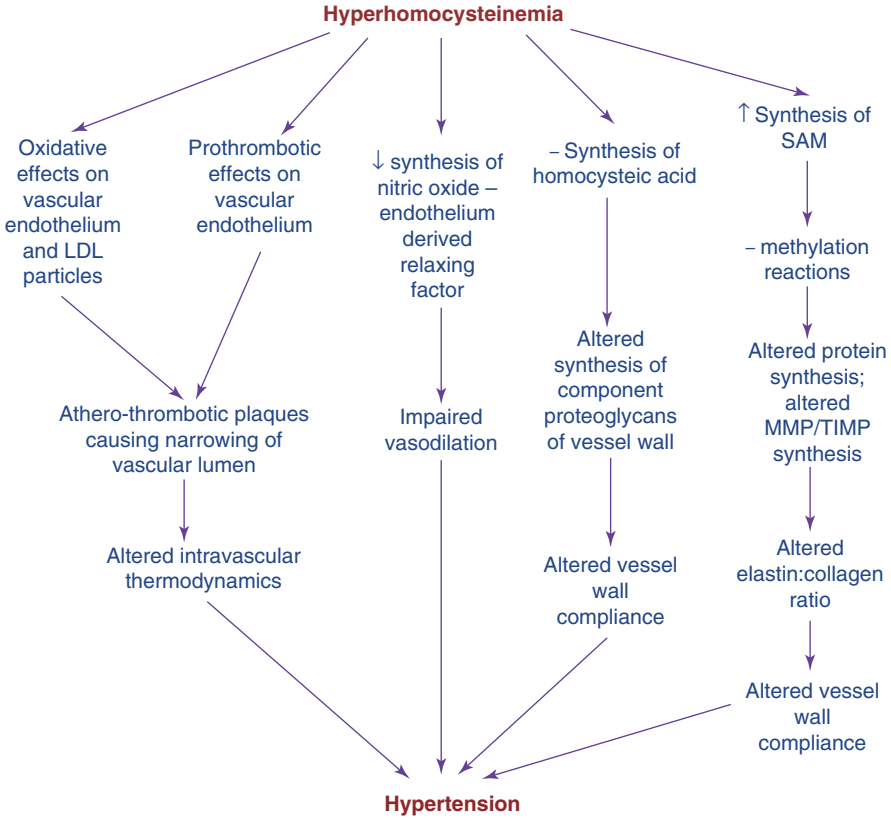

Fig. 4.1 Pathophysiology of hypertension due to homocysteinemia. Homocysteinemia can result in hypertension through several mechanisms affecting the vessel wall

a research letter by Bowman et al. (2006), it was demonstrated that subsequent development of hypertension was associated with a significantly higher baseline homocysteine. Atif et al. (2008) described a high homocysteine in 80 of their 100 hypertensive subjects. More recently, a study, enrolling over 2000 patients, demonstrated:

– That homocysteine directly correlated with pulse wave velocity in the aorta in hypertensive as well as normotensive subjects
– That homocysteine independently determines arterial stiffness (expressed as aortic augmentation index) in hypertensive subjects

[Pulse wave velocity (PWV) is a direct indicator of arterial stiffness—the greater the stiffness, the greater the PWV].

In 2010, Sen and Tyagi (2010) demonstrated yet another mechanism by which homocysteinemia causes vascular remodelling—through modulation of the MMP/TIMP axis. MMPs are Ca^{++}- and Zn^{++}-dependent endopeptidases, which are required for physiological as well as pathophysiological processes. Of these MMPs 2 and 9 are gelatinases. They degrade collagen IV, which is essential for vascular wall integrity, oxidizing it to cause fibrosis and, hence, hypertension.

In 2014, Xu et al. (2014) demonstrated that telmisartan (angiotensin receptor blocker used as antihypertensive drug) inhibits the homocysteinemia-induced pro-inflammatory effects in human umbilical vein endothelial cells cultured in vivo.

Narayanan et al. (2014) demonstrated that 5-aza-2′-deoxycytidine, a DNA methyltransferase inhibitor, reduced the expression of DNMT1, MMP9, TIMP1 and *S*-adenosyl homocysteine hydrolase (SAHH) and upregulated that of MTHFR, thereby mitigating the homocysteinemia-induced aortic remodelling and hypertension.

It would be pertinent to mention here that one of the products of metabolism of homocysteine and cysteine is hydrogen sulphide (H_2S). It is known to play a key role in several physiological processes, e.g. inflammation, angiogenesis, oxidant regulation, vasodilatation and neuromodulation. A derangement of the cysteine-homocysteine metabolism and clearance, with ensuing deregulation of H_2S biosynthesis, leads to hypertension. The gut microbiota also impacts the regulation of H_2S and is known to, thereby, contribute to hypertension. Weber et al. (2016) have discussed how H_2S physiology may be exploited for therapeutic intervention in hypertension, suggesting that normalizing gut bacteria could be used as an adjuvant to antihypertensive therapy. In fact, this would have a dual role in hypertension—one is by the modulation of H_2S metabolism and clearance from the circulation and the other, we postulate, could be by enhancing vitamin B_{12} production (80% is synthesized by the gut bacteria) and thereby promoting the methylation cycle of homocysteine metabolism.

Severe hypertension can lead to cerebral edema and disruption of the blood-brain barrier—a condition called hypertensive cerebropathy. However, the molecular pathways leading to this condition remain obscure. Kalani et al. (2016) demonstrated that inhibition of MMP9 by GM6001 in Dahl salt-sensitive (hypertensive) rats reduced MMP9 expression and activity and thereby the blood pressure. MMP9 expression and activity were also found to be reduced in the cerebral vessels, thus ameliorating the cerebral edema and disruption of BBB due to it. Hence, pharmacological inhibition of MMP9 may be used to treat hypertension and its associated cerebral pathology.

Hypertension is a known cause for increased cardiovascular risk in humans. Veeranki et al. (2016) demonstrated that homocysteinemia acted together with altered T cell immunity to cause hypertension and increased cardiovascular risk. They also suggested that it is not the homocysteine levels per se alone that impact progression of hypertension and cardiovascular health but a combination of homocysteine and the defective remethylation and/or transsulphuration pathways.

4.2 Homocysteine in Pregnancy-Induced Hypertension, Pre-Eclampsia and Eclampsia

In 2003, Noto et al. (2003) assessed the relationship between homocysteine and blood pressure in pregnant females from the twentieth week and the twenty-fourth weeks. On the basis of the subsequent course of the pregnancy and the final outcome, they segregated the subjects into those who were normotensive and those who had pregnancy-induced hypertension (PIH), low risk (LR), medium risk (MR) and high risk (HR). Homocysteine levels in the normotensive subjects were lower than in the other groups, and it increased with the severity of the hypertension. This difference achieved significance in the MR ($p = 0.05$) and the HR ($p = 0.01$) groups. Also higher homocysteine levels detected early in the second trimester was associated with higher risk of complications. In those presenting with pre-eclampsia, the homocysteine was significantly higher than in the normotensive group but similar to that in the HR group. However, the homocysteine levels were unable to predict whether or not the subject would develop eclampsia.

In 2016, Yelikar et al. (2016) elucidated that maternal plasma homocysteine was significantly higher in pre-eclampsia as well as eclampsia ($p = 0.001$ for both). It was also found to be significantly higher in eclampsia as compared to pre-eclampsia and was associated with the severity of the condition.

Laskowska and Oleszczuk (2011) evaluated the effect of homocysteine on intrauterine growth retardation (IUGR). They segregated their pregnant subjects into four groups—(a) normotensive with IUGR, (b) pre-eclampsia with IUGR (PRE-IUGR), (c) pre-eclampsia with appropriate for gestational age (PRE) and (d) healthy normotensive uncomplicated pregnancies (controls). They elucidated that IUGR as well as pre-eclampsia was significantly associated with higher plasma homocysteine levels. Interestingly, the highest level of homocysteine was observed in pre-eclampsia without IUGR.

Thus, the pathological interplay of homocysteine in pregnancy (complicated and uncomplicated) is probably due to endothelial cell dysfunction.

Lacunae in Knowledge

Further prospective population studies and clinical trials are required to confirm current knowledge. Special attention is needed on homocysteine modulating therapy in treatment of hypertension, especially in the pregnant females.

Clinical Message

1. Current regimen for treatment of hypertension do not allow for the possibility of homocysteinemia being a contributory factor to elevated blood pressure. Hence, in view of the above explanation, it may be beneficial to add a combination of vitamin B_{12}, folate, and pyridoxine as well as gut microbiota stabilizers to the antihypertensive regime.
2. Maternal plasma homocysteine should be measured in all pregnant women presenting with pre-eclampsia or PIH. Homocysteinemia, if present, should be treated immediately to avoid complications of eclampsia as well as adverse foetal outcomes.
3. Hydrogen sulphide and MMP9 inhibitors need to be assessed for therapeutic use in management of hypertension as well as pregnancy-induced hypertension.

Homocysteinemia and Its Neurological Effects

5

The neurological effects of homocysteinemia start in utero. Though the exact mechanism of neural tube defects in folate deficiency is not known, it is believed that the increase in homocysteine and consequent decrease in methyl donors (e.g. *S*-adenosyl methionine) probably contribute to these defects.

Decreased SAM results in hypomethylation which causes epigenetic reprogramming and overexpression of several genes. This conforms to the fact that many genes have been implicated in NTDs, with each gene defect being associated with a specific subtype of NTD (Bhargava et al. 2014). Also, adverse fetal outcomes are known to be associated with placental pathology. Prothrombotic states, including homocysteinemia, may result in placental vascular insufficiency and a compromised fetus (Haj Mouhamed et al. 2011).

Even though it is known that folate deficiency increases homocysteine, the reverse may also be true; homocysteinemia may interfere with the absorption and action of folate by interfering with the regulation of expression of the genes for folate receptor α and reduced folate carrier I (two of the four specific folate receptor/transport proteins) (Farkas et al. 2013; Steinfield et al. 2009). Thus, homocysteinemia may be at the helm of many known disorders due to folate deficiency.

Folate deficiency is known to cause neural tube defects, and it is proposed to act through the ensuant homocysteinemia. It has been shown by several scientists that periconceptual folate supplements prevent neural tube defects.

At the same time, Steen et al. (1998) have elucidated that cobalamin has an equal role in neural tube defects, being significantly low in the amniotic fluid of fetuses with NTD. Zhang et al. (2009) found that low levels of folate and B_{12} in maternal serum increased the risk of neural tube defects. It has further been demonstrated that folate supplement does not correct a deficient state; rather it corrects the metabolism of homocysteine towards the formation of methionine. Thus, it is the methionine synthase-based reaction (which is promoted by folate as well as B_{12}) which has been shown to be abnormal in women who have pregnancies with neural tube defects

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https://doi.org/10.1007/978-981-10-7632-9_5

(Mills et al. 1995). Thus, supplementing with periconceptual folate as well as B_{12} is equally important for the neurological outcome of the pregnancy.

5.1 Neurotoxicity

It is known that homocysteinemia exerts its neurotoxic effects through several mechanisms. These effects and their mechanisms could be summarized as in Fig. 5.1. These have been amply demonstrated in mice models of homocysteinemia.

Rhodehouse et al. used the $CBS^{+/-}$ mice as models of homocysteinemia and C57BL/6 as controls. They demonstrated an increased permeability of the blood–brain barrier (BBB) in the mice with homocysteinemia. Using the Morris water maze, they also demonstrated cognitive impairment in these mice (Rhodehouse et al. 2013). This was found to be mediated through NMDA (*N*-methyl D-aspartate) receptor-dependent regulation of adherens (VEC/β-catenin) and tight junctions (claudin-5) (Beard et al. 2011).

