Advances in CLINICAL CHEMISTRY

VOLUME 41

Edited by

Gregory S. Makowski

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Edited by

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VOLUME 41

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PREFACE

Volume 41 of the Advances in Clinical Chemistry series contains chapters of wide interest to clinical laboratory scientists.

In this volume we explore the role of taurine, a unique sulfur containing a conditionally indispensable amino acid, in humans. Another chapter describes the ability of bionanotechnology to use information from molecular biology, chemistry, and physics to link biological and nonbiological molecules into complex bioassemblies for the purpose of creating novel diagnostic test devices. A comprehensive review of cardiac troponins in the diagnosis of heart disease, the most common cause of death in the developed world, is also contained in this volume. In keeping with the importance of diagnostic testing in the aging population, an excellent review on leptin physiology and pathophysiology in the elderly is also presented. A review on biochemistry of wound healing provides particular insight into these fundamental mechanisms on development of appropriate clinical laboratory diagnostic tools. Another review focuses on the relevance of inflammation as a chronic disease process and the importance of appropriate diagnostic measures to assess underlying pathophysiology. Advances in testing for prostate specific antigen (PSA) is also reviewed with emphasis on biochemistry of antibody-based analytical systems. Lastly, a thorough review on the history, epidemiology, and clinical laboratory detection of human prion disease is also presented.

I extend my appreciation to each contributor of volume 41. Without question, it is only through their willingness to share personal insight that the Advances in Clinical Chemistry series remains a continuing resource for the diagnostic sciences. Once again I would like to thank all clinical scientist colleagues who participated in the peer-review process. I would like to thank Ms. Pat Gonzalez at Elsevier for her attentiveness and commitment to the Advances in Clinical Chemistry series.

I hope the first volume of 2006 will be enjoyed by the readership. Please send comments and thoughts. The input of the readership is vital to maintain the Advances in Clinical Chemistry series at the forefront of clinical laboratory science and research.

In keeping with the tradition of the series, I would like to dedicate volume 41 to my daughter Nyle.

GREGORY S. MAKOWSKI

IS TAURINE A BIOMARKER?

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1. Taurine

Taurine, a sulfur-containing amino acid present in high concentrations in mammals, plays an important role in several essential biological processes (Fig. 1). Taurine is not incorporated into protein and is the most abundant free amino acid in the heart, retina, skeletal muscle, brain, and leukocytes [1–3]. In fact, taurine reaches up to 50 mM concentration in leukocytes. It is considered to be an essential amino acid for felines and a conditionally indispensable amino acid for humans and nonhuman primates [4]. The level of cysteine sulfinic acid decarboxylase (CSD), an enzyme required for biosynthesis of taurine, is very low in the cat and low in humans and

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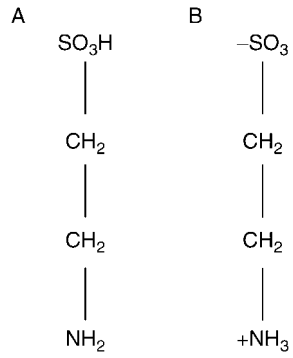

FIG. 1. Structure of taurine.

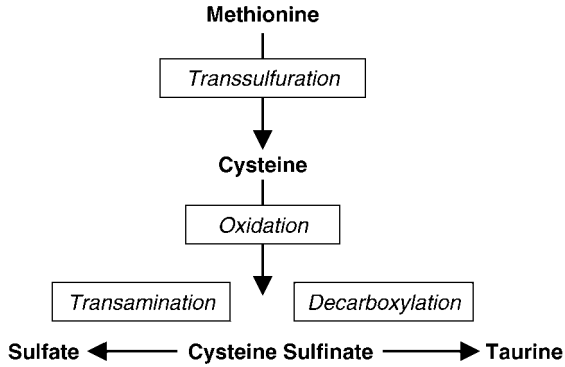

FIG. 2. Metabolism of taurine.

primates [4]. For this reason taurine has been added to infant formula as well as parenteral solutions.

Inorganic sulfate and taurine are major end products of sulfur-containing amino acid metabolism in mammals including humans [5]. The sulfur-containing amino acids, methionine, and cysteine, are taken up by mammals as constituents of proteins in foods. Through *trans*-sulfuration, methionine is converted to cysteine that is further metabolized through oxidation (Fig. 2). In addition to being used for protein synthesis, cysteine is incorporated into glutathione, converted to taurine, and is degraded to pyruvate and inorganic sulfur.

Taurine may be accumulated by cells through two mechanisms: (1) It may be synthesized from cysteine within the cells through the cysteine

dioxygenase (CDO) and CSD. (2) It may be taken up from the extracellular space through a sodium-dependent transport mediated by a specific taurine transporter (TauT).

1.1. Nutrition

Plants and vegetables contain little or no taurine while meats and fish (especially shellfish and crustaceans) contain high levels of taurine. The mean daily intake of taurine in nonvegetarians is about 58 mg. Taurine is a popular additive in health and energy drinks, such as Red Bull™, and monographed as a natural product generally regarded as safe (Gras) by the Food and Drug Administration (FDA). There have been few studies on taurine as a drug and none of them controlled. In 1984, the FDA added taurine to human infant formula. Taurine is now added in most infant formulas throughout the world. Various species have differing capacities for biosynthesis of taurine (Table 1). The nutritional importance of taurine for cats and primates has become widely accepted. As such, commercial cat food typically contains added taurine. Human infant formula and pediatric parenteral solutions both contain taurine. Clinical interest in taurine was heightened by the observations that cats fed a taurine-free diet suffered retinal degeneration [6]. In addition, it was shown that preterm human infants fed synthetic diets were becoming taurine-deficient [7]. Rhesus monkeys raised from birth on human infant formula show clearly demonstrable ultrastructural changes in the outer segments of their cone photoreceptors accompanied by reduced plasma taurine concentrations [2]. These monkeys fed formula alone had significantly reduced visual acuity compared to those fed the taurine-supplemented diet with normal visual acuity. The conclusions of these studies are that taurine is an essential amino acid for cats, rhesus monkeys, and human infants.

During pregnancy taurine accumulates in maternal tissue and is released to the fetus via placenta and to the newborn via maternal milk [8]. Taurine is

TABLE 1
Dietary Dependence on Taurine

Species	Diet	Taurine synthesis	Dietary dependence on taurine
Guinea pig	Herbivore	High	None
Rat	Herbivore	High	Low
Monkey	Omnivore	Poor	Moderate/high
Human	Omnivore	Very poor	High
Cat	Carnivore	Very poor	Absolute

accumulated in the fetal and neonatal brain. Low-maternal levels of taurine result in low-fetal levels of taurine, which can lead to growth retardation of the offspring, impaired perinatal development of the CNS and the pancreas.

The findings of Wharton *et al.* [9] suggest that the recommendations for taurine content of infant formulas should be reconsidered. These data demonstrated that low-plasma neonatal taurine was associated with lower scores on the Bayley mental development index at 18 months and the WISC-R arithmetic subtest at 7 years. They provide an important additional example of apparent long-term effects of short-term early differences in nutrient intake.

Studies on taurine status in patients (adults) receiving long-term parenteral nutrition have shown that marginal taurine intake in this patient population results in taurine deficiency [10]. Specific groups of individuals are at risk for taurine deficiency and may benefit from supplementation, such as patients requiring long-term parenteral nutrition, and those with chronic hepatic, heart, or renal failure [11].

1.2. Reproduction

The role of nutritional taurine in feline pregnancy and outcome has revealed an increased reproductive loss in taurine-deficient cats [2]. Frequently, fetuses born from taurine-deficient mothers are resorbed or aborted and kittens at term are frequently stillborn or have low-birth weight. Kittens from taurine-deficient mothers show a range of neurological problems, including abnormal hindlimb development, thoracic kyphosis, and an abnormal gait. Changes in kittens include persistence of cells in the cerebellar external granule cell layer and mitotic figures indicating that cell division is still taking place, along with extensive abnormalities present in the visual cortex. Reduced intrauterine taurine along with greatly reduced taurine in the milk significantly decreases tissue concentrations of taurine. Pregnancy and outcome were normal when pregnant cats were fed the same diet supplemented with taurine [2]. There have been reports of pediatric problems in children for strict vegetarians who consume little to no taurine [12, 13]. These problems are difficult to attribute solely to taurine deficiency as these problems are associated with malnutrition, but a role for immunologic and other consequences of taurine deficiency cannot be ruled out.

Taurine has been considered an essential nutrient for cats due to low levels of CSD, the rate-limiting enzyme for taurine biosynthesis. A diet containing 0.05% taurine is considered "normal" (Table 2). There are profound effects on tissue taurine concentration on female cats consuming less than 0.05% dietary taurine. Studies by Sturman [2] have shown that the reproduction performance of cats fed 0.05% taurine is equivalent to that of cats fed proprietary diets. However, in cats fed taurine-deficient diets (0 or 0.01%) the

TABLE 2
CONCENTRATION OF TAURINE IN TISSUES AND FLUIDS OF ADULT CATS FED A PURIFIED DIET OR A TAURINE-SUPPLEMENTED DIET

	Dietary taurine	
Tissue	0%	0.05%
Liver	0.60 ± 0.48	8.50 ± 3.33
Kidney	0.92 ± 0.45	5.15 ± 1.91
Lung	2.11 ± 1.63	8.28 ± 2.60
Spleen	1.46 ± 0.70	7.34 ± 2.44
Heart	1.67 ± 1.04	12.0 ± 2.7
Gastrocnemius	0.82 ± 0.38	5.84 ± 1.02
Diaphragm	0.67 ± 0.57	5.49 ± 2.33
Plasma	7.6 ± 6.1	127 ± 53

Values are means $\pm$ SD from 10 to 30 cats. Tissue values are expressed as μmol/g wet wt, and plasma values are expressed in μM. Adapted from Sturman [4].

reproductive performance of females was poor. They frequently had stillborn or low-birth weight kittens with severe neurologic abnormalities. These studies indicate that taurine deficiency has a profound adverse effect on pregnancy in cats. The mechanism for this effect is, however, unknown. Female cats consuming taurine-free and 0.01% taurine diets developed retinal degeneration by 6–8 months. Newborn rhesus monkeys fed formula without added taurine showed changes in the cone photoreceptors [2].

One study demonstrated that preterm human infants receiving taurine supplementation had more mature brain stem auditory evoked responses and a significant reduction in the interval between stimulus and response associated with higher plasma taurine concentrations than unsupplemented infants [14]. These data emphasize the importance of taurine for brain development.

1.3. CARDIOVASCULAR SYSTEM

There have been several studies on taurine as a biomarker in cardiovascular disease. There are a number of promising studies in both animals and humans of the beneficial effects of taurine on the cardiovascular system. As arteriosclerosis is now recognized as an inflammatory disease, this will be covered in Section 1.7. Particularly promising studies have been conducted in the spontaneously hypertensive rat (SHR) model of hypertension [15–17]. In the SHR models, controlled studies have shown a clear therapeutic effect of supplemental taurine. Similar effects including dose-response effects of taurine have been shown in the DOCA salt rats, Dahl rats, and the

renovascular hypertensive rats [15]. Simultaneous addition of 1% taurine has prevented ethanol-induced hypertension in rats [15]. In addition to these rat model studies, there have been several reports of beneficial effects of taurine treatment of hypertension in humans [18–20].

The mechanism of action of taurine is undoubtedly complex and needs further study. Despite this uncertainty, studies by Fugita and Kohashi [18, 19] indicate a clear increase in plasma taurine, following oral taurine treatment. Studies to date indicate that taurine does not act through one specific mechanism but rather through simultaneous effects on several interrelated cardiovascular processes. Taurine may act through active transport and independently as an osmolyte similar to sodium and calcium that are pivotal in many cellular processes. The most promising leads on mechanism of action of taurine in hypertension, left ventricular hypertrophy, and congestive heart failure have pointed to the effect of taurine on calcium, attenuation of angiotension II signaling [16], and effects on superoxide dismutase (SOD) [21].

In addition to these well-studied mechanisms, perturbations in the cytokine network need to be considered in the evaluation of taurine's effect on the cardiovascular system. Of interest was that the most common cause of death in SHR rats was pneumonia (74%) [17], which suggested a weakening of the immune system. Evidence has suggested a role for ACTH and the HPA axis in the SHR rat. A number of studies have shown an effect of taurine on proinflammatory cytokines [22]. Interleukin-1 (IL-1), IL-6, and tumor necrosis factor alpha (TNF-α) are proinflammatory cytokines known to be integrated into circadian rhythms and the HPA axis.

Myocardial failure (dilated cardiomyopathy) in domestic cats has been associated with low-plasma taurine concentrations [23]. With timely intervention, this condition was reversible by nutritional taurine therapy. This finding led to the fortification of commercial cat foods with additional taurine [24].

1.4. CNS

The brain is an organ that contains a high concentration of taurine [1]. Fetal brain taurine concentrations are high, similar to that in newborn babies but decrease as development progresses. Interestingly, there are considerable differences in regional brain taurine concentration. The olfactory bulb has the highest taurine concentration followed by the cerebellum and cerebral cortex [2]. The retina has taurine concentrations in the millimolar range, which are approximately 10–30 times higher than the brain. Taurine is found primarily in the Purkinje neurons of the cerebellum and astrocytes. Using an antibody to taurine, Lu [25] demonstrated a significant loss of taurine in Purkinje cells in taurine-deficient cats.

Taurine fulfills many of the criteria for a neurotansmitter. The inhibitory action of taurine has been reported to be exerted by activation of GABA A receptors and glycine receptors [26–29]. The role of taurine and the GABA B receptors is, however, unclear. Glutamate is the major excitatory neurotransmitter in the brain. In the CNS, calcium plays a key role in mediating glutamate excitotoxicity. Idrissi *et al.* [26, 27] demonstrated that taurine acts downstream of glutamate receptor activation through the regulation of cytoplasmic and intramitochondrial calcium homeostasis thereby preventing neuronal damage associated with excitotoxicity. Thus, taurine plays a significant role in neuroprotection.

Taurine had been shown to induce long-lasting potentiation (LLP) of excitatory synaptic potentials as evidenced by the enhancement of synaptic efficacy and axon excitability in rat hippocampal slices [30].

1.5. Taurine as an Osmolyte

One factor that maintains total body taurine pool size is the renal resorption by the proximal tubule [31]. Taurine is the only amino acid in which the pool size is regulated by the kidney with large amounts excreted into the urine (if there is adequate dietary taurine). If dietary taurine is reduced, the excretion is reduced. Data indicate that intracellular taurine concentration elicits changes in the activity of the taurine transporter.

Osmolytes are accumulated by cells in hypertonic conditions and released when cells are shifted to an environment of lower osmolarity. A few reports indicate that taurine is accumulated in hypertonic fluid and released into isotonic or hypotonic fluid. Taurine, a nonperturbing osmolyte, is accumulated in kidney medulla, brain, and other tissues of hypertonic experimental animals [32]. By accumulating a nonperturbing osmolyte like taurine to balance extracellular hypertonicity, cells are protected from the perturbing effects caused by high-intracellular electrolyte concentration. In the kidney, most filtered taurine is reabsorbed in the proximal tubule by a sodium and chloride-coupled transporter that has been well characterized in brush border membrane vesicles [33]. The activity of the TauT in the brush border of the proximal tubule contributes to whole-body homeostasis of taurine. When animals are fed a diet deficient in taurine or sulfur-containing amino acids, reabsorption of taurine by the kidney is increased and excretion of taurine in the urine is reduced [34]. Brains of rats made severely hypernatremic were shown to contain higher taurine concentration compared to brains of rats maintained under isotonic conditions [35]. Brain interstitial and cerebrospinal fluids (CSF) are normally in osmotic equilibrium with blood plasma and other body fluids [36]. However, in pathological state like hypertonic blood plasma and subsequent severe neurological disorder,

hypertonic blood plasma causes osmosis-driven water movements from brain fluids toward blood plasma through brain capillary endothelial cells and choroid plexus epithelial cells. As brain fluids subsequently become hypertonic from brain cells toward interstitial fluid through the cell membrane, the resulting cell shrinkage leads to alteration of various brain cell functions, as well as perturbation of spatial interrelationships between brain cells that cause the neurological disorders. It is commonly observed that neurological disorders are greatly reduced when plasma osmotic imbalance develops progressively. These clinical observations indicate that brain cells possess osmoprotective adaptive mechanisms [37, 38]. In hypertonic animals, organic osmolytes, such as taurine and myo-inositol, are increased in brain. Subsequently, osmoprotective genes like TauT and sodium/myo-inositol transporter are increased. Osmoprotective genes thus expressed in brain tissue appear unregulated by hypertonicity. In contrast, these osmolytes are decreased in hypotonic animals [39].

1.6. Pancreas

You and Chang [40] have reported that taurine-supplementation protects rats from steptozotocin-induced diabetes in a dose-dependent fashion. Using an immunochemical peroxidase–antiperoxidase technique they showed a small protective effect at 1% taurine supplementation on insulin-immunoreactive cells [41]. The effect was greater at 2% and 3% taurine-supplementation. There was no difference from nondiabetic controls.

Data has shown that taurine is associated with diabetic disease. Animal studies have shown that taurine administration reduced diabetes-associated alterations in the retina, lens, and peripheral nerves. Taurine also inhibited oxidative stress in fructose-fed insulin-resistant rats [42]. These data suggest a potential use of taurine as an adjunct in insulin resistance. Nutritional taurine given early in the life of nonobese diabetic mice altered islet development, reduced insulitis, and delayed the onset of diabetes [43]. Twenty percent of taurine-treated female mice were free of diabetes after 1 year.

Patients with poorly controlled diabetes mellitus (DM) have a high excretion of taurine [44]. In these patients, taurine levels have been shown to be decreased in both plasma and platelets. Platelet aggregation, considered a factor in diabetic complications, was found to be decreased with taurine supplementation. Despite these promising findings, taurine failed to improve kidney complications associated with insulin-dependent DM [44].

Due to its preventive role in reducing alterations in pancreas programming, Franconi *et al*. [44] discussed the possibility of taurine supplementation during pregnancy. The concept of fetal origin of adult disease has been

demonstrated and is evident with respect to taurine deficiency in pregnancy and the perinatal period [8]. In animal models, offspring of diabetic mothers display impaired glucose tolerance and insulin resistance upon reaching adult age. Taurine supplementation to pregnant mothers may prevent this "fetal programming" and thus be beneficial to subsequent generations.

A low-taurine diet during fetal or early postnatal life can lead to abnormal pancreatic β-cell development [45]. Studies by this group demonstrated that taurine increased glucose sensitivity of uncoupling protein 2 (UCP) that is unregulated in obesity-related type 2 diabetes. Taurine may increase mitochondrial Ca^{2+} influx and enhance mitochondrial metabolic function.

1.7. Taurine as an Immunomodulator

A large number of studies have indicated taurine and its chloramines metabolite, taurine chloramine (Tau-Cl), have antiinflammatory properties. Evidence has indicated that atherosclerosis is likely an inflammatory process with particular emphasis on deleterious effects of TH1 immunity and interferon-gamma (IFN-γ) in progressive disease [46]. Gupta *et al.* [46] have shown that IFN-γ knockout mice were protected from atherosclerosis in the ApoE knockout mouse model of atherosclerosis. Insulin resistance, a major predisposing factor in atherogenesis, was intimately correlated with the production of the proinflammatory mediator TNF-α [47]. Interestingly, TNF-α has been shown to be markedly downregulated by Tau-Cl [22]. Oxidation and oxygen radicals have also been implicated in atherogenesis. Both have been shown to be powerfully downregulated by Tau-Cl [48]. Low-density lipoprotein (LDL) or "bad" cholesterol is known to activate the macrophage scavenger receptor with subsequent production of TNF-α and other proinflammatory cytokines. C-reactive protein (CRP), an acute-phase reactant and inflammatory marker, has been shown to be an independent risk factor for coronary artery disease, the most lethal form of atherosclerosis, in statin-treated patients [49, 50]. The level of homocysteine, another sulfur-containing amino acid, has also been shown to be an additional independent risk factor for coronary artery disease [51]. Taurine is intimately involved in sulfur and homocysteine metabolism, and taurine and its chloramine metabolite are potent downregulators of inflammation. As such, controlled clinical trials should be performed to evaluate the effect of taurine (with 3 different regimens and delivery systems) on biomarkers of cardiovascular disease. This research would be particularly worthwhile because of the promising results of taurine in atherogenic animal models, including rats [52, 53], mice, and hamsters [54].

Deficiency of dietary taurine results in significant abnormalities in the immune system of the cat [55]. Taurine-supplementation has been documented

to prevent oxidant-induced injury in several animal models. Data has shown that Tau-Cl, a metabolite of taurine, may downregulate production of proinflammatory cytokines, leading to a significant reduction in immune response.

1.7.1. *Taurine Supplementation*

Taurine has been found in very high concentrations in tissues exposed to elevated oxidant levels. Several *in vivo* models of oxidant-induced damage have been studied using taurine to protect against subsequent inflammation. Hamsters pretreated with supplemented taurine and exposed to nitrogen dioxide did not show typical pathology associated with nitrogen dioxide damage [56]. Similarly, taurine and niacin reduced the inflammation and fibrosis resulting from bleomycin treatment in another animal model [57]. In fact, our laboratory has demonstrated that ozone-induced rodent lung inflammation was decreased by pretreatment with 5% taurine (in drinking water) for 10 days prior to ozone exposure [58]. This study demonstrated that the number of inflammatory cells and hydroxyproline, markers for inflammation and fibrosis, respectively, was significantly reduced in taurine-treated rats compared to untreated controls. Thus, the maintenance of tissue taurine level was critical to the prevention of oxidant-induced injury in several animal models.

1.7.2. *Taurine Deficiency*

For cats and primates, deficiency of dietary taurine has resulted in abnormalities in development of the CNS, retinal and tapetal degeneration, as well as, significant changes in the cardiovascular and reproductive systems. These changes were also accompanied by abnormalities in the immune system. Data from our laboratory has shown that a lack of dietary taurine in cats results in significant leukopenia, a shift in the percentage of polymorphonuclear leukocytes and mononuclear leukocytes, an increase in the leukocyte count, and a change in leukocyte sedimentation characteristics [55]. Functional studies demonstrated a significant decrease in the respiratory burst and decrease in phagocytosis. In addition, serum gamma globulin was significantly increased in taurine-deficient cats. Histologic changes in lymph nodes and the spleen were also observed.

1.7.3. *Taurine-Chloramine*

Measuring Tau-Cl in body fluids has not been described. Chloramines are highly reactive oxidants that react rapidly with thiols, proteins, and lipids. The primary amine group of taurine reacts readily with hypochlorous acid (HOCl) formed during the respiratory burst to form Tau-Cl that is converted to sulphoacetaldehyde (spontaneously or enzyme catalyzed) and

subsequently to isethionic acid [59, 60]. Data has shown that sulphoacetaldehyde formation occurs at sites of inflammation, suggesting the production of Tau-Cl from secreted myeloperoxidase (MPO) products and taurine [61].

Because of the hydrophilic nature of Tau-Cl, it has been suggested that this molecule may be restricted to the extracellular milieu [62]. However, there has been evidence that Tau-Cl can be taken up by red blood cells via anion exchange and RAW 264.7 cells (a murine macrophage cell line) via active transport [22], as well as, lung epithelial cells [62]. Evidence has demonstrated that transchlorination through chloramine exchange between taurine, glycine, and histamine will influence cell reactivity of these oxidants [63]. The authors also presented data for the impermeability of Tau-Cl in HUVEC and Jurkat cells.

Our hypothesis is that supplemental taurine in the drinking water increases the available taurine both systematically and at sites of inflammation. Leukocytes capable of generating HOCl from hydrogen peroxide and chloride via the myeloperoxidase pathway have intracellular taurine concentrations of 20–50 mM. Taurine reacts with HOCl to produce the less reactive and long-lived oxidant Tau-Cl (Fig. 3). Our laboratory and others [22, 64–66] have shown that Tau-Cl, a stable oxidant, can downregulate the production of proinflammatory cytokines, leading to a significant reduction in the immune response. This chapter describes the inhibition of proinflammatory mediators, such as nitric oxide, TNF-α, and prostaglandin E_2, by Tau-Cl in activated rodent cells [58]. It was also demonstrated that Tau-Cl suppressed superoxide anion, IL-6, and IL-8 production in activated human polymorphonuclear leukocytes [48] (Table 3). The production of IL-6, IL-1β, and IL-8 also decreased in lipopolysaccharide-activated

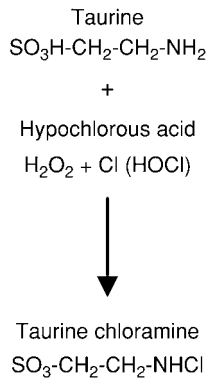

FIG. 3. Formation of Tau-Cl.

TABLE 3
EFFECT OF TAURINE CHLORAMINE ON CYTOKINE PRODUCTION (% INHIBITION) IN HUMANS

	Tau-Cl (0.1 mM)	Tau-Cl (0.4 mM)
Macrophages		
TNF-α	0	50
IL-6	0	72
IL-8	30	38
IL-1β	0	80[a]
Lymphocytes		
IL-6	0	99[a]
IL-8	20	60[a]
IL-2	29	97[a]
Proliferation (PHA)	29[a]	80[a]
PMN		
IL-6	0	100[a]
IL-8	0	60[a]

Note: Control is IL production in absence of Tau-Cl.
[a] Statistically significant ($p < 0.05$).

adherent monocytes by Tau-Cl. These data demonstrated that the ability of Tau-Cl to modulate the immune response is not species specific and extends to human leukocytes. Tau-Cl has also been shown to reduce IL-6 and IL-8 produced by fibroblasts-like synoviocytes isolated from patients with rheumatoid arthritis [67]. In these studies, Tau-Cl diminished the activity of NF-κ B and to a lesser extent that of AP-1 transcription factor. This mechanism was also demonstrated by Barua *et al.* [68]. Overall, the presence of taurine in leukocytes and the ability to form Tau-Cl in the presence of neutrophils coupled with effects on regulating nonadherent, and adherent human leukocytes suggest a central role for taurine and its chloramines metabolite in regulating immune response.

1.8. TAURINE AS A BIOMOLECULE

Taurine takes part in few chemical reactions. To date, taurine has not been found as a component of a protein or nucleic acid, and its precise biochemical regulatory mechanisms remain unclear. Conjugation with bile acids in the liver is the only major and well-documented reaction for taurine. However, studies from Suzuki *et al.* [69] demonstrated the first evidence that taurine is a constituent of biologic macromolecules. This novel finding provides significant insight into the biological function of taurine. In this study, two novel taurine-containing modified uridines in human and bovine mitochondrial

tRNAs were identified. These nucleotides were found to be synthesized by a direct incorporation of taurine that was supplemented in the medium. This study found an absence of taurine-modified mitochondrial uridine in the cells with mitochondrial diseases (MELAS and MERRF). It is hoped that these findings will lead to development of therapeutic strategies as well as provide fundamental clues for understanding the biological function for taurine.

2. Taurine as a Biomarker

The ideal biomarker or biological measure should be reliable, reproducible, noninvasive, simple to perform, and inexpensive. Samples for biological measures should be easily obtained from physiological fluids such as blood or urine. Establishing a biomarker should include confirmation by independent labs conducted by qualified investigators with results published in peer-reviewed journals. Taurine levels in physiologic fluids have been useful for both diagnosing pathology and establishing a disease modifying therapy. In the specific case of taurine, it is important that patient information include nutritional supplementation as well as information on disease status and medications.

Taurine has been measured in biological fluids due to the importance of this simple amino acid and its relative ease of determination. Taurine has been measured in animal models of disease as well as a variety of human conditions. However, it remains unclear as to how taurine should be used as a biomarker and in which situations this measurement would be a good prognostic or diagnostic indicator.

2.1. Blood and Urine Taurine Levels

A simple and reliable method is needed to assess disease activity and monitor therapy in polymyositis (PM) and dermatomyositis. Chung *et al.* [70] used *in vitro* proton magnetic resonance spectroscopy (MRS) to determine if urinary metabolites of taurine could be used as reliable markers for these diseases. In this study, taurine levels (along with creatine) were found to be significantly increased in PM/DM versus normal control patients. Taurine is a major end product of amino acid metabolism and is excreted in the urine [71]. In fact, urinary taurine has been proposed as a potential biochemical marker of total body protein status. Waterfield *et al.* [72] suggested that decreased protein synthesis leads to an increased pool of amino acids available for taurine synthesis, which in turn would lead to elevated urinary taurine. Increased urinary taurine in PM/DM, stroke, and alcoholic myopathy may, therefore, indicate reduced levels of protein accretion in skeletal muscles.

2.2. Milk Taurine Levels

Lactating mammals secrete substantial amounts of taurine in their milk, especially, during the first few days after birth (Table 4). The presence of taurine in infant formula and breast milk is important in neurodevelopment. Improved determination of taurine by high-performance anion-exchange chromatography with integrated pulse amperometric detection has determined the taurine content of breast milk averages 18 mg/liter [73]. In human milk, glutamine and taurine are the prevalent amino acids, accounting for about 50% of the total free amino acids [74]. In infant formula the total free amino acid fraction was about 10% less than that of human breast milk. This difference is mostly represented by taurine. Recommendations for the nutrient contents of term infant formulas do not include a minimum content of taurine. Formulas have contained taurine for almost 20 years and appear well tolerated with a maximum of 12 mg/kcal. The maximum amount observed in human milk is about 25% greater than that contained in formula. In contrast, preterm infant formula contains a minimum content of 5 mg/kcal.

2.3. Tissue Taurine Levels

In vitro NMR spectroscopic examination of tissue extracts can be combined with appropriate pattern-recognition and visualization techniques, in order to monitor characteristic metabolic differences between tissue classes. When such techniques were applied to 88 breast tissue samples, 49 of which were malignant, higher concentrations of taurine were detected in the

TABLE 4
Concentration of Taurine in Milk in Various Species

Species	Taurine in milk, μmol/100 ml
	Less than 5 days after birth
Cat	288 ± 14 (10)
Pig	56 ± 6 (6)
Dog	231 ± 27 (9)
Rhesus monkey	61 ± 6 (9)
Chimp	48 ± 13 (3)
Human	41 ± 7 (13)
Rat	63 ± 8 (6)
Sheep	68 ± 10 (3)
Cow	31 ± 5 (7)

Values are means ± SE of number of samples given in parentheses. Adapted from Sturman [2].

malignant samples [75]. The highest concentration of taurine was found in grade 2 and 3 tissue samples. This study postulated that taurine is a potential indicator of tumor aggressiveness because it is an osmoregulator and marker of increased cell proliferation.

Diagnosis of arrested or progressive form of hydrocephalus has a critical impact on treatment but has remained difficult [76]. Using a rat model of hydrocephalus and MRS, this study demonstrated decreased taurine concentration as well as a change in glutamate, GABA, and other cerebral metabolites. Two weeks after induction of hydrocephalus, taurine levels were significantly reduced in the cerebrum. It has been suggested that increased intracranial pressure and outflow resistance causes cell edema that can be compensated by taurine release. The authors concluded that impaired astrocyte metabolism, measured by *in vivo* MRS, might serve as an early indication for operative treatment.

Intracerebral microdialysis enables the retrieval of endogenous substances from brain fluid and is a sensitive technique for detection of abnormalities in patients with subarachnoid hemorrhage. The studies of De Micheli *et al.* [77, 78] have suggested that sustained high levels of glutamate and taurine, when associated with increased lactate production may predict development of irreversible ischemia. Microdialysis may be a useful tool for early detection of impending spasm-induced ischemia. Additional observations from this group showed that taurine levels in brain tumor tissue and adjacent parenchyma (extracellular) were significantly elevated. Tumor taurine levels were correlated to the degree of cell proliferation thus suggesting an association with edema.