One of the mechanisms, as shown in Fig. 4.1, is through activation of MMP 9 by competitive binding of homocysteine to the postsynaptic GABA-A receptors, thus antagonizing this receptor and leading to activation of MMP-9, while at the same

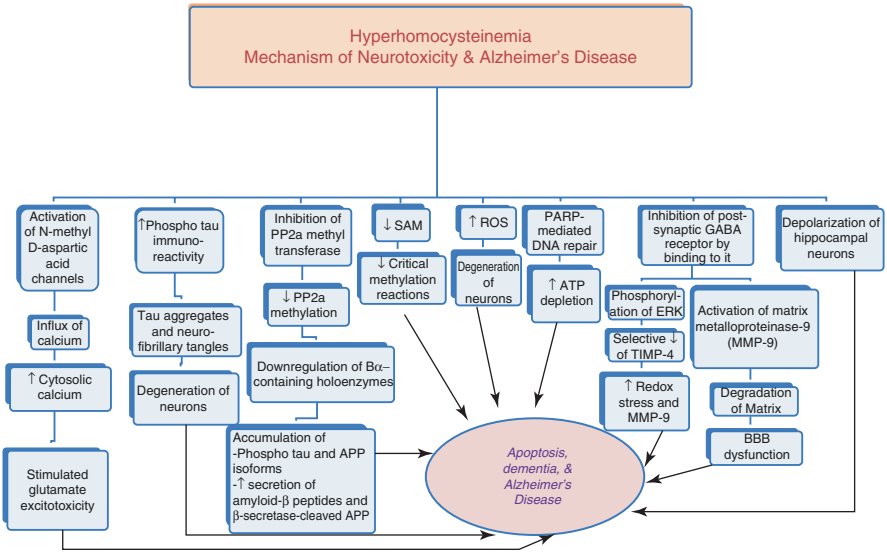

Fig. 5.1 Neurotoxicity of homocysteinemia. The neurotoxic effects of homocysteinemia are numerous impacting several molecules and mechanisms, as shown in this figure. The sequelae are, therefore, varied, ranging from neural tube defects to epilepsy to apoptosis and dementia. Through several mechanisms, homocysteinemia affects the neural tissue (including its vasculature) to result in varied pathologies through neurodegeneration and apoptosis. Homocysteinemia can result in cognitive decline and finally Alzheimer's disease through three major mechanisms—increased MMP-9, increased tau aggregates and degradation of hippocampal neurons (Modified from: Bhargava S, Bhandari A, Choudhury S. Role of homocysteine in cognitive impairment and Alzheimer's disease. IJCB 2017; DOI https://doi.org/10.1007/s12291-017-0646-5)

time, there is decreased activity of MMP-2 and TIMP-4. This has been very eloquently demonstrated by Lominadze et al. in their experiment on MMP 9 knockout mice (Lominadze et al. 2012).

In view of the experiments confirming the role of MMP-9 in homocysteinemia-induced cognitive dysfunction, we compared the auditory cognitive abilities (new object recognition test (NORT)) of $CBS^{+/-}MMP\text{-}9^{-/-}$ ($CBS^{+/-}$ mice in whom the MMP-9 gene was ablated) with those of $CBS^{+/-}$, $MMP\text{-}9^{-/-}$ and wild-type (WT) mice. It was observed that indices of cognitive abilities significantly improved ($p = 0.006$ for discrimination index and $p = 0.003$ for recognition index as compared to $CBS^{+/-}$) by ablation of the MMP-9 gene (Bhargava et al. 2014).

5.2 Cognitive Impairment, Dementia and Alzheimer's Disease

The processes of memory, learning, reasoning, attention, problem solving, decision making and language are grouped together in the term 'cognition'.

"With the current increase in geriatric population due to increased longevity, cognitive decline is becoming more prevalent and there is a need to investigate the possible causes, especially modifiable ones, so as to be able to institute preventive measures (Bhargava et al. 2017)."

A report by McCaddon et al. as early as 1998 demonstrated that early onset of Alzheimer's disease was associated with high homocysteine levels as opposed to normal homocysteine in age-matched controls without Alzheimer's disease (mean Hcy of patients = 21.9 μmol/L; mean Hcy in controls 12.2 μmol/L; $p < 0.0001$).

Wang et al. (2001) did a 3-year follow-up on individuals ≥75 years of age who were not on any vitamin supplements, specifically vitamin B_{12} and folate. Serum levels of the vitamins were measured in these randomly selected 370 subjects. Three years later, it was elucidated that those with lower B_{12} (<150 pmol/L) and folate (<10 nmol/L) were at twice as much risk (RR = 2.1; 95% CI) of developing Alzheimer's disease than those with normal levels of these vitamins. In those subjects who had a good baseline cognition, this risk was even higher (RR = 3.1; 95% CI). Even when the cutoffs of B_{12} and folate were increased to 250 pmol/L and 12 nmol/L, respectively, the pattern did not change.

Hippocampal width is known to decrease with age. That there is a relation between this decrease in hippocampal width and homocysteine was demonstrated by Williams et al. (2002) when he showed that in patients of Alzheimer's disease, there was an association of homocysteine with the atrophy of the medial temporal lobe of the hippocampus.

One of the well-known features of Alzheimer's disease is the presence of amyloid plaques. Impaired DNA repair sensitizes the hippocampal neurons to amyloid toxicity. In an in vitro study, Kruman et al. (2002) showed that in the presence of methionine and folate deficiency, the consequent homocysteinemia resulted in increased DNA damage, decreased DNA repair, increased β amyloid and increased apoptosis of the hippocampal neurons.

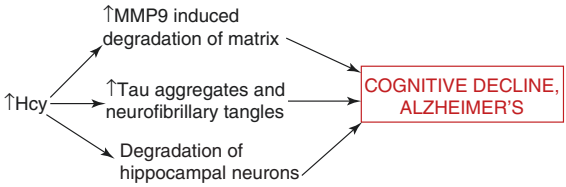

Fig. 5.2 Mechanism of homocysteinemia leading to cognitive decline and Alzheimer's disease. Homocysteinemia (**a**) induces MMP-9 expression and thereby increased degradation of the brain matrix, (**b**) enhances phosphorylation of tau protein and thereby their aggregation and formation of neurofibrillary tangles and (**c**) causes degradation of the hippocampal neurons resulting in altered functioning of the hippocampus. All these result in cognitive decline of varying degrees

It has been described that about 22% of the geriatric population has cognitive impairment. Of these 10–15% of those who have homocysteinemia progress to dementia, whereas only 1–2.5% of those without homocysteinemia proceed to dementia (Plassman et al. 2008; Edland et al. 2002; Petersen et al. 2001). As is evident in Figs. 5.1 and 5.2, occurrence of Alzheimer's disease due to homocysteinemia may be described through several of the mechanisms of neurotoxicity.

Several scientists have described homocysteinemia as an independent risk factor for cognitive impairment. Quadri et al. (2004) demonstrated that subjects with relative folate deficiency, i.e. those in the lowest folate tertile, showed a significant reduction in cognitive abilities and had an odds ratio of over three for development of mild cognitive impairment and dementia, whereas those with homocysteinemia had an odds ratio of more than four for the same.

Another study associated high homocysteine, coupled with low B vitamins, with decreased cognitive function (Tucker et al. 2005). Malouf et al. showed that asymptomatic elderly subjects with homocysteinemia when given 800 mcg of folate per day exhibited a marked improvement in global functioning ($p = 0.033$), memory storage ($p = 0.006$) and information processing speed ($p = 0.016$).

Similarly, when patients of cognitive impairment with homocysteinemia, already being treated with cholinesterase inhibitors, were given 1 mg of folate per day, they demonstrated a better overall response ($p = 0.02$) along with an improved Nurse's Observational Scale for geriatric patients ($p = 0.002$) (Malouf and Grimley 2009). In a 4-year follow-up study of 816 dementia-free subjects, Ravaglia et al. elucidated that homocysteinemia conferred a hazard ratio of 2.08 for dementia ($p = 0.002$) and 2.11 for Alzheimer's disease ($p = 0.011$) (Ravaglia et al. 2005).

Figure 5.3 delineates, in short, the mechanism involved in homocysteinemia-induced cognitive decline. Homocysteinemia, initially, inhibits the postsynaptic GABA receptor and induces increased synthesis of MMP 9. This results in the degradation of the brain matrix. Homocysteinemia also promotes the phosphorylation of the microtubule-associated tau proteins.

As these processes continue, the phosphorylated tau proteins aggregate and ultimately form neurofibrillary tangles.

At the same time, homocysteinemia increases the expression of the amyloid precursor protein, a membrane protein of the neurons, leading to an accumulation of this

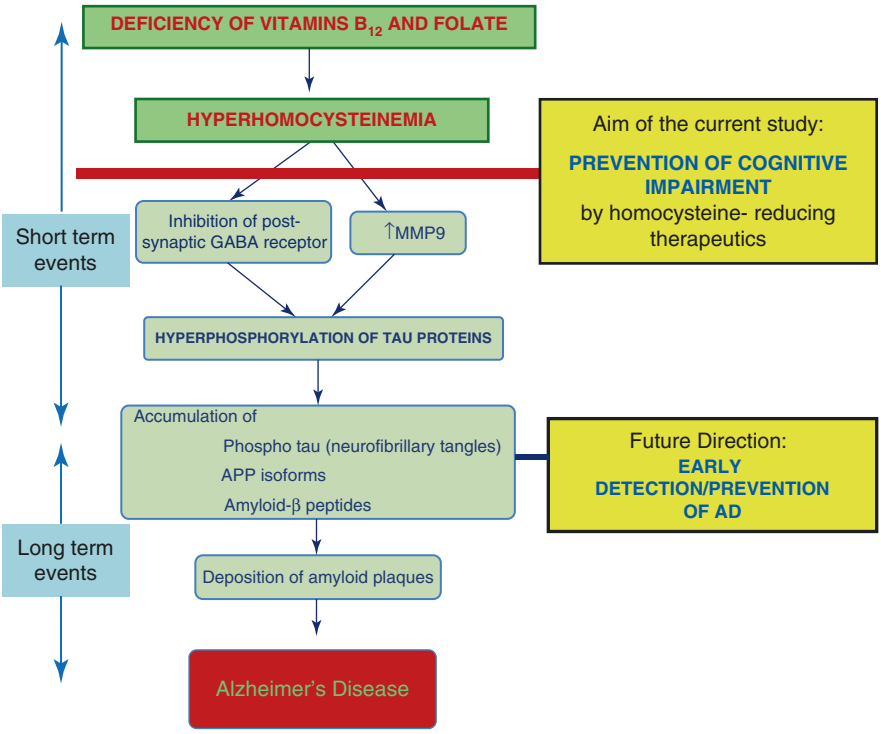

Fig. 5.3 Long-term and short-term events in the progress of vitamin (B_{12}, B_6, folate) deficiency and homocysteinemia-induced cognitive decline and its prevention. The inhibition of postsynaptic GABA receptors and the increased MMP9 production due to homocysteinemia with resultant hyperphosphorylation of tau proteins could be described as the early or preclinical events in cognitive decline; accumulation of the phosphorylated tau proteins and amyloid-β peptides into neurofibrillary tangles and amyloid plaques could be described as the successive long-term events which ultimately manifest as cognitive decline or Alzheimer's disease

protein with resultant formation of amyloid plaques which have been implicated as a cause of Alzheimer's disease. Both these processes can take several years.

But is there evidence that treating homocysteinemia with B_{12} and folate prevents further cognitive decline? McCaddon (2006) presented a case series showing a cessation of decline as well as improvement in cognitive abilities after supplementation with these vitamins. His results are summarized in Table 5.1.

Aisen et al. (2008) conducted a 2-year case-control study on 409 subjects with mild to moderate Alzheimer's disease (MMSE = 14–26) and divided them into two groups—one receiving high doses of B vitamins (5 mg folate, 25 mg B6 and 1 mg B_{12}) and the other receiving only a placebo. Only 168 subjects completed the study successfully. After 2 years of this treatment, the subjects were measured homocysteine and the cognitive subscale ADAS-cog (Alzheimer's disease cognitive scale). The results showed that the vitamin supplements significantly reduced brain atrophy probably by reducing homocysteine but did not significantly affect cognition.

Table 5.1 Effect of vitamin supplements on homocysteine and cognitive scores

Case no.	Homocysteine in µmol/L	MMSE or alternate score	Vitamin supplement with duration	Repeat MMSE (or alt score)	Repeat homocysteine in µmol/L
1	20.1	12/28	B_{12} and folate 1 month	28/30	7.5
2	27.5	Moderate to severe dementia	B_{12} and folate 3 months	Mild confusion	6.6
3	15.6	12/28 (6CIT)	B_{12} and folate 3 months	28/30	9.6
4	14.6	8/28 (6CIT) 16/39 (TICs-m)	B_{12} 6 months	21/39	8.3

6CIT six-item cognitive impairment test, *TICs-m* telephonic interview for cognitive status modified (Modified from: Bhargava et al. IJCB 2017; doi:https://doi.org/10.1007/s12291-017-0646-5)

Similarly, a meta-analysis of all double-blind, placebo-controlled randomized trials from the Cochrane Dementia and Cognitive Improvement Specialized Register Group revealed that, in healthy elderly subjects with some form of cognitive impairment, vitamin supplements with folate (with or without B_{12}) significantly reduced homocysteine but did not significantly impact cognitive parameters (Malouf and Grimley 2009).

Hence, it may be suggested that more studies are required to ascertain the effects of homocysteine on cognition and prevention of neurofibrillary tangles and amyloid plaques. Yet, homocysteinemia should be treated early to prevent its long-term effects. It may, thus, be possible to prevent, or at least delay, dementia, Alzheimer's disease or any other form of cognitive impairment. Experimental evidence of the effects of early management of homocysteinemia in terms of delay in cognitive impairment needs to be established.

5.3 Autism and Neural Tube Defects

By virtue of its definition, autism could be a subset of cognitive impairment as it includes deficits in social communication and relationships, verbal communication, language impairment and repetitive/restrictive behaviour. Tu et al. (2012), in their preliminary prospective cohort study on the role of amino acids in autism, elucidated a significantly higher plasma level of homocysteine in autistic children as compared to age- and sex-matched controls. This could be a risk factor or an association due to the poor eating habits and food selectivity of these children, resulting in multivitamin deficiencies including deficiency of B_6, B_{12} and folate which are known to increase homocysteine. Ali et al. (2011) had also reported similar findings. Kaluzna-Czaplinska et al. (2011) demonstrated significantly increased levels of homocysteine in the urine of autistic children.

Autism has also been linked to intrauterine and postnatal folate deficiency. Schmidt et al. (2012) demonstrated that in the first month of pregnancy, mothers with normal children ($n = 278$) had a significantly higher intake of folate than those

with autistic babies (n = 429). With a mean folate intake of ≥600 μg, the risk of autistic spectrum disorders was markedly reduced (adjusted OR = 0.62; p = 0.02), and with increasing folate there was a further significant reduction in risk (p = 0.001). They also demonstrated that the association between high risk of autistic spectrum disorders and low folate was highest among those mothers who had a variant methylene tetrahydrofolate reductase gene (MTHFR C677T). Frye et al. (2013) demonstrated the presence of folate receptor autoantibodies in the serum of 75% of the 93 autistic children enrolled in their multicentric study at Arkansas, New York and Melbourne. They postulated that autism spectrum disorders could be a result of cerebral folate deficiency due to the folate receptor autoantibodies. Many of these autistic children improved on leucovorin[1] therapy.

Similarly, low vitamin B_{12} levels have also been demonstrated in autistic children. Zhang et al. (2016) found serum vitamin B_{12} levels threefold lower in autistic children as compared to age- and sex-matched controls. Since deficiencies of both folate and B_{12} have been implicated in autism, it has been suggested that the mode of action of these deficiencies in causing autism is through the increased levels of homocysteine. Enough studies do not yet exist to substantiate this statement, and those that exist have included very few subjects. Also, these studies are equivocal as to the relationship between homocysteinemia and autism.

Neural tube defects (NTDs) are a failure of closure (or reopening) of the neural tube (the precursor of the central nervous system) during development of the fetus. Since the neural tube closes around the 27th day of conception and the pregnant women generally becomes aware of her pregnancy only after the third week of conception, preventing NTDs by folate intake at that time would be ineffectual. Hence, it has been suggested that periconceptual folate supplement is of prime importance (Gupta and Gupta 2004). It is yet unclear how folate deficiency causes an NTD, but it has been suggested that folate deficiency interferes with MTHFR and MS-dependent reactions, leading to homocysteinemia and decreased methyl donors. Also, folate is required for purine and pyrimidine synthesis and thereby DNA synthesis; with a deficiency of folate, therefore, neither DNA synthesis nor its methylation proceeds normally, leading to epigenetic modification of the DNA and consequent developmental defects (Feng et al. 2013).

As mentioned above, functionally diverse genes including regulators of actin dynamics, cell adhesion, electron transport and DNA repair have been implicated in the causation of NTDs, such that each gene is associated with a specific NTD. The disruption of these genes is believed to be caused by alterations in their methylation due to homocysteinemia consequent to deficiency of folate and B_{12} (Wallingford et al. 2013).

In addition to NTDs and the thrombotic effects on the placental vessels, homocysteinemia has been shown to have direct deleterious effects on the developing embryo, e.g. reduced embryo survival rate, decreased cell proliferation and decreased protein expression of the Pax 1/9 and Sox 9 genes in mesenchymal nuclei (Kobus et al. 2013).

[1] Leucovorin is a formyl derivative of tetrahydrofolate and is easily converted to the reduced folate derivatives. It, therefore, functions as the vitamin in absence or deficiency of the latter and allows for some purine/pyrimidine syntheses and, thus, DNA synthesis. Hence, it circumvents the effects of folate deficiency.

It has also been demonstrated that the development of the vascular beds is impaired in embryos exposed to homocysteinemia, unlike the controls as well as the folate-deficient embryos. There was a significant reduction in vascular area of the embryos treated with homocysteine. These vascular beds were comprised of mainly small diameter vessels; in contrast, the controls and folate-deficient embryos had mostly medium-diameter vessels. There was also a reduced expression of VEGF-A (vascular endothelial growth factor-A) and VEGFR-2 (vascular endothelial growth factor receptor-2). It is likely that this altered vasculature, along with the altered expression of the growth factor and its receptor, lead to impaired cell proliferation and a consecutive cascade of events resulting in developmental defects of the cardiovascular system (Oosterbaan et al. 2012).

5.4 Epilepsy

γ-amino butyric acid (GABA) is an inhibitory neurotransmitter in endothelial cell layer. Homocysteinemia is known to be a risk factor for neuroinflammatory and neurodegenerative diseases.

It has been demonstrated by Tyagi et al. (2007) that homocysteine competitively binds to the GABA receptor-A on the postsynaptic neuron. Homocysteinemia, therefore, causes inhibition of this inhibitory neurotransmitter. Also, it acts via the extracellular signal-related kinase (ERK) signalling pathway and thereby leads to increased redox stress and matrix metalloproteinase-9 activity and reduced activity of tissue inhibitor of metalloproteinases 4 (TIMP 4). The result is disruption of blood–brain barrier, increased microvascular permeability and increased excitatory neuronal impulses. One of the manifestations is epilepsy (Fig. 5.4).

In 2009, Tyagi et al. (2009) treated the cultured brain endothelial cells with muscimol, a GABA receptor A agonist and showed that it restores TIMP 4 activity and mitigates homocysteine-induced activation of MMP-9 as well as redox stress, confirming that in the brain, homocysteine acts through competitive inhibition of the GABA receptor A.

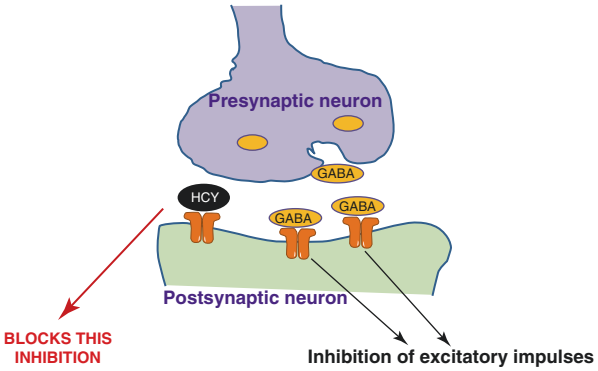

Fig. 5.4 Homocysteinemia and epilepsy. In the presence of homocysteinemia, the GABA released by the presynaptic neuron has to compete with homocysteine for attachment to the GABA receptors on the postsynaptic neurons. Hence their inhibitory effect is mitigated with resultant increased excitatory impulses and epilepsy

5.5 Parkinson's Disease

The role of homocysteinemia in other neurological disorders is controversial. In Parkinson's disease (PD), it has been established that homocysteinemia occurs in patients who are being treated with L-DOPA, the best known treatment for Parkinson's disease. This is because L-DOPA gets methylated via catechol-O-methyltransferase (COMT) which utilizes a methyl group from the remethylation cycle of homocysteine metabolism, enhancing the reaction towards homocysteine formation, thus leading to homocysteinemia (Miller et al. 1997). This may perpetrate the progression of Parkinson's due to the advent of neuropsychiatric events, cerebrovascular disease and other comorbidities as Hcy is a risk factor for neurological and vascular diseases. Also, homocysteine is an NMDA receptor agonist, which leads to neurotoxicity and dyskinesias called L-DOPA-induced dyskinesia (LID). Hence, it has been suggested that Parkinson's patients on L-DOPA should be given concomitant tolcapone, a COMT inhibitor, to prevent homocysteinemia (Müller and Kuhn 2006; Muller 2008). Kocer et al. (2016) performed an experiment to assess the effectivity of these COMT inhibitors in these patients. They observed that though homocysteine was raised in the group of patients being treated with L-DOPA and normal in those being treated with only dopamine agonists, the difference was not statistically significant. They also observed that the COMT inhibitors did not seem to prevent the development of homocysteinemia.

Zoccolella et al. (2010) published an overview of 30 studies which were involved in establishing the role of homocysteine in Parkinson's disease. They observed that the relationship between MTHFR genotype and serum concentrations of the vitamins B_{12} and folate (the major determinants of circulating homocysteine levels) was inconclusive—MTHFR genotype-related results were contradictory, and the vitamins' deficiency was associated with only an insignificantly increased incidence of Parkinson's disease. The in vitro studies showed that homocysteine resulted in a dose-dependent depletion of dopaminergic mesencephalic neurons; the in vivo brain administration of homocysteine was found to result in motor and behavioural changes similar to those seen in Parkinson's. Thus, the possibility that homocysteinemia may contribute to the causation of Parkinson's disease is still uncertain.

5.6 Multiple Sclerosis

Multiple sclerosis (MS) is considered to be an autoimmune inflammatory condition characterized by demyelination in the central nervous system with axonal degeneration and neuronal loss.

Ramsaransing et al. demonstrated that:

- Vitamins B_{12} and folate were not significantly different in controls and patients of MS.
- After correcting for the vitamin levels, there was a significant difference in homocysteine concentrations in controls and patients of MS (4.5 μmol/L higher in patients).
- Homocysteine was not significantly different in patients of MS with or without progressive disease.