2.4. Aging and Taurine

Despite the high levels of taurine in the CNS, few studies have been performed, which examine effect of aging on taurine content and CNS function. A report, however, examined changes in CNS amino acids in rodent models of aging [79]. This study found significant decline in glutamate and taurine in specific brain regions and demonstrated a link between age-related reductions of striatal taurine and a loss of dopaminergic neurons. These authors also found that in a stroke-prone model of SHRs on taurine-deficient diets the rats were significantly impaired in Morris maze performance when compared to those supplemented with taurine in the drinking water (unpublished observations). Another report has shown that a significant correlation exists between low-CSF taurine concentration and decrements in performance of Alzheimer patients [80]. The CSF levels of taurine have also been reported to decline in Parkinson's disease that affects the striatum [81]. An age-related loss of taurine could contribute to the decline in

dopaminergic function seen in aging. Because aging has been associated with oxidative damage and taurine can serve as an antioxidant, supplemental taurine may serve as an endogenous antioxidant to confer protection against aging. As such, blood or urinary taurine levels could serve as a biomarker for aging.

2.5. Myocardial Ischemia and Taurine

Rupture of cell membranes by oxygen radicals is characteristic of ischemia/reperfusion (I/R) injury. Due to its high concentration in skeletal muscle, release of taurine may be a useful biochemical marker of I/R injury. In fact, Nanobashvili *et al.* [82] found that cell membrane rupture through stimulated lipid peroxidation promoted leakage of intracellular taurine, leading to increased plasma taurine after reperfusion. This finding may be considered as prognostically unfavorable in terms of organ function reversibility. They hypothesized that if membranes are disrupted or the sodium-dependent transporter is compromised, intracellular taurine may leak into the bloodstream and lead to elevated plasma taurine. In their rabbit model, plasma taurine was found to be a sensitive marker of skeletal muscle I/R injury. They concluded that plasma taurine might provide useful diagnostic and prognostic information. It is noteworthy that a clinical role for taurine has now emerged in human trials of taurine administered prior to coronary artery bypass graft and heart valve surgery [83].

3. Conclusions

Future studies are clearly needed to address individual and genetic variations in absorption, transport, metabolism, and excretion of taurine, as well as, the effects of dietary taurine supplementation. Studies under normal physiologic steady state conditions need to be performed and comprehensively compared to pathologic conditions in which taurine has been implicated. These disease states include neurodegenerative, diabetic, cardiovascular, and neoplastic conditions, as well as aging. Studies to date with the large health food industry in humans, including infant supplementation, indicate taurine to be safe for human consumption. Future studies require controlled trials to evaluate taurine's possible therapeutic potential in disease states. In this regard, careful attention to and standardization of taurine regimens under a variety of physiologic and pathologic conditions will be required.

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APPLICATION OF NANOSCALE BIOASSEMBLIES TO CLINICAL LABORATORY DIAGNOSTICS

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1. Introduction

It is becoming increasingly clear that the new science of bionanotechnology will create a variety of devices that will improve and augment existing diagnostic techniques.

This new field uses information from molecular biology, chemistry, and physics to link biological and nonbiological molecules into complex

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bioassemblies not normally found in nature. Many of the advances that are being made are at the proof-of-concept stage and have not yet found clinical applications. Even so, progress in the several approaches being explored suggests that those applications will soon appear. In this review, we summarize progress in this area.

1.1. Nanoscale Bioassemblies

In this review, we use the term bionanotechnology to describe the science that supports the construction of nanoscale bioassemblies, however, it is also being described in the literature as the sciences of nanobiotechnology [1], nanobiological assemblage [2], supramolecular devices [3], and supramolecular assembly [4]. In order to understand the scope of this new field it is useful to define nanoscale bioassemblies as they are envisioned here. First, to be considered nanoscale the bioassembly must be under 100 nm in its largest dimension. Second, naturally occurring bioassemblies like multisubunit protein molecules (e.g., RNA polymerase II) or multisubunit ribonucleoprotein assemblies (e.g., the ribosome) are excluded from consideration. These naturally occurring entities provide models for how the rules of assembly, as they have been worked out in biochemistry and molecular biology, are applied in the field. Simply purifying such an assembly for use in a bioassay, for example, does not fall under the rubric bionanotechnology. The creation of chimeric proteins that self-assemble into multisubunit aggregates after expression in bacteria or eukaryotic cells is also excluded. The nanoscale bioassemblies under consideration here are always artificial, require some steps of assembly outside a living system, and in many cases require purification of several components prior to assembly in addition to purification of the products after the assembly process. Moreover, while it is sometimes difficult to give an exact structural description of the product assemblies, we confine our consideration to those that are uniform. Thus, assemblies that can be viewed as polydisperse (e.g., surfactant-based vesicle like particles [5, 6]) are not considered in detail.

1.2. Scaffolds

In general, the assemblies produced by bionanotechnology are built on a molecular scaffold that provides a platform for the arrangement of functional groups. Scaffolds used at the nanoscale can be classified into four general groups, those that are made up of: (1) protein molecules, (2) nucleic acids, (3) organic chains and polymers, and (4) aggregated or crystalline inorganics.

2. Protein Scaffolds

Two types of protein scaffolds have been used; both take advantage of the capacity of proteins to self-assemble based on biospecific protein–protein interaction. These assemblies can take the form of interlocked connectors or closed shell display surfaces. Both forms of interaction have been extensively studied in biology and basic rules of assembly have been developed.

2.1. Closed Protein Shells

A variety of surfactants can be used to form interesting nanoscale vesicle-like particles (e.g., polymersomes [6]), however, proteins offer, perhaps, the best characterized systems for the self-assembly of uniform closed shells. The rules for assembly of closed shells of interacting protein domains have been studied largely in connection with understanding the nature of virus capsids. In general the rules for assembly are those first worked out by Caspar and Klug [7]. They proposed that viruses produced capsids with icosahedral symmetry (Fig. 1A). The required twofold, threefold, and fivefold rotational axes are universally found in viral capsids but platonic icosahedral geometry is not necessarily present. Subunits displaying complementary protein–protein interaction interfaces arranged pentagonaly are found at each of the 12 vertices of the shell through which the fivefold rotational axes are located. The surface of the shell between the vertices is then tiled with subunits displaying these same complementary protein–protein interaction surfaces hexagonally. Hexagons and pentagons are in general composed of a set of capsid proteins arranged so as to form subunits with threefold symmetry that can serve as roughly triangular components of both the pentagons and the hexagons. When hexagons are formed they are roughly planar. When pentagons are formed, the missing triangular element is formed by moving the vertex at the center of the pentagon out of the plane. Since the protein facets forming the structure are effectively triangular, the structures are generally deltahedra. For deltahedra with icosahedral symmetry, the number of triangular facets can be enumerated.

If a unit value is assigned to the length of each side of the triangular elements, then the system can be described by the square of the distance between any two adjacent pentagons. This parameter T is called its triangulation number. It is given by $T = f^2(h^2 + hk + k^2) = f^2P$, where h and k are any two nonnegative integers including zero having no common factor, and f is any integer. Each icosahedron will have $20T$ triangular facets, 12 pentamers, and $10(T - 1)$ hexamers. P is called the class of the hexamers.

Molecular simulations [8, 9] of the viral assembly process show that icosahedral structures are thermodynamically favored when the collection

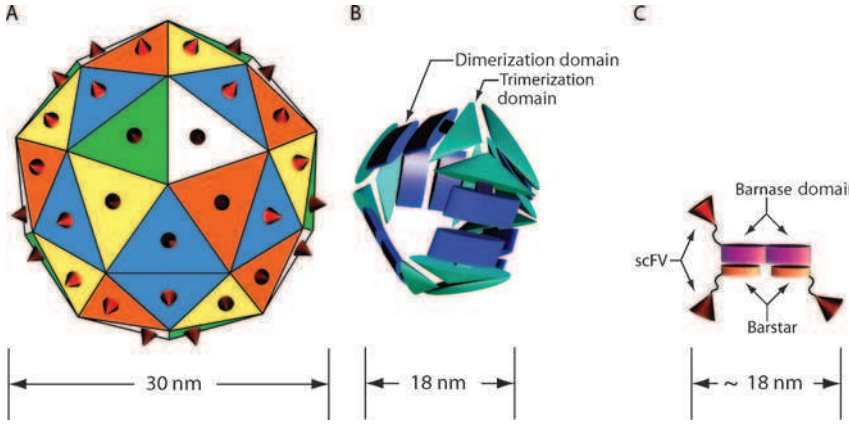

FIG. 1. Protein scaffolds. Icosahedral virus capsids, engineered nanohedra, or linked protein–protein interaction domains can serve as scaffolds for bioassembly formation. For the icosahedron (A) protein subunits form deltas (triangular facets) that can self-assemble into pentamers and hexamers to form the icosahedron. In this system as many as 60 points of attachment (cones in the schematic) have been demonstrated [10, 11]. For engineered nanohedra, tiles have been assembled along dimerization (square) or trimerization (triangle) interfaces. Restricting the angle between the fusion proteins to approximate the 54.7° required for tetrahedral symmetry allowed tiling of a closed shell of 12 subunits [13]. Although modification for ligand attachment has not yet been explored with this system, the closed structure shown (**B**) has 12 potential points of attachment that might be available for bioassembly construction. Linked protein domains (**C**) have also been used as scaffolds [14]. In the example shown, a fused antibody scFv fragment was linked to a barnase–barnase fusion dimer, and two barstar scFv fusion proteins were linked to it via a protein–protein interaction to form a trivalent system.

of protein subunits forming the facets is allowed to adopt two forms of intersubunit interaction so that both pentamers and hexamers form from the same collection of subunits at a set equilibrium ratio. The existence of a facile equilibrium permitting the formation of pentamers and hexamers at biological temperatures appears to be essential for this process to occur [8, 9].

In light of these natural properties, viral capsids are uniquely equipped to provide self-assembling quasi-spherical protein scaffolds for nanotechnological applications. To date most nanotechnological applications in this area have utilized Cowpea Mosaic Virus.

In Cowpea Mosaic Virus capsids, the triangular facets are composed of two subunits. A small subunit contains one of the interacting domains (A domain) and the large subunit contains two interacting domains (B and C domain). Together the two subunits form a triangular facet that spontaneously aggregates to form the minimal icosahedral structure with a triangulation number

of one ($T = 1$). It is composed of 12 pentagons, 60 triangular facets, and 120 protein subunits. However, because each of the triangular facets is composed of three formally nonequivalent protein domains it resembles an icosahedron with a $T = 3$ triangulation number. In this case, however, the concept of quasi-equivalence [7] gives pseudo threefold symmetry to the system, and it is termed pseudo $T = 3$ or $p = 3$.

This capsid carries a lysine residue with enhanced chemical reactivity in each of the 60 facets in the icosahedron [10]. Fluorescein *N*-hydroxysuccinimide ester and fluorescein–isothiocyanate react selectively with this lysine residue at a ratio of 60–70 dye molecules per viral particle up to a ratio of about 100 dye equivalents per capsid subunit. At higher ratios they react with additional amino acids on both the inside and the outside of the capsid. Surface exposed regions of the capsid identified during studies of antigen presentation by the virus could be altered by *in vitro* mutagenesis so as to display highly reactive cysteine-containing loops in each triangular facet of the protein capsid [11]. These reactive cysteines were successfully modified with fluorescein, rhodamine, biotin, and 900-nm diameter gold-particles. These modified viruses have also been attached to gold surfaces in precise patterns using scanning probe nanolithography [12].

In the examples given above, the symmetry of a purified virion is exploited in the formation bioconjugate cages. However, symmetry in protein–protein interaction can also be exploited in the design of cages that do not normally occur in nature (Fig. 1B and C). For example, in initial studies with computer aided design of protein shells, Padilla *et al.* (13) formed fusion proteins in which a protein–protein interaction domain from a protein that normally dimerizes (M1 matrix protein of influenza virus) was fused through a short alpha helical linker to a protein–protein interaction domain from a protein that normally trimerizes (bromoperoxidase). Geometrically, the resulting subunit was designed so as to display an element that is best described as a curved delta. This element spontaneously assembled to form a spherical shell comprising 12 subunits in tetrahedral symmetry. Using these principles, it may be possible to construct spherical shells comprising 24 identical subunits in octahedral symmetry and 60 identical subunits in icosahedral symmetry by placing the interaction domains at appropriate angles with linkers designed to provide the desired angle [13].

2.2. Linked Protein Domains

Linked arrays [14], filaments [13, 14], nanoscale tubes [5], and vesicles [5] can also be formed with fused protein–protein interaction domains [13, 14] or surfactant peptides [5], however, in these cases one must choose protein–protein interaction domains that exhibit high interaction affinities in order

to achieve the necessary stability [13], and only the linked arrays are homogeneous [14].

The tight interaction exhibited by the streptavidan tetramer meet this criterion. The biotin binding sites on the streptavidin tetramer are displayed in a roughly tetrahedral arrangement. Its tight interaction with biotin has permitted it to link proteins together [15, 16]. When engineered antibodies conjugated with biotin are linked to the binding sites, a multivalency effect results in an enhancement of almost 35-fold in binding avidity [17]. In immunology, the terminology for the aggregate binding constant is the avidity, while the intrinsic combining affinity of a single site is termed the affinity. In the barnase–barstar system (Fig. 1C), the tight interaction between the RNAse barnase and its proteinaceous inhibitor barstar is employed to display barnase–antibody fusions. In this case both dimeric and trimeric bioassemblies are possible [14]. Each assembly has yielded an enhancement in antigen binding avidity even though the fragments are displayed on a flexible dimer or trimer [14].

3. DNA Scaffolds

3.1. Single-Stranded Tethers

DNA-tethered fluorophores are clearly the most successful nanoscale bioassemblies currently in use in diagnosis. They have generally been used as fluorogenic probes in two formats: TaqMan® quantitative PCR probes and molecular beacons (Fig. 2). The TaqMan concept was initially applied to the release of radioactivity from 5′-end-labeled DNA that carried a 3′-phosphate so that it could not be extended during the polymerase chain reaction. The sequence was chosen to be homologous to the target amplicon so that it would hybridize to one strand prior to the extension step in the PCR. Once extension begins, the 5′-exonuclease activity of the Taq polymerase degrades the sequence as extension moves past the hybridization site releasing 5′-end-labeled fragments. The initial publication [18] utilized ^{32}P as end-label and showed that its release was proportional to the generation of the expected product with increasing cycle number in the PCR. Tethered fluorophores were used early on in this application [19]. For this purpose, the fluorophores linked by DNA are chosen so that they are quenched by Förster resonance energy transfer [20]. This is a nonradiative energy transfer to an acceptor through induced-dipole–induced-dipole interaction. It occurs when the emission spectrum of the donor overlaps the absorption spectrum of the acceptor. The transfer rate is inversely proportional to the sixth power of the distance between the fluorophores and is therefore a very short-range interaction. At

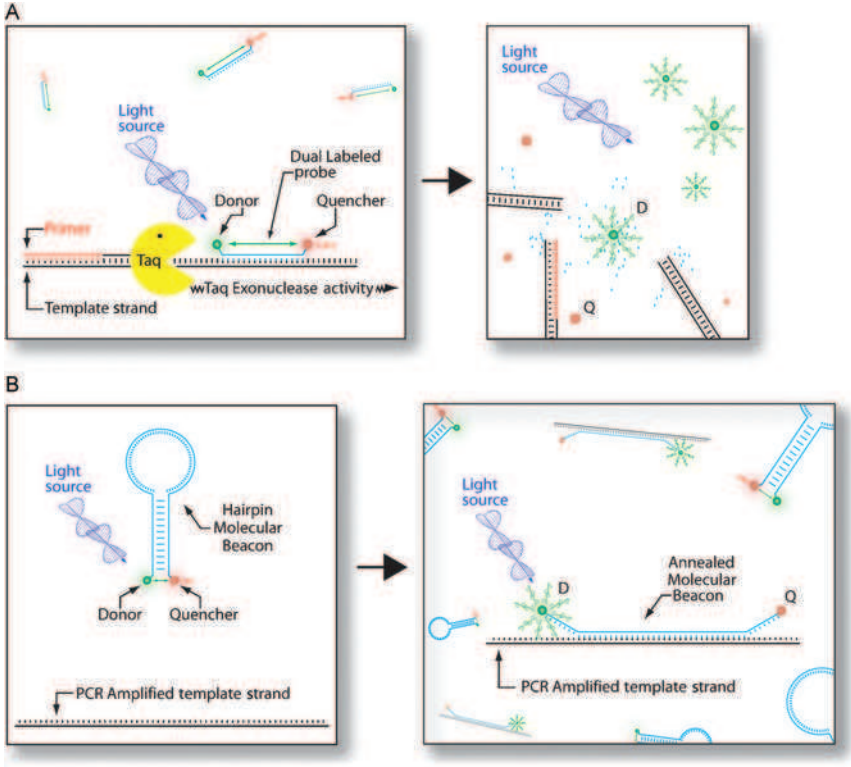

FIG. 2. DNA tethers. Fluorophores tethered by DNA scaffolds have already been widely employed in diagnostic testing. In the TaqMan® system (A) the fluorophores linked by DNA are chosen so that they are quenched by Förster resonance energy transfer [20]. The tether itself is designed to hybridize by Watson–Crick complementarity to one of the amplified product strands of the PCR. The 5′-exonucleolytic activity of the Taq polymerase degrades the DNA tether releasing the 5′-fluorophore to solution where it is no longer quenched. This permits the acquisition of data in real time, so that initial target DNA concentrations can be related to a threshold cycle number in the PCR. In the Molecular Beacon approach (B) the fluorophores are tethered to a DNA strand that contains a short tandem repeat at each end of the tether so that the DNA forms a hairpin loop that brings the fluorophores into close apposition so that they are quenched by resonance energy transfer at lower temperatures where the hairpin is stable. In quantitative PCR applications, the region of the loop is chosen so as to hybridize to one of the single-strands of the amplicon during the annealing step in the PCR. Hybridization requires that the short terminal repeats denature so that the longer loop can form a duplex. This opens the structure and moves the fluorophores apart so that quenching is diminished [26, 27]. Molecular Beacons appear to remain intact during the reaction and simply yield a more intense fluorescence as the number of amplified target strands increases [30].

the Förster radius (generally 2–6 nm) the transfer is 50% efficient. The dyes employed with this PCR technique are efficiently quenched at distances in the range of 8 nm, permitting tethers in DNA probes to have lengths of about 24 nt. As with the end-labeled system [18] the 5′-exonucleolytic activity of the Taq polymerase degrades the DNA tether releasing the 5′-fluorophore to solution where it is no longer quenched. This permits the acquisition of data in real time, so that initial target DNA concentrations can be related to a threshold cycle number in the PCR. A number of instruments are commercially available for this purpose. The technique has now been broadly applied in diagnosis, and it can be anticipated that the number of such applications will increase rapidly in the near future. Among the successful diagnostic tests that have been based on this principle are tests for trisomy 21 [21], sex chromosome aneuploidies [22], HIV type 1 [23], and the detection of cancers of different types [24, 25].

In the molecular beacon format tethered fluorophores contain a short tandem repeat at each end of the tether so that the DNA forms a hairpin loop that brings the fluorophores into close apposition where they are quenched by resonance energy transfer. They remain quenched at lower temperatures where the hairpin is stable. In quantitative PCR applications the region of the loop is chosen so as to hybridize to one of the single-strands of the amplicon during the annealing step in the PCR. Hybridization requires that the short-terminal repeats denature so that the longer loop can form a duplex. This opens the structure and moves the fluorophores apart so that quenching is diminished [26, 27]. Terminal repeats of the molecular beacons are chosen so that they do not hybridize with the target, and the beacon is displaced during the primer extension step in the PCR. In spite of the apparent 5′-flap endonuclease activity of the native Taq polymerase [28], molecular beacons do not appear to be degraded during amplification when native Taq polymerase [26] or its variant Taq Gold® [29] are employed in quantitative PCR procedures. Thus, they appear to remain intact during the reaction and simply yield a more intense fluorescence as the number of amplified target strands increases [30]. As with the TaqMan approach, molecular beacon PCR methodology has yielded a variety of diagnostic applications. For example, hepatitis A [31] and hepatitis B virus have been accurately detected in human specimens [32, 33], as has *Candida dublinienis* [34]. Moreover, expression levels of human chorionic gonadotropin have been determined to have prognostic value in breast cancer [35] using this method. In comparing the two techniques the molecular beacon approach appears to be superior to the TaqMan approach in selectively detecting single nucleotide polymorphisms [29].

Tethered fluorophores have also been used in the construction of a nanotechnological device. In this system, strand displacement was used to alter the

position of two dye molecules, tetrafluoro-flourescein (TET) and carboxytetramethylrhodamine (TAMRA), at the ends of a DNA strand. The two dye molecules begin the cycle in a 40-bp duplex containing a central 2-nt gap placing the fluorophores approximately 13 nm apart, where quenching by resonance energy transfer is minimal. Each end of the duplex has a single-strand overhang of 24 nt that can pair with incoming strands to form a duplex. When a 56-nt strand is added to the system it hybridizes with the two 24 mers at the ends of the gapped duplex and brings them together causing the gapped duplex to fold so that the fluorophores are brought into apposition extinguishing their fluorescent signature by resonance energy transfer quenching. In this state the 56 mer presents an 8-nt single-strand overhang that can serve to begin hybridization to a 56-nt displacing strand that is fully complementary to the previously added 56 mer. Once the displacing strand is added, a 56-bp duplex is released from the system and the 40-bp duplex containing a central 2-nt gap returns to the extended conformation. In effect the system cycles between an open and closed set of molecular tweezers. Cycling could be monitored by using polyacrylamide gel electrophoresis (PAGE) to detect the open and closed forms of the assembly or in real time by using changes in fluorescence associated with the conformational changes [36]. The system could be cycled as many as 7 times. However, bleaching of the fluorophore and the accumulation of unusual structures dampen the signal at high cycle numbers [36]. Similar devices based on other DNA structural interconversions [37, 38] have also been described.

3.2. Open and Closed Edged Assemblies

Quenched molecular beacons commonly employed in quantitative PCR applications are an example of the use of a single-stranded DNA scaffold carrying reporter fluorophores [24, 25]. Although this application requires the extended form of the nucleic acid to participate in a hybridization reaction, one might expect that DNA and RNA aptamers might also serve as single-stranded albeit uniquely self-associated scaffolds for chemical modification and molecular targeting. Certainly the potential is there, because the stable and metastable conformation spaces for both single-stranded DNA and RNA are quite large. Proteins with 20 amino acids and multifaceted potential for hydrogen bond and hydrophobic bond formation have the largest such space. RNA, because of its 2′OH, has more stable conformers than DNA when the four standard bases are considered [39], even the study of DNA aptamers [40] suggests that DNA could also be used for this purpose. With few exceptions [41], single-stranded nucleic acids have not been exploited as aptamer scaffolds.

On the other hand, duplex DNAs that spontaneously form stable or immobile junctions have been used as scaffolds [42, 43]. Junctions with three, four, or as many as six arms have been studied [44, 45]. These properties of DNA have been exploited in the construction of molecules with the connectivity of platonic solids [46–48] and Borromean Rings [49]. The stable junctions used in these bioassemblies are analogs of recombination intermediates in biology. Double crossover analogs have also been assembled into scaffolds. Here the crossover interconnections constrain the DNA into nanoscale planar units with complementary edges suitable for association during tiling [43]. Introducing a hairpin into the double-crossover molecules, permitted the use of atomic force microscopy (AFM) in the demonstration of an expected nanoscale periodic pattern in the product [43]. Here, however, the degree of extension in the product could not be easily controlled, so the tiled product was nonuniform.

DNA double-crossover molecules linked by a short duplex have also been adapted to sense salt concentration changes (Fig. 3) by using the B–Z transition in a short region of duplex DNA [50]. Resonance energy transfer between dye molecules at the ends of each double crossover was used to sense the position of the switch. In this system, both the rigidity and the bulk of the double crossover are important in fixing the distance between the fluorophores as the duplex region twists them from beyond the Förster radius in the Z-DNA form to close apposition in the B-DNA form (Fig. 3).

3.3. Ordered Display

Studies of molecular evolution suggest that many of the most fundamental molecular elements of living systems are relics of prebiotic systems. Gibson and Lamond [51] have suggested that early nucleic acids served as organizing centers for nucleic acid based catalysts. In that proposal, Watson–Crick complementarity of adaptor strands on the catalysts was suggested as the driving force for ordering the catalysts in logical metabolic orders along a longer linear nucleic acid strand. These prebiotic metabolosomes [51] have been mimicked by first coupling biotinylated DNA to biotinylated proteins via streptavidin and then ordering the coupled proteins to single-stranded DNA complementary of the coupled DNA fragments (Fig. 4). The DNA streptavidin–protein complexes, thus, ordered along a guide RNA or DNA strand [52, 53], form an ordered linear assembly analogous to the prebiotic metabolosome. Nitrilotriacetic acid (NTA) derivatized oligodeoxynucleotides linked by nickel chelation to His_6-tagged proteins might also be used in this application [54], as could the DPDPB {1,4-Di[3′-(2′-pyridyldithio) propionamido]-butane}-cross-linking system [4]. However, this later system would require engineering each protein so that it displayed a reactive

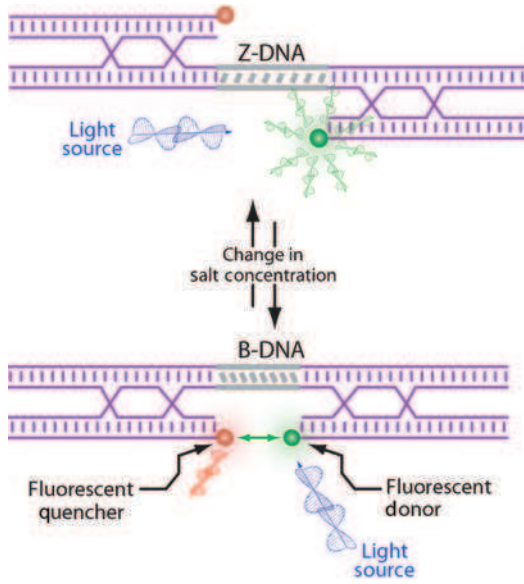

FIG. 3. A two-state switch using the B–Z transition in DNA double crossovers. In this device a spatial shift in the terminally bound fluorescent dyes, occurs when the system undergoes a conformational change from right-handed B-DNA to left handed Z-DNA upon an increase in salt concentration. This releases the flourophore from resonance energy transfer quenching [50]. The double crossovers (Xs in the schematic) bind two short strands tightly to the longer strand so that the fluorophores are locked in the positions shown.

solvent-accessible cysteine residue and a thiol modified adaptor nucleic acid. Although the resulting assembly contains duplex DNAs, single-stranded DNA serves as the scaffold for the assembly of the linked proteins. In principle, only the length of the guide strand limits the number of available orders in a system of this type. In practice, systems with as many as four ordered proteins have been assembled along a linear guide RNA or DNA [52].

Duplex DNAs can be made into linear, branched, closed, or even interlocked forms. Thus, sequence specific proteins can be used to target fusion proteins to these scaffolds to form complex bioassemblies. It is desirable to use proteins that can form a sequence specific covalent link to DNA. Of the available proteins only the DNA (cytosine-5) methyltransferases and the Cyclization REcombination (CRE) recombinase from bacteriophage P1 have well-characterized sequence specificity. CRE recognizes the 34 base pair loxP site. The enzyme forms a stable covalent intermediate that does not undergo further reaction [55] when it attacks DNA at a symmetrical (loxA) recognition site containing 3′-DNA nicks.

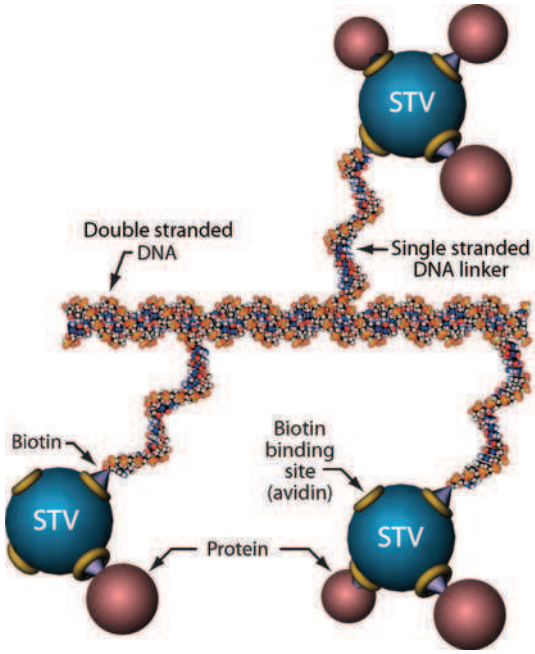

FIG. 4. Ordered linear bioassemblies. Proteins have been ordered in linear fashion using single-stranded RNA and DNA guides as scaffolds for DNA linked proteins based on Watson–Crick complementarity. In this system the streptavidin (STV) molecule is bound to a terminal biotin on a DNA tether that also contains a region complementary to a distinct region of the guide. The open valences on the STV molecules are used to link interesting proteins to the tether for ordering on the guide molecule [52].

DNA (cytosine-5) methyltransferases with many DNA recognition sequences have been characterized, permitting the ordering of fusion proteins (Fig. 5) of many types [56]. It is possible to covalently trap these enzymes at sites on duplex DNA structures containing 5-fluorocytosine. Multiple recognition sites in linear or branched DNA structures (Fig. 5) have been targeted in the construction of bioassemblies [56–58]. In addition, different methyltransferases (e.g., M · *Hha*I, M · *Eco*RII, and M · *Msp*I) have been targeted to their specific recognition sites in DNA-scaffold bioassemblies [56–58]. As with most of the scaffolds under consideration here, the resulting bioassemblies can be modified with a variety of targeting and detection chemistries. With the methyltransferases, it is possible to create fusions with other proteins of interest, including peptides that bind to receptors for directed targeting of the nanostructure [59].

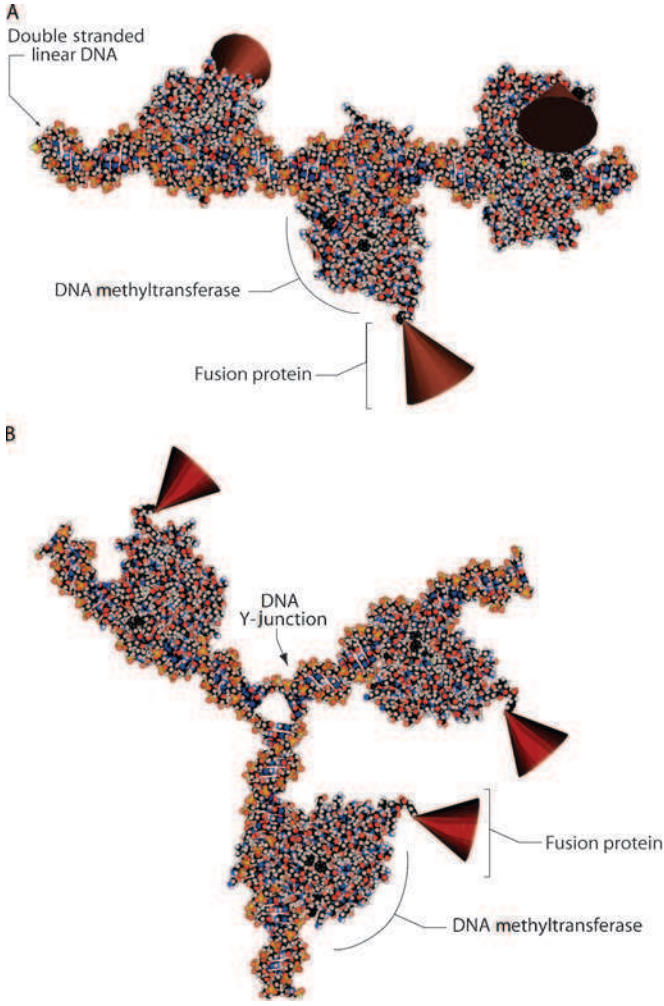

FIG. 5. Ordered linear and branched bioassemblies. DNA methyltransferase fusion proteins are covalently linked at methyltransferase recognition sites containing 5-fluorocytosine along (A) a linear DNA scaffold [57] or (B) a Y-junction DNA scaffold [58]. Cones represent the positions of fusion peptides and proteins.