Thus, homocysteinemia does not seem to have a role in pathogenesis or progression of disease in these patients. Instead, it seems to be a result of the disease process being produced in excess of homocysteine rather than having a decreased removal (as implied by the similar vitamin levels in the controls and the patients).

Astrocytes have been demonstrated to be activated during the course of the disease, and these are known to produce and secrete homocysteine. Hence, it may be possible that homocysteinemia seen in MS may be secondary to activation of astrocytes and disruption of the blood–brain barrier (Ramsaransing et al. 2006).

There are a few reports that indicate that this raised homocysteine in MS patients may lead to the increased occurrence of vascular and other neurological comorbidities.

5.7 Peripheral Neuropathy

Since homocysteine affects the nervous system through a variety of mechanisms, several scientists performed experiments to elucidate its role in peripheral neuropathy.

Bruce and Young (2008), in their community-based study on 483 adults, established through multivariate logistic regression that, after correction for age, sex, low socio-economic status, low education, HbA_{1c} and smoking, homocysteine was an independent risk factor for peripheral neuropathy. Also, it was found that it exacerbates existing peripheral neuropathy per se as well as diabetic peripheral neuropathy.

Similarly, Jianbo et al. (2011) demonstrated that plasma concentration of total homocysteine was associated with diabetic neuropathy independent of the traditional risk factors.

Luo et al. (2013) demonstrated the presence of elevated plasma homocysteine in the absence of any other identifiable aetiology in a group of patients with peripheral neuropathy. They termed this condition IHIN (isolated homocysteine-induced neuropathy). Electrophysiological studies suggested large fibre neuropathy with demyelination and axonal denervation.

Shandal and Luo (2016) demonstrated that sensory deficits were the predominant components of IHIN.

Further studies are required to better understand this entity and improve its management.

5.8 Down's Syndrome

The first clinically identified human syndrome that was shown to be of chromosomal origin was Down's syndrome. Being trisomy 21, it bears a relation to homocysteine metabolism as the gene coding the transsulfuration pathway enzyme, CBS, resides on this chromosome. The phenotype of Down's syndrome

is, hence, opposite to that of homocysteinemia, which is attributed to the extra copy of the CBS gene present on the third chromosome 21. The metabolic consequence, as demonstrated by Pogribna et al. (2001), is that more homocysteine is directed to the transsulfuration pathway and less goes through the remethylation cycle. This results in a functional folate deficiency state. There is also hypoactivity of methionine synthase secondary to the removal of its substrate (homocysteine) by CBS.

As a consequence, there is less regeneration of methionine from homocysteine and less SAM, which are important for appropriate protein synthesis required especially for the processes of growth, immunity and hormone synthesis. Thus, there is a consistent milieu of hypomethylation due to the decreased synthesis of SAM and SAH. It has been suggested that this may contribute to the pathology of Down's syndrome.

In 1997, Yu et al. (1997) analysed chromosome 21 in patients with Down's syndrome. They found a high proportion of densely methylated interspersed repetitive sequences. These have been postulated to be a compensatory mechanism for down-regulation of the overexpressed genes on this chromosome.

In 2014, Nandha Kumar et al. (2014) studied the association of homocysteine and folate levels in controls as well as 108 children with Down's syndrome with or without congenital heart defects and neural tube defects. They observed that the homocysteine was significantly lower in the children with Down's syndrome as compared to the controls. At the same time, those with congenital heart defects had homocysteine levels that were significantly higher than in those without these defects. Homocysteine was lower in those with neural tube defects than those without these defects, but this was not significant. Folate levels were lower in the presence of both types of defects—congenital heart defects and neural tube defects—but this too was not significant. Hence, they concluded that the congenital heart defects seen in Down's syndrome could be a result of pathology due to altered homocysteine metabolism.

5.9 Psychiatric Disorders

So far, we have been talking about the effects of homocysteinemia. However, Levine et al. (2008) demonstrated the effect of post-traumatic stress disorder (PTSD) on circulating levels of homocysteine. They included 28 male patients of PTSD and compared them to 223 controls. The increased level of homocysteine was significant ($p < 0.001$); on applying logistic regression analysis to the data, the duration of PTSD was able to predict the homocysteine.

Psychiatric disorders differ from neurological disorders in that the former are mostly a sequel of the social environment, whereas the latter is the result of some somatic pathology. Consequently, the former is unaccompanied by physical signs like stroke, haemorrhage, etc. whereas the latter has definite physical signs and symptoms.

5.9.1 Neuropsychiatric Disorders

Neuropsychiatric disorders are a group of psychiatric conditions that are attributable to altered pathophysiology in the nervous system. Even though these disorders are likely to have multiple genetic and environmental causes, addressing each causative factor would result in improved management.

5.9.2 Schizophrenia

As described above, homocysteinemia has a multipronged effect on neurobiology, and it blocks the normal neuroinhibitory impulses mediated by GABA. The neuropathology of some neuropsychiatric disorders has been identified, e.g. schizophrenia is associated with oxidative stress and hypoactivity of the *N*-methyl-D-aspartate (NMDA) receptors in the brain. Glutamate interacts with these receptors and mediates postsynaptic excitation of neural cells. Homocysteine is known to interact with glutamatergic transmission in the brain. It stimulates the NMDA receptors leading to an increased influx of calcium into the neurons which culminates in neurotoxicity and apoptosis (Ho et al. 2002). As shown in Fig. 5.1, homocysteine also causes oxidative stress and aberrant DNA methylation. This would explain the implication of homocysteinemia in *schizophrenia*.

In the presence of low glycine levels, homocysteine acts as an antagonist within the glycine site of the NMDA receptors, thus exhibiting a neuroprotective effect at normal concentrations, but still being toxic at higher concentrations (Lipton et al. 1997). Alternately, in the presence of high glycine levels (as occurs in head trauma and stroke), even low homocysteine becomes toxic because it acts in synergism with the glycine (Alam et al. 1998). Thus, homocysteine has a dual effect on these receptors.

5.9.3 Depression

The possible connection between depression and homocysteine-methyl donor pathways was first described by Reynolds and his colleagues in the early 1970s and 1980s (Reynolds et al. 1970, 1984; Reynolds and Stramentinoli 1983). Several subsequent population studies reported high homocysteine in depressive disorders. In a meta-analysis, Bressa (1994) demonstrated that *S*-adenosyl methionine (SAM) functioned as an antidepressant, showing that mood can be altered by alterations in the homocysteine pathway.

Here, one would like to mention the two studies that did not find a correlation between homocysteine and depression. One study included 478 non-depressed, 100 mildly depressed and 122 severely depressed women. All three categories of subjects exhibited vitamin B_{12} deficiency (14.9%, 17% and 27%, respectively) but showed no association between homocysteine and depression (Penninx et al. 2000). In the other study, those with a lifetime diagnosis of major depression exhibited significantly lower serum and RBC folate levels as compared to those without

depression, but there was no association between homocysteine and depression (Morris et al. 2003).

Later, studies established that depressive episodes may predict the development of cardiovascular disease. It was proposed that since homocysteinemia is one of the risk factors of cardiovascular disease, it may be the co-occurring metabolic disruption between this condition and depression (de Jonge et al. 2014). The Rotterdam study of older men and women observed that high homocysteine and deficiencies of B_{12} and, to a lesser extent, of folate were associated with depression (Tiemeier et al. 2002). Similarly, the Hordaland study of Norway elucidated that in older men and women, high homocysteine coupled with the T/T allele of the MTHFR gene was associated with depression (Bjelland et al. 2003). Yapislar et al. (2012) observed raised levels of homocysteine and platelet aggregation along with low levels of nitric oxide in patients of panic disorder and major depressive disorders, indicating the role of homocysteine and its mechanism of action in these disorders. Ford et al. (2013) demonstrated that the memory and cognitive disabilities observed in depression were due to homocysteinemia.

5.9.4 Bipolar Disorder

Two common polymorphisms in the *MTHFR* gene (C677T and A1298C), which have been associated with schizophrenia, may also increase the risk of bipolar disorder. A meta-analysis was conducted associating MTHFR polymorphisms with the major psychiatric disorders (schizophrenia, bipolar disorder and unipolar depressive disorder). 29,502 subjects with MTHFR C677T and 7934 subjects with MTHFR A1298C were included. It was elucidated that all these psychiatric disorders shared a vulnerability due to the MTHFR 677TT genotype with an odds ratio of 1.26 for the TT versus the CC genotypes (Peerbooms et al. 2011). Another study on 120 patients of bipolar disorder examined the MTHFR and the CBS gene polymorphisms. They found an association of bipolar disorder with the CBS T833C genotype but not with the MTHFR C677T genotype (Permoda-Osip et al. 2014).

A point worthy of note is that patients of bipolar disease may have higher circulating levels of homocysteine if treated with sodium valproate or lamotrigine. The former has been found to inhibit methionine adenosyltransferase and the latter to weakly inhibit dihydrofolate reductase—both resulting in reduced functional folate levels despite normal folate levels in circulation (Baek et al. 2013).

Lacunae in Knowledge

Though more data is required in all aspects of neurological effects of homocysteinemia, two areas need more attention from researchers: (a) whether food fortification or early institution of vitamin supplements in the elderly age group would reduce the incidence/progress of AD and dementia and (b) whether there is a therapeutic role of vitamins in neuropsychiatric disorders.

Clinical Message

1. Since homocysteinemia and folate deficiency are implicated in many neural tube defects and autism, these should be measured in all prospective mothers before as well as during pregnancy so that timely action may be taken to avoid these defects. Ideally, periconceptual folate supplements are recommended. At the same time, vitamin B_{12} status must also be ascertained and the vitamin supplemented if required.
2. It would be pertinent to advise measurement of circulating homocysteine in all cases of stroke, cerebrovascular accident, cognitive impairment, dementia, Alzheimer's disease, epilepsy and peripheral neuropathy especially diabetic neuropathy. In fact, circulating homocysteine should be measured in all elderly patients along with vitamin B_{12} and folate.
3. If these levels are elevated, then treating with an appropriate vitamin supplement (preferably containing all three modulating vitamins—folate, pyridoxine and vitamin B_{12}) would enable the clinician to reduce the morbidity by eliminating one confounding factor.
4. Diagnosing AD or MCI is just the tip of the iceberg; preventing further cognitive decline or, better still, improving cognition is the current therapeutic target. Keeping a control on the circulating levels of homocysteine could be one step towards achieving that target.
5. Equally pertinent would be the measurement of plasma homocysteine in Parkinson's disease with the aim of reducing homocysteine if it is found elevated to avoid comorbidities. Even better would be the concomitant administration of tolcapone to patients receiving L-DOPA as treatment for PD.
6. Also, since anticonvulsants are a heterogenous class of drugs with a common ability to cause folate deficiency by a variety of mechanisms (e.g. reduced intestinal absorption, increased metabolism of folates in liver, altered activity of some enzymes involved in one-carbon transfer, etc.), adding folate to the anticonvulsant regimen would be advisable (Lambie et al. 1985).