3.4. NETWORKED DISPLAY

The streptavidin–biotin system has also been used to join duplex DNA into networks [60]. Since the streptavidin molecule has the capacity to bind four biotins, it can be used to link as many as four DNA biotinylated DNA

fragments. Thus, a bioassembly can be formed from a network of DNA molecules linked by streptavidin molecules. Here, the strepavidin should serve as a node of degree 4. However, when these assemblies were generated, only 2–3 streptavidin connections [61] in circularized or networked oligodeoxynucleotides could be detected with gel electrophoresis and scanning force microscopy (SFM). This suggests that steric hindrance may prevent complete occupation of the streptavidin nodes in end-on DNA connections. As with the formation of protein [13] and peptide-based filaments [5], the resulting assemblies are extended but nonuniform.

4. Organic Polymer Scaffolds

4.1. Cross-Linked Display

Tethering multiple moieties with short synthetic chains was explored early on in attempts to increase antigen binding avidity in antibodies [62, 63] and binding avidity for multivalent ligands [64–66] of different types. The results were encouraging in that the multivalency effect generally enhanced binding [62, 63, 67]. While these systems are perhaps more properly viewed as cross-linking elements, they have produced divalent, trivalent [63], tetravalent [68], and even octavalent (dendritic) systems [64]. The multivalency effect has been most often interpreted in terms of the higher probability of a productive initial binding event (higher local concentration) followed by cooperative binding to adjacent binding sites (low entropy loss associated with the subsequent binding events), coupled with the corresponding slow release of the system due to the requirement for simultaneous multiple binding-site dissociation.

4.2. Dendrimer Display

Dendrimers are branched polymers that are synthesized from a central core compound that anchors polymerization (Fig. 6). The core generally displays multiple linking functionalities to which branching monomers are added sequentially in generations. Adding the first round of branches to the core brings the system to generation G0, adding the second round to G1, and so on (Fig. 6). After the last round of synthesis, the branched elements display a roughly spherical surface with a variety of surface functionalities that can be used for attachment of almost any desired bioconjugate. Dendrimers with a variety of core (e.g., diethylamine), branch [e.g., poly(amido amine), lysine], and surface chemistries (e.g., amine, succinamic acid) are now commercially available as scaffolds, with generation levels ranging up to 10.

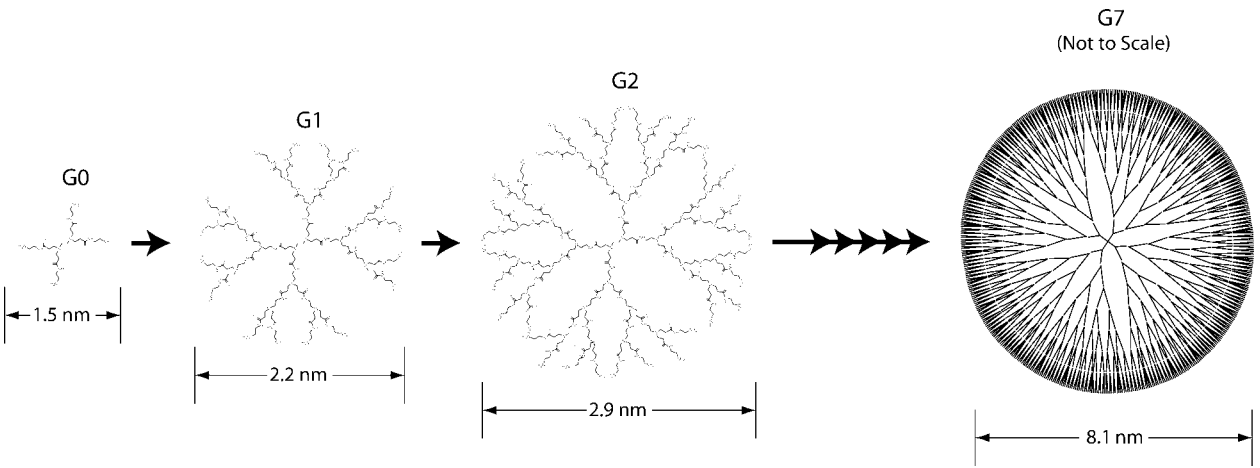

FIG. 6. PAMAM dendrimers. The chemical structures of dendrimers composed of poly (amido amine) (PAMAM) monomers are shown at low generation numbers, and schematically at generation seven. The G0 dendrimer shown is a tetravalent linker.

Dendrons (asymmetric or half-dendrimers) having 2, 3, 4, or 9 branches from a single point of attachment to the nucleic acid have been used as organic scaffolds for the attachment of multiple fluorophores to nucleic acids. In this application they have successfully enhanced the detection of herpes virus [69]. Enhanced detection of antigens via radiolabeled dendrimers linked to antibodies is also possible [70]. Folate receptors on tumor cells have also been targeted by using folate ligands linked to generation 3 [71] and generation 5 [72] polyamidoamine dendrimers. Dendrimers of this size (about 5 nm in diameter) appear to adopt a spherical shape in solution [73]. However, part of the capacity for strong binding exhibited by dendrimers appears to result from their capacity to adopt a flattened shape upon contact with a substrate [74].

While enhanced binding is generally seen with these multivalent systems, a corresponding enhancement of biological response is not always observed. Thus, cytokines linked to generation 5 polyamidoamine dendrimers did not enhance the induction of human interleukin-3 (hiL-3) dependent *in vitro* cell proliferation over that observed with the free cytokine [75]. The conjugated cytokines, did however, retain their ability to bind cell surface hIL-3 receptors [75]. Other biologically active ligands have been linked to dendrimers [70, 76, 77].

5. Inorganic Scaffolds

5.1. Quantum Dots

Crystalline inorganics can offer another form of nanoscale scaffold with very interesting properties. The most useful of these properties are those that follow from the quantum confinement effects exhibited by nanoscale clusters of atoms. Quantum confined 3D clusters are generally called quantum dots (Fig. 7). Their properties include absorption and emission energies that are a function not only of the atomic composition but also of the effective size and shape of the particle. For noble metal clusters of less than 100 atoms *ab initio* treatment at the Hartree-Fock level of theory allows one to view the clusters as a single molecule with HOMO-LUMO energy differences (band gaps) that are a decreasing function of the size of the cluster [78]. For gold quantum dots (Fig. 7A) with as many as 31 atoms, the band gap appears to be inversely proportional to the cube root of the number of atoms in the cluster [79]. For semiconductor crystals (Fig. 7B), electronic structure calculations arrive at a similar molecular orbital picture for groups of atoms ranging in size from several hundred to as many as 10,000 atoms. Here, however, the methods are semiempirical and generally rely on the effective mass approximation [80] method for calculating band gaps although other methods have been

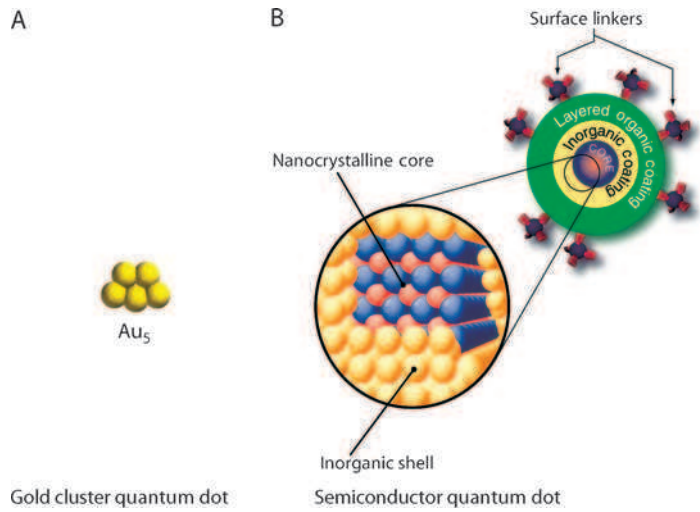

FIG. 7. Schematic of the structure of quantum dot scaffolds for bioassembly formation. Quantum dots may be a small cluster of noble metal atoms like the Au_5 cluster (A) or the larger crystalline cluster of semiconductor material (B). Quantum dots formed from clusters of small numbers of gold atoms are soluble [79] and low-energy structures can be predicted with *ab initio* techniques [106, 107]. The structure shown in (A) was obtained with a density functional calculation [107]. Semiconductor nanocrystals are insoluble. When they are used in biological applications they are coated with an inorganic shell on which a layered organic coating is deposited. Functional groups on the organic layer (e.g., carboxylic acids) serve to link targeting molecules or generalized surface linkers like streptavidin.

developed [81, 82]. For larger roughly spherical particles, the band gap energies are roughly proportional to the inverse of the square of the quantum dot diameter [82, 83].

The important concept that emerges from both theory and experiment is that quantum dots can be viewed as single molecules with broad absorption energies and sharp emission energies that can fall in the visible spectrum. Moreover, unlike organic fluorophores (e.g., fluorescein) they are very stable to continuous excitation. At high excitation intensities they blink as they undergo cycles of photoinduced ionization and neutralization. This process can also cause them to blueshift in emission wavelength indicating that surface oxidation effectively shrinks the particle [84]. Recent progress suggests that blinking can be suppressed under appropriate conditions [85].

Although noble-metal quantum dots can be water-soluble when they contain a small number of atoms [79] most of the semiconductor-based quantum dots are insoluble in water. Solubilization is often achieved by coating the dot with a layer of ZnS [86]. In general, trioctylphosphine

(TOPO) [83, 87] is used to terminate growth of the crystal and the phosphine is exchanged in a reaction with diethylzinc and hexamethylsilathiane to produce a ZnS coating (Fig. 7). The ZnS surface coating can then be reacted with mercaptoacetic acid to give a coating of carboxylic acid groups that solubilize the dot and are available for coupling reactions. Coupling reaction schemes have proliferated to the point where almost any desired moiety can be linked to a quantum dot (Fig. 7). A variety of passivated and coated products are commercially available, notably quantum dots linked to streptavidin.

Given the available bioconjugate chemistries, the whole range of targeting systems is being explored. Cell surface markers have been detected with antibodies [88–90], peptides [91, 92], and growth factors [93] linked to quantum dots. Expressed chimeric proteins bearing an avidin tag have been detected with biotinylated quantum dots [94]. Moreover, specific chromosomes [95] and specific genes on chromosomes [96] have been detected with nucleic acids linked to quantum dots. In this latter application, fluorescent *in situ* hybridization (FISH), quantum dots appear to have considerable potential in clinical diagnostics. The commercially available streptavidin coated quantum dots can be used directly in an existing FISH protocol using biotinylated 2′-deoxyuridine 5′-triphosphate (dUTP) labeled DNAs as nucleic acid probes. Organic flourophores linked to streptavidin generally bleach significantly after as little as 30 min of exposure to their excitation wavelength, making the method semiquantitative and nonarchival. Streptavidin coated quantum dots were seen to be brighter and were much less susceptible to photo bleaching under the same conditions with as much as 80% of the initial signal present after 30 min of illumination [96]. Together, these properties suggest that it may be possible to develop more quantitative and perhaps even archival FISH methods using quantum dots.

5.2. Photonic Crystals

Photonic crystals can take advantage of the dependence of diffraction wavelength on lattice spacing [97]. They have been used in diagnostic assays for small molecule analytes like glucose [97]. Thus far, however, these particular systems have not been adapted as multipurpose scaffolds in biology nor have available photonic band gap materials adapted in this way [98]. These structures restrict optical photon emission by blocking available optical emission modes [99].

Noble metals like gold, form quantum dots when they form clusters with small numbers of atoms. However, larger clusters exhibit characteristic surface plasmon resonance that is coupled in aggregates of the particles [100, 101] making monitoring aggregation possible in the near infrared and

visible regions of the spectrum. Gold is a natural substrate for bioconjugate linkage based on sulfhydryl coupling. Thus, it has served as a scaffold for bioassemblies displaying DNA, RNA [102], and proteins. Aggregation has been used to detect analytes in blood [103], and a novel albeit complex application termed the bio-bar code amplification assay has been able to detect very low levels of prostate specific antigen (PSA) in blood [104]. It has also been used to correlate the presence of very low levels of amyloid-derived diffusible ligands (ADDLs) in spinal fluid [105] with a diagnosis of Alzheimer's disease. In this assay, short duplex oligodeoxynucleotides are linked to a nanoscale gold scaffold, which also carries a secondary polyclonal antibody designed to decorate a target protein after it binds to a monoclonal antibody linked to magnetic beads. After separation of the magnetic complex from the specimen, the system is heated to denature the multiple duplex oligodeoxynucleotides on the nanoscale gold scaffold. The large number of single-stranded bar-code DNAs, thus released, represent a significant amplification of the signal from bound antibody that can easily be detected with the PCR or with light scattering. In the light-scattering technique, the bar-code single-strands are used to link silver-coated gold particles carrying oligodeoxynucleotides complementary to one end of the bar-code single strand to oligodeoxynucleotides in a microarray that are complementary to the other end of bar-code DNA [104, 105].

6. Discussion

There is no real bright line between biotechnology and bionanotechnology when one considers tethered fluorophores, tethered targeting elements, or tethered ligands. They are nanoscale bioassemblies in every sense. However, when the application (e.g., quantitative PCR detection of DNA) requires that the tethered fluorophores be released from resonance energy transfer quenching by enzymatic cleavage or induced conformational change, in most cases very little structural information is required for correct construction of the required probes. This ease of application, commercial availability of custom synthesis for the bioassemblies themselves, and the wide availability of the fluorescence detection systems involved have lead to an extraordinary bloom of diagnostic applications for these bioassemblies. Tethered antibodies and ligands have generated far fewer applications in part because of the lack of wide commercial availability for such products and in part because single antibody avidities are adequate for most applications in immunochemistry.

In a similar fashion, the commercial availability of quantum dots and photonic crystals is generating a bloom of exciting applications in

diagnostics, largely because these systems are becoming available in increasingly flexible formats. Dendrimers may soon enjoy the same growth in diagnostic applications based on their capacity for tight surface binding. Bionanotechnological scaffolds like the closed protein shells and the duplex DNA scaffolds are relatively new, and their potential has yet to be developed. Scaffolds integrating ordered proteins and nucleic acids are now being developed.

7. Conclusions

In each of the current applications to diagnostics, bionanotechnological devices play a largely passive role. Cell surface targeting with an antibody, a growth factor, or a small molecule ligand achieves a new level of sophistication, however, it is still a passive approach. While the induced conformational changes associated with the binding of dendrimers or molecular beacons are somewhat more complex responses to the local environment, they are still largely passive mechanistically. The goal of bionanotechnology is somewhat larger. One envisions not only the specificity and telemetry that a soft-landed radio beacon on the moon might exhibit but also the capacity for detailed and serial analyses of the landing site that a soft-landed robot might exhibit. No one expects that robotic control of a molecular device will be available anytime soon, but a device based on one of the scaffolds described earlier might be preprogrammed to report several findings, once its initial target was located. Dynamic devices that change color with time of incubation based on the presence or absence of secondary or tertiary cellular markers within a population exhibiting a primary marker would be of considerable utility. Dynamic nanoscale devices of this type await the application of the rules of assembly associated with the scaffolds described earlier and perhaps the discovery and application of new rules of assembly and new scaffolds.

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CARDIAC TROPONINS: CLINICAL AND ANALYTICAL ASPECTS

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1. Abstract

Heart disease remains the most common cause of death in the developed world with 1 in 10 patients still dying of a myocardial infarction. With the advent of assays to measure cardiac troponins, the diagnosis and prognostication of acute coronary syndromes (ACS), including myocardial infarction, has greatly improved. The cardiac troponins are now considered the 'gold standard' biochemical test for the diagnosis of acute myocardial infarction. One assay for cardiac troponin T and numerous for cardiac troponin I are available. It is the aim of this review to describe the structure and biochemistry of the cardiac troponins and outline their clinical significance in the context of its role in diagnosis, prognosis, risk stratification and monitoring treatments and interventions. The measurement of cardiac troponins will also be described together with pertinent issues of standardization, point-of-care/near-patient testing and quality assurance. The factors affecting cardiac troponin measurement in the clinical laboratory setting is also discussed.

2. Introduction

Heart disease remains the most common cause of death in the developed world with 1 in 10 patients still dying of a myocardial infarction (MI) [1]. With the advent of assays to measure cardiac troponins, the diagnosis and prognostication of acute coronary syndromes (ACS), including myocardial infarction, has greatly improved. A historical perspective of the development of the use of cardiac biomarkers has recently been published [2]. Briefly, Katus *et al.* [3] were the first to describe the measurement of cardiac troponin T (cTnT). This was followed by Bodor *et al.* [4] who described the development of the cardiac troponin I (cTnI) assay, building on the work of Cummins *et al.* [5], both for the diagnosis of MI. After a number of classical studies, the cardiac troponins are now considered the ''gold standard'' biochemical test for the diagnosis of acute MI (AMI). Furthermore, it is becoming increasingly recognised that the clinical laboratory and the clinical biochemist have an

important role to play in clinical cardiology. In the near future, the apparent chasm between the laboratory and the bedside will finally be bridged.

It is the aim of this review to describe the structure and biochemistry of the cardiac troponins in some depth but without resorting to details at a specialist level. The clinical significance of cardiac troponins in the context of its role in diagnosis, prognosis, risk stratification, and monitoring is also reviewed. The measurement of cardiac troponins is briefly described with pertinent issues of standardization, point-of-care testing, and quality assurance discussed. Finally, the factors affecting cardiac troponin measurement in the laboratory setting will be summarized. Although other cardiac markers still have a role to play in many situations, this review will strictly focus on the cardiac troponins.

3. Structure and Biochemistry of Troponins

The troponin complex was first described in 1946 by K. Bailey in a letter to *Nature* [6] but it was the work by Ebashi *et al.* [7] that showed that the contraction of striated muscle and not smooth muscle was regulated by a special protein complex, now known as the troponin located on actin filaments. With the development of techniques, such as site-directed mutagenesis, studies have yielded new details about the structure of the troponin complex.

The troponin complex consists of three subunits:

- *Troponin C (TnC)*: The component that binds calcium and regulates the activation of thin filaments during contraction by removing troponin I inhibition. It has a molecular weight of 18 kDa [8].
- *Troponin I (TnI)*: The inhibitory subunit that inhibits ATPase activity of actinomyosin. Its molecular weight is 22 kDa and is encoded for by chromosome 19q13.3 [9].
- *Troponin T (TnT)*: The component that plays a structural role and binds the troponin complex to tropomyosin. TnT is also involved in activating actinomyosin ATPase activity. Its molecular weight is 37 kDa and its gene is present on chromosome 1q32 [10].

A comprehensive and detailed account of the structures and sequences is available elsewhere [11, 12]. Here a brief overview is provided of each of the subunits and their coordinated functioning. An appreciation of the structure and biochemistry is important as it lays the foundation for the development of sensitive and specific assays for the measurement of cardiac troponins. It also forms the molecular basis underlying the regulation of muscle contraction.

The description below applies equally to both cardiac troponins and skeletal troponins unless otherwise stated.

3.1. Troponin C

TnC is the calcium-binding component of troponin. There are two known isoforms: a fast isoform is found only in the skeletal muscle, whereas a slow isoform is found both in the skeletal and cardiac muscle [8]. TnC has four motifs having helix-loop-helix structures and is part of the family of proteins known as EF-hands [13]. This family of protein consists of a 12-membered loop flanked by α-helices and has a high affinity for calcium. They function either as calcium buffers (parvalbumins, calbindin) or as calcium-dependent triggers (calmodulin, TnC). The general three-dimensional structure has been described as a "dumbbell-like" form (see Fig. 1) because it has two globular domains connected by a long central helix [12].

TnC consists of three main components:

- *N-terminal globular domain*: This domain contains two calcium-specific (because it does not bind magnesium), low-affinity (compared to C-terminal) calcium-binding sites.
- *C-terminal globular domain*: This domain contains two sites that are able to bind both calcium and magnesium with very high affinity. However, they do not directly participate in the regulation of muscle contraction but have an important structural role in fixing TnC to other components of the thin filament.

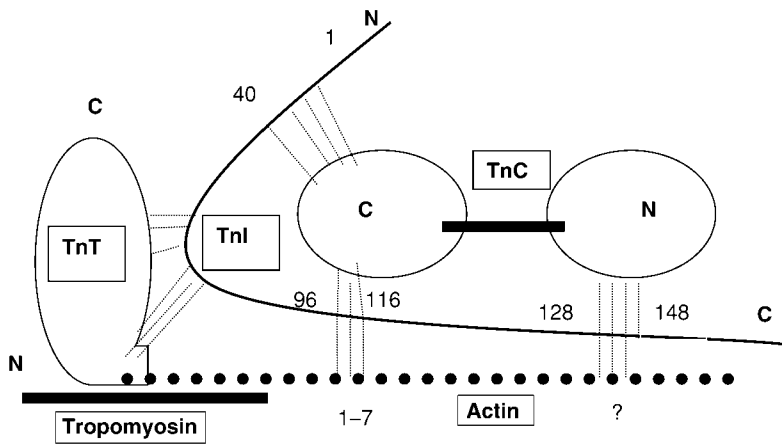

FIG. 1. Schematic of the interactions between the components of the troponin structure and the actin thin filament protein. TnT is shown as a "comma-like" structure, TnC is presented as a "dumbbell-like" structure, and TnI is depicted as a "hook-like" structure. The sites of interactions are shown by dashed lines and the residue number starting from N-terminal. For detail refer to text. ? implies sites of interaction are unknown. Distances are all apparent and not drawn to any scale.

• *Central α-helix*: This is the semiflexible hinge connecting the two globular domains. It plays a role in the conduction of signals between the two globular domains via conformational changes.

The initiation of muscle contraction begins with the saturation of the regulatory sites of TnC by calcium. Calcium binding is accompanied by conformational changes in the structure of TnC. In general, in the absence of calcium the calcium-binding sites are in the so-called closed configuration and the hydrophilic residues of the helices flanking the calcium-binding sites form contacts with each other and are effectively shielded from the solvent. After saturation with calcium, the helices belonging to the calcium-binding sites move further apart from the central helix, exposing the hydrophobic residues to the solvent and the calcium-binding sites are now in the open configuration. This mechanism is characteristic for skeletal TnC [14].

For cTnC, due to nonconservative replacements in the first calcium-binding site, it cannot bind calcium. However, the second site plays a role in regulating cardiac contraction. It has been shown that the replacement of the first 41 residues of the skeletal TnC by the corresponding residues of cTnC makes the properties of the chimerical protein similar to cTnC [15]. Thus, this portion is vital for the regulation of cardiac contraction.

TnC interacts with TnI and this interaction is increased in the presence of calcium. X-ray diffraction studies indicate that TnI winds around the TnC molecule [16]. The interaction between TnC and TnI is shown in Fig. 1. TnC does not directly interact with TnT, although this is now being disputed [17].

3.2. Troponin I

3.2.1. *Structure and Function*

TnI is the inhibitory component that inhibits actinomyosin ATPase activity. It is a linear structure with five α-helices. Three isoforms have been described for striated muscle. Two isoforms are characteristic for the fast and slow skeletal fibres respectively, and one isoform for cardiac muscle. The skeletal TnI consists of 181 amino acid residues, whereas the cTnI consists of 211 amino acids. It is a polar protein with an excess of positively charged residues. Three distinct genes encode for the three different isoforms. The expression of TnI is dependent on the stage of ontogenesis – both cardiac and slow skeletal isoforms are expressed in the heart of the human fetus. After birth, the expression of the slow skeletal isoform is stopped and the expression of cTnI is enhanced. It has been reported that by the ninth month of life, cTnI is expressed exclusively [18].

The inhibitory activity of ATPase is enhanced in the presence of tropomyosin and abolished in the presence of a fully calcium-saturated TnC [12]. TnI

contains two inhibitory sites. The main site (residues 96–116, see Fig. 1) has a hairpin form. It inhibits ATPase activity and interacts with actin (residues 1–7) and TnC. The second inhibitory site is present on residues 128–148. The fact that the inhibitory activity of TnI is increased in the presence of tropomyosin suggests a direct interaction of TnI and tropomyosin, although the exact sites of interaction remain unknown [19].

In the absence of calcium, that is, during relaxation, the inhibitory sites of TnI interact with actin, whereas in the presence of calcium, that is, during contraction, these sites interact with TnC. TnI and TnC are oriented in an antiparallel fashion. As shown in Fig. 1, the first inhibitory site (residues 96–116) of TnI and the N-terminal part of TnI (residues 1–40) interact with the C-terminal globular domain of TnC. The C-terminal part of TnI interacts with the N-terminal domain of TnC. In the absence of calcium, TnC usually interacts with TnI, which in turn binds to actin via its inhibitory and actin-binding sites (residues 96–116 and possibly 140–148) and thereby inhibits actinomyosin ATPase activity. Binding of calcium to the regulatory sites of TnC enhances the interaction of TnC with TnI and this causes the dissociation of the main inhibitory site of TnI from actin, which disinhibits actinomyosin ATPase activity.

The interaction of TnT and TnI lies between residues 1–40 and 96–148 of TnT (Fig. 1). This juxtapositioning with TnI allows any conformational signal induced by calcium binding to TnC to be transferred to TnT and then to tropomyosin.

3.2.2. *Phosphorylation of TnI*

The phosphorylation of TnI modulates the interaction among the components of the troponin complex. This is especially important for the cTnI. *In vitro*, three protein kinases (PKs) are known to phosphorylate TnI. Phosphorylation by calcium-phospholipid-dependent protein kinase C (PKC) results in a decrease in actinomyosin ATPase activity without significant change in its dependence on calcium concentration. Thus, *in vivo* this ultimately decreases the power of cardiac contraction [20]. The cyclic nucleotide-dependent protein kinases – both cyclic adenosine monophosphate (cAMP) (PKA) [21] and cyclic guanosine monophosphate (cGMP) – dependent protein kinases (PKG) [22] phosphorylate TnI. *In vitro*, both phosphorylate the same sites of TnI. Phosphorylation of TnI by PKA results in a decrease in the sensitivity of the contractile apparatus to calcium, causing a phosphorylation-induced decreased affinity of TnI to TnC. For cTnI, phosphorylation of ser-23 and ser-24 results in significant changes in TnI structure and this affects the interaction of TnI with TnC and the binding of calcium by the regulatory sites of TnC. Thus, the phosphorylation of ser-23 and ser-24 of cTnI by both PKA and PKG decreases the calcium sensitivity of the contractile apparatus.

The contractile activity of the heart depends on the coordinated phosphorylation of both TnI and TnC and other membrane proteins such as phospholamban, phospholeman, and so on. The interested reader can obtain a comprehensive account of the regulation of muscle contraction by the troponin complex in a review by Farah and Reinach [12]. It is, however, important to note that the phosphorylation of TnI plays a vital role in the regulation of the contractile activity of the heart. It has been suggested that different pathologies, such as heart failure and MI, have a differential affect on TnI phosphorylation. However, at present it is not possible to relate the different forms of pathologies to TnI phosphorylation. This is clearly an avenue for future research and much progress is being made in this particular area.

3.3. Troponin T

3.3.1. *Structure and Function*

TnT is the component that interacts with tropomyosin. It plays an important role in muscle contraction regulation. Its structure can be described as "comma-shaped" (Fig. 1) and it is located in the groove of the actin helix and extends along the thin filament.

Multiple TnT isoforms exist, formed by alternative splicing of the primary RNA transcript in a developmentally regulated manner [23]. The human cTnT contains four isoforms, three of which are expressed in the fetus and one is characteristic of the adult heart [24]. It is believed that the reexpression of embryonic forms of TnT, both at the mRNA level [24] and protein level [25], occurs during heart failure. Furthermore, in end-stage renal failure it was believed that cTnT isoforms were reexpressed in skeletal muscle [26, 27] and were probably the basis of the associated peripheral myopathy [28] but this theory has now been challenged [29]. This is discussed further in Section 5.1.

The N-terminal part has an abundance of negatively charged residues, whereas the C-terminal has mainly positively charged residues [17]. This confers TnT with a "polarity" and hence at physiological salt solution TnT tends to aggregate.

Since the discovery that TnT and tropomyosin interacted in an intricate way [30], it has now been shown that they form a triple-coiled coil stabilized by hydrophobic interactions [31]. TnT contains three tropomyosin-binding sites. The N-terminal chymotryptic peptide (also known as T1 fragment, residues 1–158) interacts with tropomyosin in a calcium-independent manner with the C-terminal of one and N-terminal of another tropomyosin dimer. The other two sites interact with tropomyosin in a calcium-dependent way, thus providing a calcium-dependent regulation of muscle contraction. The second and third sites of interaction are on residues 156–227 and 227–259 respectively [12].

The TnI binding site of TnT is in the C-terminal region. The interaction depends on the redox state of cys-48 and cys-64 of TnI. Oxidation of these residues or chemical modification decreases the interaction of TnT and TnI. Interaction of TnT and TnI is by forming a triple-coiled coil structure [32].

The interaction with TnC remains controversial and sufficient information was lacking at the time of writing. However, the N-terminal globular domain and central helix of TnC (residues 1–100) play a role in the interaction with TnT [17].

TnT plays a crucial role in "fixing" the troponin components to actin-tropomyosin complex. It also links adjacent tropomyosin dimers such that the N-terminal of TnT interacts with the C-terminal end of one and N-terminal end of another tropomyosin dimer. TnT plays a role in activating actinomyosin ATPase activity in the presence of calcium, by its direct interaction with TnC. In the absence of calcium, the troponin-tropomyosin complex is fixed on the actin filament via TnI. In the presence of calcium, the contacts of TnI and actin are weakened, enabling contraction.

3.3.2. *Phosphorylation of TnT*

TnT kinase has been isolated in skeletal and cardiac muscle [33, 34] and it phosphorylates the isolated and complexed TnT at ser-1 residue. The enzyme belongs to the casein kinase II family. Phosphorylase kinase and PKC also phosphorylates TnT at various sites. The physiological role of TnT phosphorylation remains unclear. However, phosphorylation of TnT by the α-isoform of PKC results in a decrease in the actinomyosin ATPase activity and calcium sensitivity. This causes a decrease in the affinity of phosphorylated TnT to the actin-tropomyosin complex and may affect the contractile activity of the heart [35].