7. In Down's syndrome, there is no homocysteinemia—the contrary, in fact. However, there is a functional deficiency of folate. Hence, it may be prudent to modify the nutrition in these subjects by supplementing two substances: (a) methionine-rich diet (to promote the production of SAM and SAH) and (b) folinic acid (to bypass the folate cycle).
8. It is evident that homocysteinemia plays a role even in the aetiology of neuropsychiatric disorders. Moreover, drugs used for some of these conditions cause homocysteinemia by various mechanisms. Hence, circulating homocysteine levels should be monitored in these patients, too, and homocysteine-lowering therapy given to them to decrease the probability of comorbidities.
9. In addition, it would be:
   (a) Beneficial to start vitamin B supplements in the elderly so as to prevent homocysteinemia-induced changes, thereby minimizing cognitive decline
   (b) Advisable to ensure periconceptual intake of folate as well as B_{12} to prevent NTDs and autism

Homocysteinemia in Nephrology Practice

6

6.1 Homocysteine in Renal Disease

In chronic renal disease, especially end-stage renal disease, there are a myriad of alterations in the metabolism of proteins and amino acids. Homocysteine metabolism is also altered resulting in an associated increase in its circulating levels.

In 1987, Smolin et al. (1987) conducted a study measuring the alteration in plasma-free and protein-bound sulphur-containing amino acid levels in patients undergoing maintenance hemodialysis. They observed that protein-bound homocysteine levels are elevated in both pre- and post-dialysis plasma and did not change significantly with dialysis, so that some of the morbidity of chronic renal failure [CRF] may be attributed to raised homocysteine levels, due to decreased renal excretion of homocysteine.

Like Smolin et al. (1987), Wilcken et al. (1988) too observed that homocysteine levels are increased in chronic renal insufficiency. They explored the interrelation between folic acid and methionine metabolism in these patients. They selected renal transplant subjects with varying degrees of renal insufficiency and elevated plasma homocysteine. Through the sequential administration of pyridoxine, folic acid and vitamin B_{12} to these subjects, they demonstrated that only folic acid lowered homocysteine levels significantly, probably by folate enhancement of remethylation of homocysteine to methionine. They concluded that since elevated homocysteine is associated with premature vascular disease, folic acid may reduce cardiovascular risk in chronic renal insufficiency.

Plasma homocysteine is directly correlated to estimated glomerular filtration rate (eGFR). When the eGFR reaches 60 ml/minute, mean plasma homocysteine nears 12 μmol/L. By the time end-stage renal disease (ESRD) has set in, prevalence of homocysteinemia is 85–100%. Even during the hyperfiltrating stage, plasma homocysteine and eGFR are closely associated (Wollesen et al. 1999). The prevalence of homocysteinemia in renal transplant patients is 50–60%. Cohen and Galbraith (2001) established that renal function is a major determinant of fasting plasma

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homocysteine, and the inverse relationship between the glomerular filtration rate and plasma homocysteine is present throughout the whole range of renal function. On analysis of the plasma concentrations of the various cofactors and substrates of homocysteine metabolism, and the effects of different therapies, they also observed that an abnormal folate metabolism may be the cause of homocysteinemia in uraemia. Even though the exact mechanism of correlation of GFR and plasma homocysteine is not established, yet it has been clinically ascertained that homocysteinemia does not cause renal insufficiency; however, the reverse may be true, i.e. renal insufficiency causes homocysteinemia (Samuelsson et al. 1999; Sarnak et al. 2002; House et al. 1998). Since homocysteine is a vasculotoxin, as described in the earlier sections of this compilation, it is a significant determinant of the risk of cardiovascular disease in chronic renal failure patients. It would also be responsible for any other systemic toxicity in these patients.

It is important, therefore, to understand the normal physiology of kidney function and homocysteine excretion (Fig. 1.1). The kidney plays a major role in homocysteine metabolism, though the mechanism of this role is controversial. Normally, the glomerulus filters over 500 μmol of homocysteine a day. Of this, at least 85% is reabsorbed, and only 15% is excreted resulting in a urinary concentration of about 6 μmol/L. House et al. demonstrated that in conditions of normal renal function, the excess homocysteine is metabolized in the renal tissue through the transsulfuration pathway by the action of the CBS enzyme, whereas the remethylation cycle has a very limited role. They also demonstrated that under conditions of acute homocysteinemia produced by the infusion of L-homocysteine, the renal uptake increases fourfold and was equivalent to 50% of the infused dose. At the same time, renal excretion was negligible.

Similarly, nitrous oxide-induced homocysteinemia resulted in a threefold increase in renal reabsorption of homocysteine with little excretion (House et al. 1998). It has not yet been elucidated as to which part of the kidney tubules

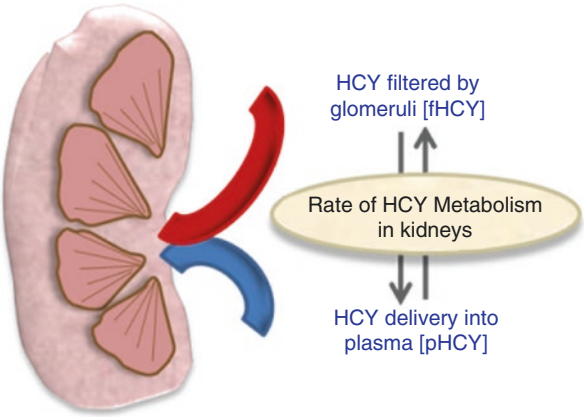

Fig. 6.1 Renal handling of homocysteine

homocysteine is reabsorbed from. Since the plasma homocysteine is maintained despite the increased filtration and reabsorption of homocysteine in the kidneys, Wollesen et al. hypothesized that the kidney may modulate the pathways of homocysteine metabolism in response to the amount of homocysteine reabsorbed.

Thus, under conditions of increased GFR when more homocysteine is reabsorbed by the kidney tubules, the transsulfuration and the remethylation (to some extent) pathways are upregulated; conversely, when GFR decreases, intrarenal homocysteine metabolism is downregulated, resulting in a maintenance of the amount of homocysteine exported to the plasma (Wollesen et al. 1999). As renal function decreases, homocysteine increases, and most patients on dialysis experience mild to moderate homocysteinemia. This is the major contributory risk factor for the 30 times higher incidence of cardiovascular disease-related death in ESRD patients as compared to the normal population (Foley et al. 1998). Guttormsen et al. (1997) demonstrated that homocysteinemia seen in renal disease is the result of decreased renal clearance as a result of the decrease in functional renal mass, and not due to an increased delivery of homocysteine to the plasma. Friedman et al. (2001) have suggested an alternate hypothesis which indicates a role of as yet unidentified uremic inhibitory substances that block normal extrarenal homocysteine metabolism.

Yet there are reports of direct renal damage due to homocysteinemia. The homocysteinemia-associated modulation of MMP/TIMP axis leads to excessive oxidation of collagen IV (through MMPs 2 and 9, the gelatinases) which is essential for the integrity of the glomerular basement membrane. The oxidized collagen gets deposited in the membrane leading to glomerulosclerosis (Sen et al. 2008).

Lacunae in Knowledge

More prospective studies are required to elicit the exact interrelation between renal dysfunction and homocysteinemia and how much benefit may be derived from vitamin supplements, especially towards reduction of the incidence of comorbidities. The role of H_2S (described later) also needs to be established so that it may be used to its maximum therapeutic benefit.

Clinical Message

Since homocysteinemia is known to contribute to the severely increased cardiovascular morbidity in ESRD patients and also to enhance glomerulosclerosis, it would be imperative to administer homocysteine-lowering vitamins to them.

Homocysteinemia and Oncology

7

7.1 Homocysteine and Neoplasias

As folate is shunted towards actively multiplying cells in neoplasias, less of it is available for the remethylation cycle with resultant accumulation of homocysteine and alteration of the methylation-dependent specific protein synthesis. Thus, homocysteine and tumour markers would increase simultaneously with the advancement of the neoplastic process. This is associated with other biochemical changes as well, e.g. oxidative stress, folate deficiency, aberration of DNA methylation and production of homocysteine thiolactone. Hence it has been suggested that homocysteine may be used to monitor carcinogenesis in patients on treatment to assess the effectivity thereof.

Because of the importance of homocysteine thiolactone metabolism in the growth of normal tissues, and because of abnormal metabolism of homocysteine thiolactone in malignant cells, McCully suggested that its derivatives would have antineoplastic properties. In 1989, he injected a single dose of 2.5 mg/kg of *N*-homocysteine thiolactone derivative directly into subcutaneous human pancreatic adenocarcinoma neoplasms in athymic mice and demonstrated a 50% inhibition of growth without any toxic effects. At the same time, several studies have demonstrated a positive correlation of homocysteine levels in the blood with susceptibility to neoplasias. For example, Wu et al. (2014) showed a positive association of polymorphisms of the genes transcribing methionine synthase (A2756G), methionine synthase reductase (A66G) and cystathionine-β-synthase (C1080T, C699T) with breast cancer. Since these polymorphisms are associated with higher plasma Hcy concentrations, this would indicate that Hcy might be a metabolic risk factor for this cancer. Hosseini (2013) also demonstrated a significant association of breast cancer risk with MTR A2756G polymorphism. Contrary reports also exist which demonstrate an absence of significant association between some cancers (non-small cell lung cancer) and polymorphisms associated with altered Hcy metabolism and consequent homocysteinemia (Senses et al. 2013). Zhu et al. (2013) conducted a meta-analysis on the association of several MTHFR and MS alleles with neoplastic

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processes. They concluded that, in the Asian female population, MTHFR 677T allele may enhance the risk of cervical cancer, but, in Caucasian females, it plays a protective role. However, it is suggested that the association between MTHFR A1298C and MS A2756G polymorphisms with cervical tumorigenesis is limited.

In late 1999, Zhang et al. (1999) found that the excess risk of breast cancer associated with alcohol consumption was reduced by adequate folate intake, its mechanism of action probably being through its effect on homocysteine metabolism.

Thus, the molecular ramification between homocysteinemia and carcinogenesis (or protection against cancer) would require further detailed study to understand the role of each of these polymorphisms and the consequent homocysteinemia in neoplasias arising from different cell lines.

However, several antimetabolites, used for treatment of various types of cancers, alter the rate of homocysteine metabolism. Among these, the most noteworthy are methotrexate and 6-azauridine triacetate.

It has been observed that methotrexate enhanced the homocysteine egress from both nontransformed and malignant fibroblasts in culture (Ueland et al. 1986). Also, it decreases intracellular levels of reduced folates, notably 5-MTHF (Allegra et al. 1986; Baram et al. 1987). Hence, among the reactions requiring reduced folates, the 5-MTHF-dependent salvage of homocysteine to methionine would be particularly impaired in the presence of methotrexate. Thus, intracellular homocysteine levels would rise, causing an increased egress of homocysteine into the extracellular fluid as well (Svardal et al. 1969; Svardal et al. 1986).

6-azauridine triacetate interferes with de novo synthesis of uridine-5′-monophosphate and increases the urinary excretion and plasma levels of several amino acids including homocysteine (Slavik et al. 1969) and, therefore, predisposes to thromboembolism which resolves on withdrawal of the drug (Shupak et al. 1977).

Lacunae in Knowledge

There is no clear-cut consensus as to the involvement of homocysteine as a risk factor for neoplasias, even though sometimes it has been implicated and sometimes announced as a protective factor. Hence, there is an immense requirement of further data in this area of homocysteinemia.

Clinical Message

Yet, in view of its altered metabolism in the presence of specific antineoplastic agents, it has been suggested that plasma homocysteine levels may be used as a prognostic marker in the follow-up of cases of neoplasias who are being administered these specific antineoplastic agents. It is also important in these cases to administer homocysteine-lowering vitamins to reduce the occurrence of comorbidities, especially those related to thromboembolic phenomena.

Homocysteinemia and Otology

8

8.1 Homocysteinemia and Otology

The cochlea is a perfect example of the matrix effects of homocysteinemia as most of its effects are exhibited through modulation of the extracellular matrix in this organ. To be able to clearly understand these effects, it would be pertinent to give a short description of this organ.

The cochlea is a convoluted compartmentalized snail shell-like structure with its lumen containing three fluid-filled compartments. In two of these, the fluid functions as a pressure transmitter, perceiving the sound waves as pressure changes and transmitting them to the third compartment which houses the very sensitive organ of Corti. Here the pressure changes are converted to electrical impulses which then travel along the auditory nerve to the brain. The organ of Corti is comprised of very specialized cells—the hair cells—which are present in two layers (the outer and the inner layers) embedded in an equally specialized extracellular matrix (ECM). These hair cells along with the ECM constitute the actual sound impulse detectors and transmitters of our ears. Any alteration in these cells or this matrix would therefore negatively impact our ability to deal with the impulses perceived. Since homocysteinemia alters the ECM through the MMPs, especially MMP-9, it would follow that homocysteinemia impacts hearing as well. Also, as discussed in the section on neurological effects of homocysteinemia, hearing could also be impaired by the effect of homocysteinemia on the neurons associated with the function of hearing (Fig. 8.1).

Kundu et al. demonstrated that as compared to their wild-type counterparts, CBS+/− mice exhibited elevated levels of MMPs 2, 9 and 14 and decreased concentrations of TIMPs 1, 2 and 3 in the brain and cochlea. Also, since it is known that acute as well as age-related hearing loss occurs as a sequel of altered blood flow to the inner ear, they proposed that homocysteinemia probably affects the cochlear microcirculation in the same way that it affects the BBB, as described in the previous sections.

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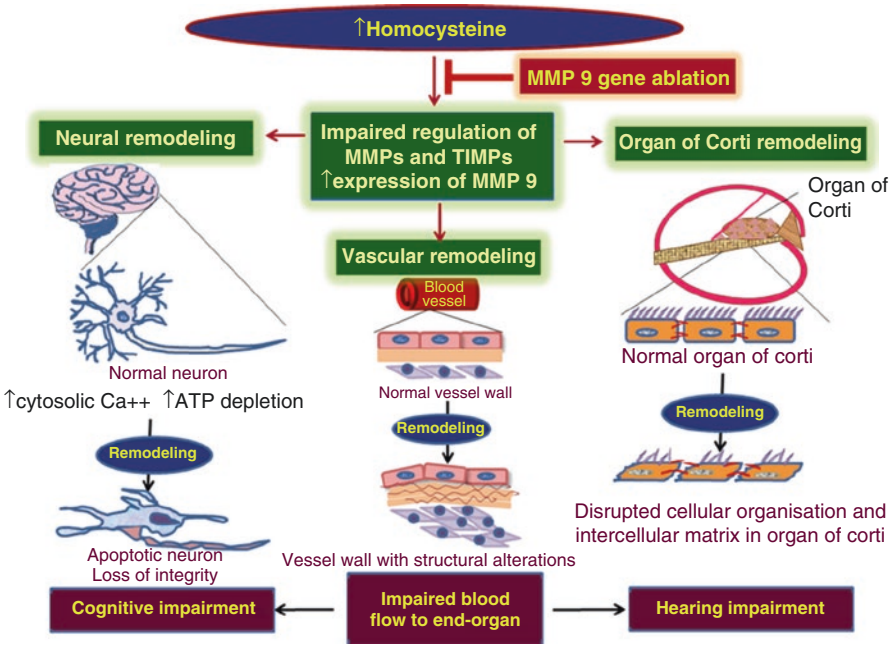

Fig. 8.1 Mechanism of homocysteinemia-induced MMP-9 causing vasculo-cerebral and vasculo-cochlear remodelling

So far as the role of MMPs and TIMPs is concerned, in normal hearing they maintain a very fine balance. This was exemplified by Setz et al. (2011) who demonstrated that gentamicin (ototoxic, specifically for the organ of Corti) increased the expression of MMPs 2 and 9 in the organ of Corti, whereas exposure to MMP inhibitors resulted in hair cell death in the wild-type mice (those without homocysteinemia). Thus, it emerged that the proportionate expression of MMPs and TIMPs is all-important for normal hearing.

The cochlea actually lends itself to a variety of alterations due to trauma and homocysteinemia as there are several types of tissue within this structure (Thome et al. 1984). Hu et al. (2012) demonstrated the differential response of apical versus basal epithelium of cochlea to insult; the apical expression of MMPs was more upregulated as compared to that in the basal region. The apex transduces low-frequency sounds, whereas hearing of the higher frequencies is attributed to the basal region. Hence, hearing loss due to homocysteinemia starts in the lower frequencies as opposed to age-related hearing loss (presbyacusis) which starts at the higher frequencies. Another reason is that due to the apex being the distalmost in terms of blood supply, it is the first to get affected by aberrations of the microcirculation in the stria vascularis, and hence low-frequency hearing loss precedes high-frequency loss due to homocysteinemia.

Martinez-Vega et al. (2014) demonstrated that C57BL/6 mice (normal) fed with a folate-deficient diet for 8 weeks had sevenfold lower circulating folate levels and threefold higher circulating homocysteine levels. These folate-deficient mice exhibited severe hearing loss as measured by the auditory brainstem recordings and apoptotic cochlear cells. In the cochlea, the enzymes of homocysteine metabolism were reduced, and there was a 30% increase in protein homocysteinylation. Also, redox stress was evident in the form of decreased expression of catalase, glutathione peroxidase 4 and glutathione synthetase genes coupled with an increased expression of manganese superoxide dismutase and several other related enzymes and proteins. Thus, the severe hearing loss in these folate-deficient mice was attributable to homocysteinemia.

In view of the experiments confirming the role of MMP-9 in homocysteinemia-induced cochlear and cognitive dysfunction, we compared the auditory brainstem recordings (ABR) and cognitive abilities (new object recognition test—NORT) of $CBS^{+/-}MMP\text{-}9^{-/-}$ ($CBS^{+/-}$ mice in whom the MMP-9 gene was ablated) with those of $CBS^{+/-}$, $MMP\text{-}9^{-/-}$ and wild-type (WT) mice. It was observed that ABR and cognitive abilities significantly improved ($p = 0.0004$ for ABR, $p = 0.006$ for discrimination index of NORT as compared to $CBS^{+/-}$) by ablation of the MMP-9 gene (Bhargava et al. 2014).

In human studies, Durga et al. (2007) conducted a double-blind, randomized, placebo-controlled clinical trial on 728 older adults who had plasma homocysteine concentrations ≥13 μmol/L but who did not suffer any middle-ear dysfunction, unilateral hearing loss or pathological ear conditions not attributable to their age. It was observed that a 3-year daily supplement of 800 μg folate slowed the increase in the hearing threshold (pure tone air conduction thresholds) by 0.7 decibels ($p = 0.020$) in the lower frequencies, but did not affect the deterioration in the higher frequencies.

Gocer et al. (2009) conducted a study in 78 hearing-impaired subjects and 53 age-matched controls (no hearing impairment). The plasma homocysteine levels were significantly higher, whereas the serum concentration of vitamin B_{12} and folate was significantly lower in those with hearing impairment as compared to the controls. There was a statistically significant correlation between the pure tone audiometry and the levels of homocysteine, B_{12} and folate at 250 Hz (low frequency).

Gopinath et al. (2010) demonstrated that individuals above 50 years of age with a homocysteine >20 μmol/L had a 64% greater likelihood of hearing loss of >25 dB. This association with increased prevalence of hearing loss was also observed in individuals with low serum folate but not associated with low serum vitamin B_{12}.

At this juncture, since the inner ear is being discussed, the only study that has evaluated the effect of homocysteinemia on vertigo deserves mention. Aydin et al. (2012) studied the relation between the circulating levels of homocysteine, B_{12} and folate with peripheral vestibular dysfunction. They did not observe any correlation between these biomarkers and the three types of peripheral vestibular dysfunction—Meniere's disease, vestibular neuritis and benign vestibular positional vertigo.

Raponi et al. (2013) demonstrated that homocysteinemia may have a role in delaying recovery after acute vestibular neuritis. In their study, patients given homocysteine-lowering therapy in conjunction with specific therapy recorded a better improvement (as per the Dizziness Handicap Inventory [DHI] questionnaire) after 1 month of therapy than those given only specific therapy ($p < 0.01$).

Lacunae in Knowledge

1. Further studies need to be conducted to elucidate the optimum concentrations of various MMPs and TIMPs in the different parts of the inner ear and their correlation to homocysteinemia. Also, the role of inhibitors of MMPs and TIMPs needs to be elucidated.
2. Further studies are also necessary to establish the interplay of the B vitamins and homocysteinemia with hearing loss in other age groups.
3. There is a dearth of studies on the role of homocysteine in vestibular neuritis; this needs to be addressed.

Clinical Message

Since homocysteinemia can cause alterations in the MMP/TIMP axis in the inner ear, hearing loss of varying degrees may occur. This could be minimized by appropriate administration of the B vitamins, specifically folate.

Homocysteinemia and Bones

9

9.1 Homocysteinemia and Bones

As per Fig. 1.1, metabolism of homocysteine can be through oxidation to homocysteic acid by the action of vitamin C. This homocysteic acid is required for the synthesis of sulphated proteoglycans which are an integral part of the connective tissue in the walls of the blood vessels as well as in the bones.

Liu et al. (1997) showed that homocysteine thiolactone inhibited lysyl oxidase (by decreasing the expression of the LOX gene) and, thereby, interferes with the post-translational modification of collagen. Homocysteine selectively promotes chondroitin sulphate so that the chondroitin/dermatan sulphate ratio is maintained when homocysteine is within the biological reference interval; this homeostasis is altered in the presence of homocysteinemia (Fujiwara et al. 2008). Both these processes result in an altered collagen fibre with decreased strength.

Also, homocysteinemia is known to increase osteoclast activity, decrease osteoblast activity and activate matrix metalloproteinases (MMPs). MMPs are activated in the mitochondria by hypochlorous acid (HOCl) which is generated from hydrogen peroxide (H_2O_2) by the action of myeloperoxidase, an enzyme that is induced by homocysteinemia and oxidative stress (Fu et al. 2001). This is the pathway for activation of MMP-7. MMP-9 activation is affected through mitochondria via another pathway. Homocysteine activates and translocates calpain-1 from the cytosol to the mitochondria, increasing the intramitochondrial stress and activating MMP-9 (Moshal et al. 2006). These MMPs then degrade the extracellular bone matrix. In addition, there is a reduced blood flow to the bones due to vascular remodelling subsequent to homocysteinemia. The result is remodelling of bone matrix and increased susceptibility to fractures (Hermann et al. 2005).

In addition to remodelling of bones, it has also been demonstrated that the MMPs play an important role in healing and repair of fractures; MMP-7 and MMP-12 have been found in non-healing fractures, confirming this finding (Fajardo et al. 2010). Conversely, MMP inhibitors have been shown to aid bone resorption by causing

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cleavage of the triple helices of collagen 1, resulting in its degradation (Murphy and Reynolds 1985).

Bosch-Marcé et al. (2005) suggested that increased vascular resistance due to impaired angiogenesis, as a result of homocysteinemia, could result in decreased blood flow. Tyagi et al. (2011) demonstrated a decreased blood flow in the tibia secondary to homocysteinemia in a homocysteinemic mouse model.

Blouin et al. (2009) obtained a correlation between homocysteine and bone marrow density ($p < 0.05$), indicating that homocysteinemia may not directly alter the density but affects the quality of the bone matrix by altering the collagen cross-links. Morris et al. (2005), however, demonstrated that subjects with homocysteine ≥20 μmol/L had a lower BMD (bone marrow density) than those with homocysteine <20 μmol/L, making them more prone to fractures and osteoporosis.

In addition to increase in MMPs, bone matrix can be affected directly by the homocysteine molecule or thiol binding to the collagen which is mostly type I. Herrmann et al. (2009) demonstrated that 65% of the total homocysteine binds to collagen type I in the extracellular matrix of the bone.