3.4. Implications from the Newly Elucidated Structure of the Core Domain

Recently, the structure of the core domain of cardiac troponin in the calcium-saturated form has been published (Fig. 2) [36]. Electron microscopy [37] and low-resolution X-ray crystallography [38] studies have revealed that the troponin structure consists of two domains: the TnT1 extension and the core domain. The core domain retains most of the regulatory function of troponin [39]. It was shown (Fig. 2B) that the core domain was dominated by α-helices and is subdivided into two structurally distinct subdomains: the regulatory head (consisting of TnC residues 3–84) and the IT arm (consisting of TnC residues 93–161, TnI residues 42–136, and TnT residues 203–271). Flexible linkers making the entire molecule highly flexible connect the two domains. The authors concluded that the flexible nature must be of relevance

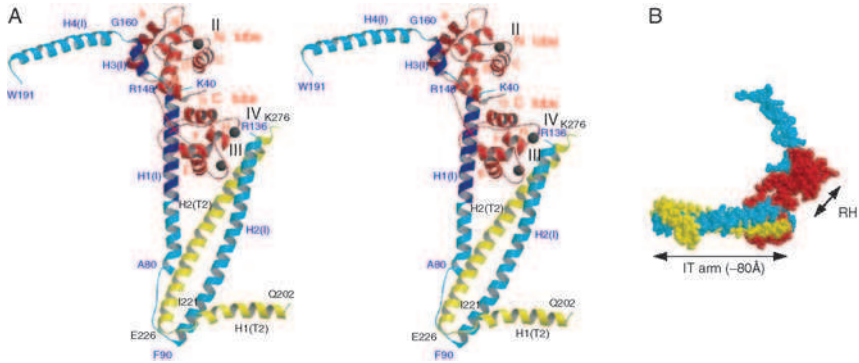

FIG. 2. Crystal structure of the core domain of human cardiac troponin in the calcium saturated form. (A) Stereo view of the Tn52KB molecule. TnC and TnT are coloured in red and yellow respectively. TnI is coloured in cyan, except for the two streches of amphilic helices (TnC binding sites), which are dark blue. The three calcium ions bound are represented by black spheres. Each helix within TnI and TnT is indicated by the helix number, whereas each helix of TnC is indicated by a capital letter. (B) A space-filling model of the Tn52KB molecule showing the regulatory head (RH) and IT arm. (Reproduced with the permission from reference 36).

to a physiological function, the implication here being that the molecular motion of troponin could be described in terms of changes of orientation of individual α-helices plus mobility of individual flexible linkers [36]. The structure of the troponin complex imply that calcium binding to the regulatory site of TnC removes the carboxy-terminal portion of TnI from actin, thereby altering the mobility and/or flexibility of troponin and tropomyosin on the actin filament. Unfortunately, the crystallization of the entire troponin molecule has not been successful to date.

This study has improved the understanding of the mechanics behind muscle contraction, but has limitations. For example, the authors note that in the Tn46K core domain preparation, 46 residues were deleted to improve the crystal quality. This deletion is, however, known to impair the ability of TnI to bind to actin-tropomyosin at low-calcium concentrations. Thus, at present it is difficult to make any valid conclusions about the overall structure of the troponin molecule *in vivo*.

3.5. Coordinated Regulation of Muscle Contraction

The thin filament consists of 7 actin monomers that interact with one tropomyosin dimer and one heterotrimeric troponin complex, which consists of TnC, TnI, and TnT. Together they make up the regulatory unit. The ratio

of troponin:tropomyosin:actin in the so-called regulatory unit is 1:1:7. The continuous thread of tropomyosin (which is an α-helical-coiled coil) is located in the groove of the actin helix. The sliding of the two types of filaments against each other is the molecular basis of muscle contraction. This sliding is induced by the interaction of myosin heads with actin.

The current understanding of the mechanism that regulates striated muscle contraction is based on the model proposed by Tripet *et al.* [40]. Briefly, in the absence of calcium the N-terminal site (residues 1–40) of TnI interacts with C-terminal globular domain of TnC. The inhibitory sites of TnI (residues 96–116 and 140–148) interact with actin. Calcium is released from the sarcolemma on depolarization upon contraction. In the presence of calcium, which binds to TnC, a conformational change is induced. The N-terminal domain of TnC interacts with residues 116–131 of TnI, forcing the inhibitory sites of TnI to dissociate from actin. The main inhibitory site (residues 96–116) then starts to interact with the C-terminal domain of TnC, that is, there is increased affinity of TnC for TnI. The TnI then moves away from the actin-tropomyosin complex, causing the release of the inhibition of actinomyosin ATPase. This allows ATP hydrolysis and therefore muscle contraction. As calcium is pumped back into the sarcoplasmic reticulum, the complex reverts to its previous conformation, inhibiting ATPase action and the muscle now relaxes (see Fig. 3) [12, 41].

3.6. Kinetics of Release, Degradation, and Clearance of Troponins

The majority of cardiac TnT and TnI are found in the contractile apparatus and released as a result of proteolytic degradation. About 6–8% [42] of cTnT and 3–8% [11, 43] of cTnI are present in the cytoplasm.

cTnT has a biphasic release pattern with an initial peak at 12 h after the onset of muscle ischemia, followed by a plateau phase lasting 48 h, and a subsequent fall to undetectable levels after 10 days [43, 44]. Successful early reperfusion leads to a more rapid peak that is generally a marker of favorable prognosis [45]. The duration of the elevation is determined by the size of infarction; small infarctions may remain elevated for as little as 7 days and with large infarcts it may remain detectable for as long as 3 weeks.

cTnI on the other hand has a monophasic release pattern. The duration of elevation is typically from 5 to 10 days but depends greatly on infarct size [46].

Regarding clearance, there is a common misconception that the cardiac troponins have a long half-life. It has been clearly demonstrated that the half-life of cTnT in circulation is about 2 h [42]. The prolonged and continuous detection of the troponins is due to its release from the myofibrillar pool as the contractile apparatus in the cell undergoes total degradation [42, 44]. The half-life of cTnI in dogs has been shown to be about 70 min with a similar release

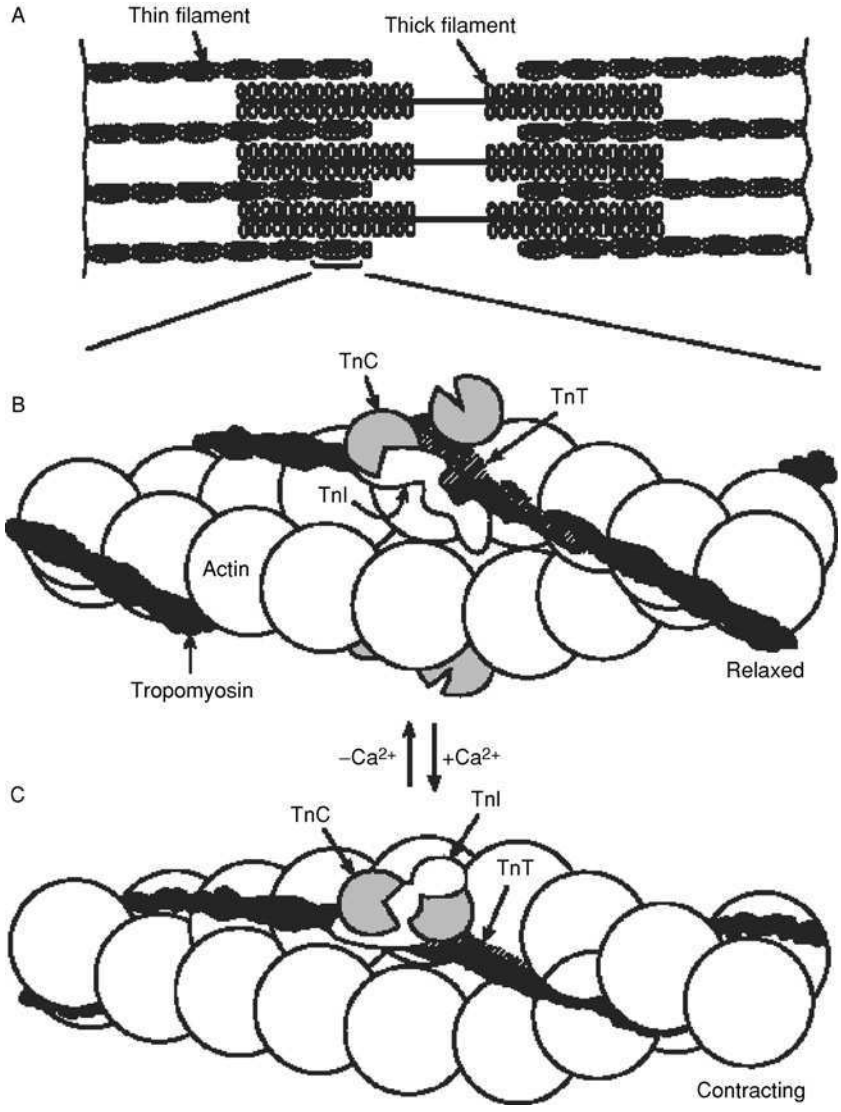

FIG. 3. The coordinated mechanism of cardiac muscle contraction. (A) Schematic of the structure of actin (thin filament) and myosin (thick filament). On depolarization of the cell membrane upon contraction, calcium is released from the sarcolemma (SR). The released calcium is bound by troponin C (TnC), which undergoes a conformational change that causes troponin I (TnI) to dissociate from actin. This in turn causes the disinhibition of actinomyosin ATPase allowing ATP hydrolysis and thereby muscle contraction (C). The removal of calcium causes the muscle to relax (B). (Reproduced with permission from reference 41).

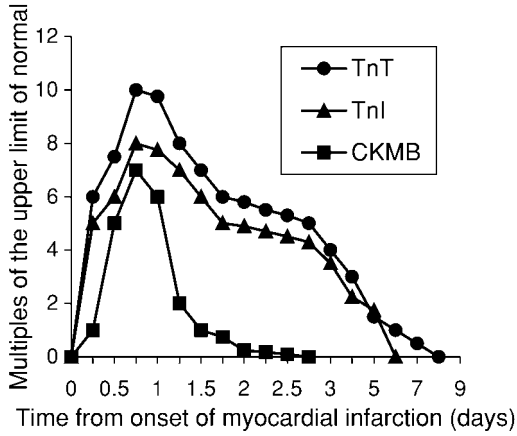

FIG. 4. Typical profile of the release kinetics of TnT, TnI, and CKMB after an acute myocardial infraction. Note that x-axis is nonlinear.

pattern to cTnT [47]. Figure 4 shows the typical release kinetics of the troponins over time after the onset of an acute myocardial infarction. For comparison, the creatine kinase-MB isoform (CK-MB) release pattern is also shown.

3.7. Cardiac Troponin Subunit Release into Serum After Acute Myocardial Infarction

For assay development and calibration, it is important to ascertain the forms of cardiac troponin subunits released into serum following an AMI. The type of subunit released will directly affect the choice of antibodies used in detection immunoassays. The antibodies used will also impact on the sensitivity and cut-off values of the assay. Thus, efforts have been made to characterise the cardiac troponin subunits released after an AMI.

Using Western blot analysis, it has been shown that cTnI occurs predominantly as free subunits, with no evidence of a troponin I-T complex [48]. In contrast, another group using specific monoclonal antibodies has shown that cTnI is released into the blood stream mainly as the troponin I-C complex with less than 10% found in the free form [49]. Wu *et al.* [50] have provided indirect evidence to support the notion that there is little free TnI in blood and that the predominant form of cTnI in blood is the troponin I-C complex.

Following an AMI, free cTnT is released into circulation together with ternary (T-I-C) complexes and other cTnT fragments [50]. The initial release is of free cTnT, with the subsequent release of a mixture of T-I-C complexes and a small quantity of free TnT.

The small amount of TnT and TnI present in circulation makes its detection by Western blotting difficult due to the presence of larger quantities of albumin and immunoglobulins. Using a novel Western blot-direct serum analysis (WB-DSA) procedure, Labugger and coworkers [51] have been able to circumvent this problem and show the forms of cardiac TnT and TnI that appear in the bloodstream of AMI patients over time. Although the authors did not quantify their data, it was clearly evident that there was modification of both the native cardiac TnT and TnI over time. Studies of ischemic-reperfused rat hearts [52] and human post-ischemic myocardium [53, 54] have shown that there are post-translational modifications such as selective degradation, covalent complex formation, and phosphorylation. Labugger and coworkers [51] showed that there are numerous cTnI products released into the bloodstream following AMI. For cTnT, there was one major and two minor products released. It was suggested that these products were generated in the diseased myocardium itself and then subsequently released into circulation upon an infarction [51]. The release of these modified products might therefore correlate to the progression of the underlying pathology and may also be highly specific for certain types of cardiac pathologies, although this remains to be clearly established.

Taken together, these studies suggest that cardiac troponins found in circulation after an AMI show modifications that reflect primary insult on the myocardium, as well as changes arising after the release of troponin in the bloodstream. Whether the presence of certain modified cardiac troponin correlates with a specific cardiovascular pathology, infarct size, and reinfarction or with a specific time post-AMI remains to be shown. One point, however, needs to be emphasized, that is, the design of immunoassays for cardiac biomarkers must reflect the underlying pathology and must also be able to detect the different forms of cardiac troponins present in serum.

3.8. From Structure to Analysis: Implications for Troponin Assay Development

It is clear from the foregoing that the structure of the troponin molecule and the various modifications that occur to it both *in vivo* and *in vitro* will greatly affect its analysis. Robust assays are indeed available, which have addressed some or all of the following issues.

3.8.1. *Cardiac Specificity*

The realization that different isoforms of troponin makes them antigenically different has led to the development of monoclonal antibody assays specific for cardiac TnT and TnI. Because a cardiac-specific form of TnC is lacking, assays have not been developed for it. The demonstration that cTnT

is expressed in regenerating skeletal muscle [55] has cast doubt on the cardio-specificity of cTnT [56] although studies have demonstrated its utility as a specific and sensitive diagnostic marker of AMI [57] and prognostic marker of the acute coronary syndrome [58, 59].

3.8.2. *Ontogenic Expression*

Certain isoforms are expressed in a developmentally regulated manner. In the human skeletal muscle, a fetal isoform is predominantly expressed and fetal cTnT is present at very low levels. The fetal isoforms are transiently expressed and disappear in adulthood. In adulthood, TnT composition of the heart may change due to expression of different isoforms during heart failure [23, 24]. There are 10 known isoforms of troponin T expressed in the mammalian heart, generated by the alternative splicing of the *TNT2* gene. Isoform 6 ($cTnT_3$) is expressed in the healthy adult heart, whereas isoform 7 ($cTnT_4$) is expressed in the failing adult heart [24]. Both TnC and TnI are expressed as single isoforms in the adult heart. The development-related changes in cTnT have lead to discussion about whether there can be reexpression of cTnT isoforms in damaged or regenerating skeletal muscle [55]. Studies of skeletal muscle from patients with regenerating skeletal muscle [60], Duchenne muscular dystrophy [61], and in end-stage renal failure [55] have shown the presence of cTnT, although others have refuted these claims (refer to Section 5.1). Thus, it is vital when designing current assays to take these facts into consideration. For the cTnT assay, it has been reported that the antibody used in the second- and now the third-generation assay is designed such that the cTnT isoforms expressed in renal diseased skeletal muscle will not cause false positive results [62]. However, false positive results continue to be seen in patients with renal failure albeit at a much reduced frequency. This is discussed fully in Section 5.1.

3.8.3. *Effects of Troponin C*

It has been shown that the presence of TnC alters the immunogenicity of cTnI [49]. This effect is calcium-dependent as the addition of EDTA could partially or totally reverse this effect. EDTA reduces the magnitude of the interaction between cardiac TnI and TnC and effectively dissociates these two components from the troponin complex. This is the basis for why EDTA plasma is not used as a sample type for most of the cTnI assay platforms available with the notable exceptions of the Vitros ECi (Ortho–Clinical Diagnostics) and Immulite (Diagnostic Products Corporation) (refer to Section 4.3.1). Also, as shown by Katrukha *et al.* [49], the forms of cTnI found in circulation include complexes with troponin T and C (T-I-C or I-C). These are likely to affect immunoreactivity leading to altered signal generation in cTnI immunoassays.

3.8.4. *Phosphorylation and Redox Effects*

As discussed in the sections above , phosphorylation is part of the regulation of the troponin complex, which results in conformational changes. Studies have demonstrated that the phosphorylation of ser-23 and ser-24 changes the conformation of the TnI molecule and affects the interaction of cTnI with certain monoclonal antibodies [63, 64]. However, at the present time it remains unknown whether the phosphorylated or unphosphorylated form is present in blood.

Human cTnI contains two cysteine residues (Cys-80 and Cys-97). The formation of disulphide bond between the two cysteine residues as a result of oxidation affects its interaction with troponin components and may also interfere with its binding to monoclonal antibodies [65]. Thus, both oxidized and reduced forms of cTnI are present in the bloodstream. Since human cTnT does not contain any cysteine group, a disulphide bond cannot be formed after oxidation.