Thus, it is evident that homocysteinemia would impair bone matrix, increasing the probability of fractures and also would impede the healing process.

Lacunae in Knowledge

There are limited human case-control studies elucidating the relationship between altered bone metabolism in the presence of homocysteinemia and the benefits of the B vitamins/metabolic modulators.

Clinical Message

In the presence of homocysteinemia, care must be taken to reduce homocysteine by appropriate vitamin supplements. Care must also be taken to prevent fractures.

When a fracture has occurred, blood levels of homocysteine and its determining vitamins must be estimated and appropriate measures taken in the presence of homocysteinemia or deficiency of folate or B_{12}.

Hydrogen Sulphide: The Body's Inherent Defence Against Homocysteinemia

10

10.1 Hydrogen Sulphide [H_2S]

Having elaborated on the pathophysiology of homocysteinemia with respect to different organ systems of the body, it would be pertinent to mention the role of hydrogen sulphide [H_2S] in the body, in general, and in vasculopathy, neural tissue and renal disease, specifically.

For every reaction that can go awry, the body has inbuilt countering mechanisms or dual regulatory pathways to minimize the subsequent pathological effects. Putting things in a simplistic perspective, for example:

- Normally, the thyroid gland secretes triiodothyronine (T3) and tetra-iodothyronine (T4) under the influence of the thyroid-stimulating hormone (TSH) which is negatively regulated by the circulating T3 and T4 levels. When the thyroid secretes less of T3 and T4 (as may occur in iodine deficiency), the body's inherent defence is to produce more TSH to counter the pathophysiology of low T3 and T4, thus causing a compensatory overaction of the thyroid tissue. Conversely, if the thyroid manufactures more T3 and T4, then the feedback mechanism ensures a decrease in the TSH. [www.thyroid.org]
- Under normal circumstances, plasma glucose levels are tightly regulated by the hormones insulin and glucagon, insulin secretion is being induced by hyperglycaemia and glucagon secretion is being induced by hypoglycaemia. This is especially important for the brain as it relies heavily on glucose for all its energy supply, but can neither synthesize it nor store it. Thus, when there is a decreased level of circulating glucose (as may occur in fasting) and a consequent decrease in the production of the main source of energy (ATP), the alternate pathways for production of glucose from other substrates (gluconeogenesis and glycogenolysis) and concomitant decreased uptake of glucose by skeletal muscles and even adipose tissue are promoted. When hyperglycaemia is the prevailing condition, the opposite happens—gluconeogenesis is inhibited, and the peripheral uptake of glucose is increased.

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There are many other ramifications of the thyroid hormones and glucose, but the above is a graphic description of the major modulators.

As may be noted from the above described regulatory mechanisms, it is the metabolite itself that is regulated rather than its pathological consequences. In the case of homocysteinemia, however, the increased production of H_2S counters the pathological effects of homocysteinemia rather than the production of homocysteine. Thus, in this case, the body's defence functions at a different level.

With these facts in mind, presented here is a brief background of H_2S and its physiological/pathological effects.

H_2S is a downstream by-product of the transsulfuration pathway by which homocysteine is converted to cystathionine through the action of cystathionine-β-synthase [CBS] which then is further metabolized to yield cysteine through the action of the enzyme cystathionine-γ-lyase [CSE]. This then proceeds towards protein synthesis through the action of 3-mercaptopyruvate sulphur transferase [3-MST], and in these pyridoxal phosphate [PLP]-dependent reactions, too, H_2S is a by-product. Thus, these three enzymes—CBS, CSE and 3-MST [all PLP-dependent enzymes]—promote the production of H_2S. These enzymes are tissue specific so that their expression varies in different tissues. For example, CBS is maximally expressed in neural tissue, especially the hippocampus and cerebellum, whereas CSE is expressed in the vascular smooth muscle, liver and kidney. On the other hand, 3-MST has only been reported as a H_2S-producing enzyme in the vascular endothelium. Thus, these tissues have some benefit in terms of combatting the pathology of homocysteinemia, as described below.

Historically, nitric oxide (NO) was the first endothelium-derived relaxing factor identified. It is a gasotransmitter that is synthesized from arginine, a reaction involving folate as a cofactor—called folate shuttle in Fig. 1.1. H_2S, too, is a gasotransmitter which smells like "rotten eggs". Both NO and H_2S are small molecules of gas generated in the mammalian cells and freely permeable to the lipid bilayer. H_2S has been associated with neurotoxicity and, as an environmental hazard, acute toxicity manifesting as loss of central respiratory drive. But later, Abe and Kimura, in their ground-breaking work on the CNS, demonstrated that H_2S was produced by the hippocampus and functioned as a neuromodulator, as well. CBS in the brain was involved in the production of H_2S which enhanced NMDA receptor activity and thereby facilitated long-term hippocampal potentiation (Abe and Kimura 1996; Kimura 2002).

Further, Kimura demonstrated that H_2S was produced in the ileum also where it exhibited smooth muscle relaxing properties. They also demonstrated that smooth muscle relaxation, induced by nitric oxide in the thoracic aorta, was enhanced by even small quantities of H_2S. Hence, it was concluded that H_2S may regulate smooth muscle tone in synergy with nitric oxide (Hosoki et al. 1997). Further studies then identified the various physiological functions of H_2S with respect to oxidative stress, hypertension, neurodegenerative disease, inflammation, vascular dysfunction, etc. Thus, H_2S was shown to function as an endogenous signalling molecule which regulates a variety of physiological processes including cellular oxidative stress.

Another important physiological function of H_2S is due to the fact that it shares many physiological processes rendered by carbon monoxide and nitric oxide, without the requirement of oxygen as substrate. Hence, it is able to sustain mitochondrial ATP generation in the hypoxic state as well.

Yet another physiological function of H_2S is the hyperpolarization of vascular smooth muscle cells so as to close voltage-dependent calcium channels. Thus, here it functions like the endothelium-derived hyperpolarizing factors (EDHFs). This might account for the more potent vasorelaxing effect that H_2S has on small mesenteric arteries as compared to its effect on the larger aorta, probably due to the fact that the contribution of EDHF to vasorelaxation is more in the smaller arteries including mesenteric artery and coronary arteries (Zhao et al. 2003).

These physiological and pathological effects of H2S are summarized in Fig. 10.1.

Before proceeding to the interrelation of homocysteinemia and H_2S, let us take a look at the half-life of both these EDHFs. Of the two, H_2S is the more stable in protein-free solution, whereas NO is scavenged within seconds by oxyhaemoglobin. H_2S can be scavenged by methaemoglobin or disulphide-containing molecules like reduced glutathione (Wang 2002; Zhao et al. 2003).

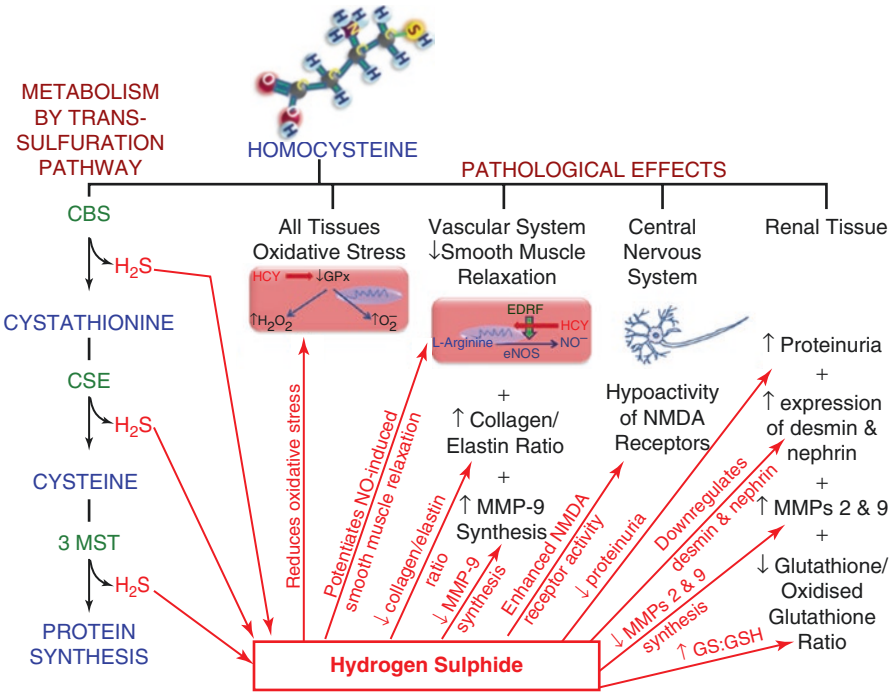

Fig. 10.1 Hydrogen sulphide counteracts several pathological effects of homocysteinemia

One of the possible mechanisms of vasculopathy due to homocysteinemia has been suggested to be the relative depletion of H_2S.

In homocysteinemia-induced hypertension, the pathology due to homocysteine includes increased vascular fibrosis due to an enhanced synthesis of collagen, resulting in an altered elastin/collagen ratio and reduced vascular wall compliance. This not only causes hypertension but also impairs the vascular relaxation induced by nitric oxide. H_2S counters these effects by reducing collagen synthesis, thereby normalizing or improving the elastin/collagen ratio and the vascular wall dynamics. This was demonstrated by Sen et al. (2010a, b). But as homocysteine increases, even these reactions are inhibited through homocysteinylation of CSE, which process alters its enzymatic activity. Chang et al. (2008), demonstrated that the activity of the H_2S-producing enzymes was lowered in hyperhomocysteinemic rats and also that there was dysfunction of the mitochondrial respiratory enzymes related to reactive oxygen species (ROS) generation. When H_2S was injected intraperitoneally, enzyme activity was restored, and oxidative stress was mitigated. Distrutti et al. reported that homocysteinemia-induced decrease in NO production by the sinusoidal endothelial cells was corrected by the perfusion of the liver with sodium sulphide—a donor of H_2S.

Thus, it was demonstrated that endogenous and exogenous H_2S upregulate VEGF and thereby attenuate hypertension as well as renal damage.

Another mechanism of homocysteinemia-induced hypertension is the release of intracellular calcium causing vascular contraction and stiffness. H_2S promotes intracellular calcium depletion and thereby prevents contractility.

Yet another mechanism of hypertensive pathology induced by homocysteinemia is by the upregulation of angiotensin 1 (AT 1) receptor-induced synthesis of MMP-9 and collagen in the vascular endothelial cells. This is also mitigated by H_2S which downregulates the expression of angiotensin-converting enzyme (ACE) in endothelial cells and, hence, reduces AT 1 receptor-induced hypertensive pathology (Sen et al. 2007).

That H_2S is actually the active pathological factor was amply demonstrated by Yang et al. (2008b) in their experiment on cystathionine-γ-lyase knockout mice. These CSE mutant mice developed hypertension at 7 weeks as opposed to 12 weeks in the case of the wild-type (WT) mice. It was noted that the brain H_2S levels were similar in both types of mice—CSE mutant and WT—indicating that CSE is not the source of H_2S in the brain, and this emphasizes that the hypertension in these mutant mice is not effected by the CNS. At the same time, they also demonstrated that the levels of the endothelial NO synthase (eNOS) did not show any decrease in the mutant mice, thus ruling out loss of eNO-mediated vasorelaxation as the cause of hypertension. To assess the therapeutic role of exogenous H_2S, all the mice were given a bolus of intravenous NaHS (sodium hydrogen sulphide, a donor of H_2S), and it was observed that both types of mice showed a decrease in blood pressure, but the magnitude of decline was more pronounced in the CSE mutants than in the WT, probably due to their heightened sensitivity to H_2S.

Just as homocysteinemia can cause reduced CSE expression, the reverse is also true. This was amply corroborated by the fact that the homozygous CSE mutants had a homocysteine nine times higher than the heterozygous mutants. It was further demonstrated that CSE was activated by calcium-calmodulin, which is the mechanism for vascular activation-mediated H_2S formation (Yang et al. 2008b).

In the kidneys, homocysteine has a dual role: firstly it is excreted by the kidney, and, hence, in cases of renal insufficiency, it accumulates with resultant homocysteinemia; secondly this homocysteinemia perpetuates further renal pathology with further deterioration of renal function. This constitutes a vicious cycle of injury and effect.

In the renal physiology, H_2S has several important interactions. By the downregulation of the synthesis of collagen (as a result of homocysteinemia-induced altered synthesis of MMPs and TIMPs) and subsequent correction of the altered collagen/elastin ratio, H_2S decreases renal fibrosis subsequent to ureteral obstruction. It also decreases the expression of inflammatory cytokines and reduces oxidative stress by preserving the metal-associated superoxide dismutases [CuZnSOD, MnSOD] in the renal tissue.

Sen et al., in their study on hyperhomocysteinemic as well as normal mice, demonstrated the following features of renal failure due to homocysteinemia and how H_2S ameliorates them (Sen et al. 2009):

a. Hyperhomocysteinemic ($CBS^{+/-}$) mice had increased proteinuria; exogenous H_2S supplementation normalized the protein excretion.
b. Increased activity of the MMPs 2 and 9 was observed in the $CBS^{+/-}$ mice with resultant apoptotic cells in the renal cortex; H_2S mitigated the MMP activities and prevented apoptotic cell death.
c. H_2S also downregulated the increased expression of desmin and nephrin (glomerular podocyte markers of proteinuria in damaged renal tissue) in the renal cortical tissue of $CBS^{+/-}$ mice.
d. There was increased superoxide production and reduced glutathione-to-oxidized glutathione ratio; this was normalized by H_2S.

In a subsequent experiment, Sen et al. (2012) elucidated that NADPH oxidase and blood pressure were upregulated by a downregulation of CSE expression, reduction of H_2S production and decreased glomerular filtration rate. Both these pathologies were ameliorated by H_2S supplementation.

Perna and Ingrosso (2012) also demonstrated that after dialysis, H_2S levels increase significantly. This probably indicates that dialysis eliminates a uremic toxin which inhibits the H_2S-generating enzymes. Hence, they suggested that chronic kidney disease in liaison with low hydrogen sulphide could be dangerous.

Sen et al. (2012) conducted yet another study to ascertain whether converting homocysteine to H_2S improves renovascular function. Triple-gene therapy of

ex vivo renal artery cells with CBS, CSE and 3-MST resulted in increased production of H2S in these cells and made them more sensitive to endothelium-dependent vasodilation as compared to the untreated cells. This triple-gene therapy also leads to the attenuation of overexpression of MMP-13 as well as of the downregulation of TIMP-1.

Clinical Message and Lacunae

1. H_2S has several clinical implications, but this text has been restricted to its interaction with homocysteinemia. With the above knowledge, it would be advisable to explore the possibility of using H_2S as a therapeutic agent for patients with a raised homocysteine, especially as it ameliorates most of the vascular and renal pathology due to homocysteinemia.
2. However, it is evident that this molecule can behave differently at different concentrations and in different physiologic/pathologic situations—like a double-edged sword. Hence, it would be prudent to conduct strictly controlled clinical trials after establishing an optimum concentration of H_2S to be delivered.

Homocysteine and Epigenetics

11

11.1 Epigenetics

When the DNA sequence of the gene was discovered as the carrier of all our traits, we could not imagine that there could be anything beyond; yet certain observations forced us to look beyond! Finally we realized that it is not the genetic make-up per se that defines an individual—it is how the genes are expressed that is the deciding factor. For almost a century now, scientists have been poking into the nooks and crannies of genes trying to decipher all the interactions and modulations that result in totally different expression of the same DNA sequence. The term "epigenetics" was introduced by Conrad H. Waddington in 1942 to describe "the interactions of genes with their environment that bring the phenotype into being". Waterland modified this description and stated that "epigenetics" is "mitotically and/or meiotically heritable and stable alterations in gene expression potential that are not caused by changes in DNA sequence".

Our DNA is made up of a string of purines and pyrimidines in a specific sequence which determines our traits. We now know that these sequences are made up of exons (which carry the code for a particular trait) and the introns (which are interspersed between the exons). It is stimuli to the *introns* that leads to expression of the *exons*. Even the stimuli are predetermined. When the stimulus changes, the expression of the gene (exon) is altered causing a difference in the final effect. If these altered stimuli are transient, they would lead to some change in the organism causing only a phenotypic modification which would not be inherited. However, when the stimuli are stable, they can be inherited, thus causing the same altered expression of the gene in the progeny as well! And this is *epigenetics.*

As you can well imagine, this changes our concept of genetic inheritance and adds an entirely new dimension to it.

Several epigenetic modifications have been identified which are stable. Of these, the most common are DNA methylation and histone modification. DNA methylation, the first recognized and most well-characterized epigenetic modification, is linked to transcriptional silencing and is important for gene regulation, development

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and tumorigenesis. Histones are the core protein around which the DNA is wrapped. Its modification can be in the form of acetylation as well as deacetylation, and its effects are, therefore, varied. To put it simply, methylation of certain DNA bases (typically the 5′ position of the cytosine ring) represses gene activity, and acetylation of histones enhances gene expression, whereas deacetylation suppresses it. But nothing in the human body, or any other living organism, is simple and straightforward as there are concomitant processes that affect each situation and each other differently—the end result is an expression of the sum effect.

DNA methylation is catalysed by DNA methyl transferase (DNMT). It occurs in normal cells where DNMT functions as a maintenance MT. This predominantly recognizes and methylates hemi-methylated cytosine-guanine repeat sequences. This pattern is transferred from parent strand to daughter strand and is thus inherited. Evidence indicates that absence of DNMTs can be lethal to the cell. Normally, genes have certain highly methylated repeat elements, including satellites (e.g. SAT2) and retrotransposons (e.g. LINEs). When there is a relative loss of methylation at these sites, it leads in genomic instability and oncogene activation. And global hypomethylation of DNA is the epigenetic hallmark of cancers, the number one killer in the world.

The purpose of giving this description at this juncture in this compilation is to emphasize that the one major methyl group donor which is involved in almost all one-carbon moiety reactions is SAM—*S*-adenosyl methionine—as shown in Fig. 1.1. Homocysteine is the precursor of methionine which is itself the precursor of SAM. When the circulating homocysteine increases, there is an upregulation of its metabolism resulting in increased concentrations of SAM and therefore, increased availability of methyl groups for epigenetic modification of the expression of genes.

12.1 Food Fortification

In the preceding chapters, it has been brought forth that the effects of homocysteinemia (due to either deficiency of folate and vitamin B_{12} or to polymorphisms of the genes of the rate-limiting enzymes of homocysteine metabolism) are widespread, involving almost all organ systems in our body. It has also been suggested that the use of folate and B_{12} supplements for all these disease states minimizes the effects of homocysteinemia, even when the cause is genetic, the efficacy of which has also been amply demonstrated in several studies. In view of this, it would seem pertinent to fortify foods with folate as well as B_{12} to protect all systems from the deleterious effects of homocysteinemia. Towards this, please find below a short history of food fortification in the world as it stands today.

In 1931, Dr. Lucy Wills (1931), who worked at the Maternal Mortality Inquiry, Indian Research Fund Association, reported that yeast extract could cure the "pernicious anaemia" of pregnancy. In the early 1940s, when the United States of America implemented food fortification with thiamine, riboflavin, niacin and iron, folate and B_{12} were not included due to lack of evidence. It was soon elucidated that the active ingredient in Dr. Wills study on yeast was folate. This leads to the World Health Organization's promotion of combined folate and iron supplementation of pregnant women in the developing countries. Six decades later, Sir Nicholas Wald and his colleagues at United Kingdom's Medical Research Council conducted a randomized controlled trial and demonstrated that NTDs were folate deficiency diseases and that folate supplements in early pregnancy could prevent them (MRC Vitamin Study Research Group 1991). At the same time, Czeizel et al. (1991) demonstrated in Hungary that there were no new cases of NTDs after folate supplementation.

After these studies and several others established that periconceptual folate deficiency resulted in neural tube defects, the Food and Drug Administration mandated that all enriched flour will be fortified with 140 μg of folate per 100 g of flour (FDA 1996). Here it would be pertinent to state that the FSIS (Food Safety and Inspection Service), in their statement of food labelling guidelines, said that

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they did not permit the addition of nutrient additives (e.g. vitamins and minerals) as per the FDA's fortification policy. Hence, fortification is done only for grains and cereals. The effectivity of this fortification was demonstrated in several trials. Honein et al. (2001) elucidated that the prevalence of NTDs among 100,000 live births reduced from 37.8 to 30.5 after mandatory folic acid fortification. De Wals et al. (2007) reported that in Canada the occurrence of NTDs reduced from 1.58 to 0.86 per 1000 live births. Pfeiffer et al. (2005) measured the biochemical indicators of B vitamin status in the American population under the National Health and Nutrition Examination Survey. They found a reduction in folate deficiency (from 16% to 0.5%), an increased prevalence of high folate levels in the elderly (from 7% to 38%) and plasma homocysteine of <9 μmol/L in 78% of the population. A few years later, it was observed that not only did the incidence of NTDs decrease but so did the prevalence of cardiovascular disease and stroke. Through several studies, this was attributed to the decrease in homocysteine due to this fortification.

Despite all these positive reports, food fortification was implemented only in the United States, Canada, Chile and Australia; the whole belt of Eurasia, where the benefits of folate supplements were first demonstrated, remained bereft of fortification.

Morris et al. (2007) demonstrated that in the elderly with B_{12} deficiency, folic acid fortification was associated with an increased incidence of B_{12} deficiency anaemia and cognitive impairment. Concern has been expressed that food fortification with folate in the presence of neoplasias can enhance the growth of these neoplasias by increasing the methionine synthase-driven reaction which results in increased availability of one-carbon moieties for methylation, specifically DNA methylation. This is proposed to enhance the methylation of the p53 gene causing a decreased production of this protein and a consequent reduction in tumour inhibition due to it. However, studies have in fact demonstrated the very opposite; Piyathilake et al. (2009) have shown a decrease in the risk of cervical intra-epithelial neoplasia after implementation of food fortification. Brouwer and Verhoef reviewed the effect of folate fortification in the presence of B_{12} deficiency and concluded that concomitant fortification with B_{12} would solve most, if not all, problems of masking of B_{12} deficiency with folate fortification. Dual fortification of food has been implemented in the United States since then.

Recently, Germany started food fortification and was followed by several European countries. However, it has not yet been implemented in India and other countries of Southeast Asia, despite reports on the continued prevalence of folate and vitamin B_{12} deficiency in these populations (Yajnik et al. 2006; Mahajan and Aundhakar 2015). Data from our lab including over 52,000 subjects (unpublished as yet) shows that in our North Indian population, over 30% have severe vitamin B_{12} deficiency, whereas over 60% have subclinical deficiency of the vitamin. This too emphasizes the need for widespread vitamin supplementation, preferably by food fortification.

The current era has hailed the importance of preventive medicine. Food fortification, in itself, is a gigantic yet simple step towards prevention of many diseases and, at the same time, even improves quality of life.

In view of this history and supporting data, developing countries should introduce food fortification with folate and B_{12} to prevent vitamin deficiency states and effects of homocysteinemia and thus alter global prevalence and distribution of many disease conditions.

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