3.8.5. *Stability to Proteolysis*

Assays that use widely spaced epitopes suffer from sample instability. The current cTnT (Roche Diagnostics Inc.) uses two antibodies that are eight residues apart (refer to Section 4.2). Thus, the cTnT assay appears to be very stable at room temperature, 4 °C, when frozen, after 5 freeze–thaw cycles, and after 5 years of storage [41]. For cTnI assays, most assays have excellent stability with the notable exception of the Access assay (Beckman Coulter) due to the selection of a C-terminal epitope that renders it amenable to degradation [66], although recently improvements have been incorporated [67].

3.8.6. *Choice of Epitopes*

As already alluded to, the choice of epitopes will directly affect the assay specificity and sensitivity. Cardiac TnT and TnI have unique amino acid sequences that differentiate them from their respective skeletal muscle isoforms, thus allowing for the development of cardiac-specific monoclonal antibodies [68, 69]. The current cTnT assay uses antibodies that recognize cardio-specific sequences that are distinct from the fetal TnT isoforms and skeletal isoforms. The antibodies also recognize T-I-C and binary complexes released into serum [50]. The various cTnI assays that are commercially available use different antibodies directed at different epitopes [41]. They have all been shown to be cardiac specific, although information regarding their relative specificity is lacking. Whether the detection of phosphorylated and in the case of cTnI, reduced forms of cardiac troponins offer any advantages in terms of correlation with the severity of an AMI or the size of an infarct remains an interesting hypothesis.

4. Clinical Significance of Measured Cardiac Troponins

4.1. Role in Diagnosis

4.1.1. *Current Diagnostic Criteria and the Implications of the Redefinition of Myocardial Infarction*

MI has traditionally been diagnosed according to the 1971 (revised 1979) World Health Organization (WHO) criteria (refer to Table 1) [70]. Using these criteria, MI is diagnosed by the documentation of two of the following three characteristics: clinical symptoms (e.g., chest pain), increase in cardiac enzyme concentrations, and a typical electrocardiogram (ECG) pattern usually involving the development of Q waves. With the advent of sensitive and specific assays that can detect very small infarcts and improved imaging techniques, there was need to reconsider the definition of MI. The current diagnostic criteria are based on the consensus document of the Joint European Society of Cardiology and the American College of Cardiology (ESC/ACC) [71] (see Table 1). The clinical, electrocardiographic, biochemical, and pathological contexts have been comprehensively reviewed and discussed in the consensus document.

TABLE 1
Diagnostic Criteria of the World Health Organization (WHO) and the Joint European Society of Cardiology/American College of Cardiology (ESC/ACC)

WHO criteria for myocardial infarction
Definite acute myocardial infarction
1. Definite electrocardiograph (ECG) or
2. Symptoms typical or atypical or inadequately described, together with probable ECG or abnormal enzymes
3. Symptoms typical with abnormal enzymes with ischemic or noncodable ECG or ECG not available or
4. Fatal case, whether sudden or not, with naked eye appearance of fresh myocardial infarction, recent coronary occlusion found at necropsy, or both
ESC/ACC criteria for myocardial infarction
Acute, evolving, or recent myocardial infarction
1. Typical rise and gradual fall (cardiac troponin T or I) or more rapid rise and fall (creatine kinase-MB) of biochemical markers of myocardial necrosis with at least one of the following:
a. Ischemic symptoms
b. Development of pathological Q waves on ECG
c. ECG changes indicative of ischemia (ST segment elevation or depression)
d. Coronary artery intervention (e.g., coronary angioplasty)
2. Pathological findings of an acute myocardial infarction
Established myocardial infarction
1. Development of new pathological Q waves on serial ECGs or
2. Pathological findings of a healed or healing myocardial infarction

What are the implications of the redefinition of MI? First, a substantially increased proportion of patients with the ACS will be classified as having had an MI. A central theme in the consensus document is that any amount of myocardial necrosis caused by ischemia should be labeled as an infarct, although the document does make it clear that the term MI should not be used without "further qualification." Such qualifications include infarct size, context of the infarct (spontaneous or after a revascularization procedure), and time of infarct (whether evolving, healing of healed). Thus, a patient previously diagnosed as having unstable angina might now be diagnosed as having had a small MI. This will have a profound effect on the health services around the world in terms of increased management and treatment costs. The increased sensitivity offered by the new definition might identify more cases, but in the long term it could be argued that the costs to society might be lower if it means that more patients are identified early and appropriate secondary prevention measures are instituted sooner. Second, because all elevated levels of cardiac troponins have been shown to have an adverse outcome (Section 3.2), the redefinition of any amount of myocardial necrosis as an MI might allow more intensive long-term patient management that might not have been previously considered necessary. Thus, an increased diagnosis rate might, in the long term, mean better prognosis for individual patients. Third, the increased specificity of the diagnostic criteria will result in the significant elimination of false-positives, thereby leading to reduced costs and wasted resources for both hospitals and primary care physicians. It goes unsaid that the cost implications might be considered high at the outset but the true cost, especially in the long run, will be more economical. Fourth, the new definition of MI will alter the recorded prevalence of the disease and might confuse efforts to follow trends in disease rates and outcomes. One way around this is to retain the established definition of MI and to continue the measurement of previous markers (CK and CK-MB in this case). This will allow continuity of data and comparisons to be made with cardiac troponins. Fifth, clinical trials relying on MI as an entry criterion or as an end point will find that the modification of the definition of MI might impact on patient selection and the conclusions obtained from the trial. It will also impact on the findings of meta-analyses undertaken. Thus, there is a need to maintain consistency at the present time until a clear precedence is set. Sixth, for the individual patient the label MI carries important implications in terms of obtaining a life insurance policy, making disability claims, acquiring a driving or piloting license, and a patient's profession might also be adversely affected. The implication upon the psychological status of the patient, as well as life-style changes that may have to be implemented after diagnosis must also be considered. Finally, to the society at large the implications include: changes in the mortality statistics, political leveraging whereby politicians might argue for changes in taxation to cover the perceived

increased health-care costs, increase in the number and duration of sick leave, and diagnosis-related grouping (DRG). It must also be borne in mind that the new definition will affect the health policies of the country and alter the preparation of clinical guidelines.

Thus, efforts have been made to monitor the situation and the following sections highlight how the use of cardiac troponins has improved the ability to diagnose the ACS and MI.

4.1.2. *The Acute Coronary Syndrome*

The acute coronary syndrome (ACS) is a term that encompasses a spectrum of clinical manifestations, resulting from a common pathophysiological mechanism (Fig. 5). From early life, lipid-rich deposits containing macrophages and T lymphocytes are laid as plaques in the coronary artery (fatty streaks). With increasing age, the lesions continue to enlarge and form a fibrous plaque that contains smooth muscle cells and may even start becoming highly vascularized. It is important to note that the inflammatory process is also an integral part of atherogenesis. Ross [72] and Lusis [73] have written an excellent account of the pathophysiological mechanism of atherogenesis.

The rupture or erosion of the atheromatous coronary plaque results in the formation of a thrombus, which may partially or completely obstruct the coronary artery. The clinical manifestation is dependent on the rupture of a

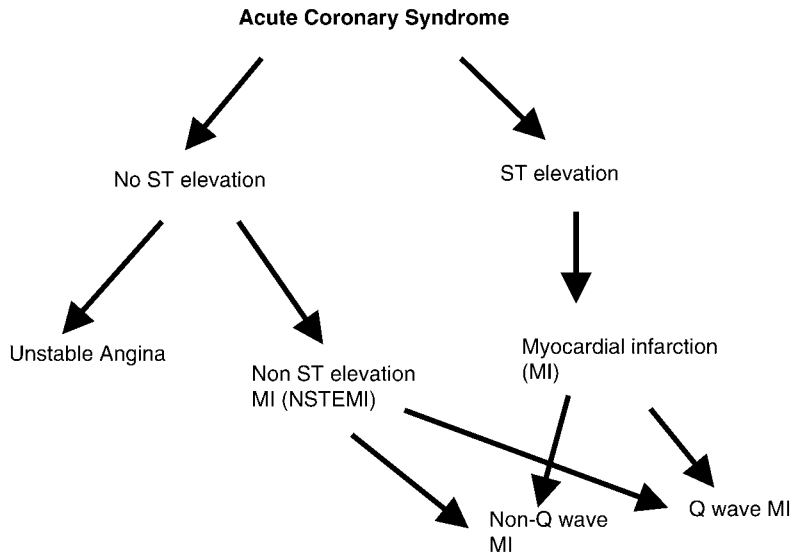

FIG. 5. The spectrum of ACS.

plaque, the resultant intraluminal thrombosis, which may cause reduced blood perfusion that usually leads to myocardial ischemia and then finally to overt myocardial necrosis [74].

Thus, the spectrum ranges from unstable angina, which is associated with reversible myocardial ischemia where patients might usually be asymptomatic to ACS with variable degrees of myocardial necrosis to frank myocardial infarction with large areas of necrosis causing left ventricular dysfunctions.

Chronic stable angina is usually caused by stenosis due to plaque deposits in the coronary arteries; the plaques are usually nonthrombotic and stable. Clinical symptoms usually occur upon exertion when the oxygen demand by the heart cannot be adequately met by the coronary blood flow; transient episodes of chest pain usually subside with rest.

Unstable angina is a heterogeneous syndrome with variable symptoms and prognosis and is caused by an unstable plaque, which causes platelet aggregation and the activation of the coagulation pathways. This results in the formation of a platelet-rich thrombus (the so-called "white thrombus") [75]. It is the local thrombosis that results in flow-limiting stenosis that causes the ischemia-associated symptoms and the characteristic non-Q wave myocardial infarction (Fig. 5). The rupturing of the plaque and the resultant extensive thrombosis, which is rich in fibrin and erythrocytes ("red thrombus"), may cause full occlusion of the coronary artery and this presents as a Q wave myocardial infarction (Fig. 5). If the myocardial cells do not receive oxygen within 10–15 min, irreversible cell necrosis occurs. Hamm and Braunwald [76] have published the classification of unstable angina previously.

4.1.3. *Myocardial Infarction*

A myocardial infarction (MI) is defined as the necrosis of cardiac myocytes caused by prolonged ischemia due to perfusion insufficiency. It is usually identified from a history of ischemia-related symptomatology (chest, epigastric, arm, wrist, or jaw discomfort/pain at rest or on exertion) and typical ECG changes. For a specific review on AMI, refer to Boersma *et al.* [77].

The cardiac troponins have been demonstrated to be the best markers for the definitive detection of MI and ACS with better sensitivity and specificity than CK or CK-MB [78, 79, 80, 81, 82, 83]. The troponins are now considered the gold standard marker of myocardial necrosis [84]. It has been previously estimated that of 889 patients in one study who met the criteria for AMI, 2.1% were mistakenly discharged from the emergency department while among 966 patients with unstable angina, 2.3% had missed diagnoses [85]. Such cases account for the largest source of successful malpractice lawsuits in emergency medicine [86]. In addition, it has been suggested that half of all MI deaths occur within the first hour of the onset of symptoms [87]. Thus, it is imperative that an accurate diagnosis is reached early so that correct patient management can be instigated.

The biochemical criteria for detecting myocardial necrosis, according to the consensus document [71], are: (1) maximal concentration of cardiac TnT and TnI exceeding the 99th percentile of a reference control group on at least one occasion during the first 24 h; (2) maximal value of CK-MB (preferably mass measurements) exceeding the 99th percentile of the value for a reference control group on two successive samples, or maximal value exceeding twice the upper limit of normal for the specific laboratory on one occasion during the first hours after the index clinical event. It is suggested that values for CK-MB should rise and fall; values that remain elevated without change are usually never due to MI. Furthermore, the ESC/ACC committee recommended that total imprecision (coefficient of variation, CV) at the cut-off level should be $<10\%$. The impact of this imprecision and the 99th percentile reference limit are discussed in Section 4.6.3.

It is worth mentioning that there is need to detect ischemia before the onset of necrosis. It is the products of myocardial necrosis in blood, that is, troponins, CK-MB, and myoglobin that clinically define an AMI, but whether these markers have the sensitivity to detect ischemia is another question. The early detection of coronary ischemia in unselected patients with chest pain remains a challenge for cardiologists, emergency department physicians, medical and clinical biochemists. There is need to detect ischemia before it progresses to necrosis because these subsets of patients have a poor prognosis [88]. Early detection of these patients would afford the opportunity to intervene early with improved mortality (refer to Jesse and Kukreja [89]).

Regarding reinfarction, CK-MB due to its rapid rate of rise and fall is used [90, 91, 92], although it has been shown that cTnT with its biphasic release pattern has equal, if not better, clinical utility [83, 93]. Myoglobin, which has the shortest half-life in circulation, has been suggested to be the best marker of a reinfarction [94, 95], although others have refuted this claim [82, 96]. There have been studies that have described the increase of troponin concentration following reinfarction [4, 97, 98]. In one very recent study, nine MI patients who had had an in-hospital reinfarction had the changes in the patterns of released cTnI and CK-MB mass analyzed [98]. An increase in cTnI concentration was detected despite an existing elevation of cTnI from a previous infarction. Overall, the profiles for the cTnI and CK-MB in these patients paralleled each other. This study, together with previous ones, lends support to the use of cardiac troponins in the detection of reinfarction. It concluded that given limited financial resources in laboratories and healthcare, it would be more cost-effective if clinicians and laboratorians considered monitoring just cardiac troponins for the diagnosis of both MI and reinfarction [98]. However, larger prospective studies are required to replicate these preliminary findings, both for cardiac TnT and TnI. Taken together, there is sufficient support for using cardiac troponins (instead of CK-MB) to detect reinfarction.

For the present time, there is an increasing body of evidence and a growing consensus that the cardiac troponins due to their sensitivity and specificity are the markers of choice for the diagnosis of ACS.

4.2. Role in Prognosis and Risk Stratification

The 1990s should have been called the decade of cardiac troponins. Whereas the early 1990s saw the emergence of the cardiac troponins as a diagnostic marker of MI, in the mid- and late-1990s, it was convincingly demonstrated in numerous studies that even minor elevations of the cardiac troponins carried poor short- and long-term prognoses. The first study to demonstrate the risk stratification potential of cardiac troponin was conducted in 1992 by Hamm *et al.* [99]. They showed that cTnT was a more sensitive marker of myocardial injury than CK-MB and was a useful prognostic indicator in patients with unstable angina. In that study, cTnT was detectable at presentation in the serum of 33 of 84 (39%) patients with acute angina at rest. Among the 33 patients, 10 (30%) subsequently died or suffered a cardiac event during hospitalization compared to only 1 who died (2%) among those with negative cTnT at presentation. This study concluded that cardiac risk during hospitalization could be estimated by measuring cTnT soon after admission to guide management. Apart from being a small study with 109 patients and mostly male (80 out of 109), this study laid the foundation for the next wave of research in the clinical utility of cardiac troponins. Other similar studies by Wu [100] and Ravkilde [101] also showed the prognostic value of cTnT in unstable angina and AMI respectively. However, these studies had one major limitation in that they were all relatively small.

In 1996, Ohman [102] and Antman [103] published landmark studies in the same issue of the New England Journal of Medicine that also had an editorial by Van de Werf [104]. Both the studies were well powered and convincingly demonstrated that cardiac TnT and TnI respectively could predict the risk of mortality in patients with ACS or AMI.

In the prospective study by Ohman *et al.* [102], (which was a sub-study of the Global Use of Strategies to Open Occluded Coronary Arteries in Acute Coronary Syndromes, GUSTO-IIa), cTnT (Roche first generation) levels above 0.1 μg/liter, measured as soon as possible after admission (median 3.5 h; interquartile range, 2.3–6.3), was shown to be associated with significantly higher mortality within 30 days in patients with AMI. When in combination with CK-MB and electrocardiography, it allowed for further risk stratification. This study verified the findings of previous observations in smaller studies, it showed that the use of a single blood sample obtained early could be used for the stratification of risk, and importantly it identified a lower threshold for increased risk. Previous studies had used a cut-off of

0.2 μg/liter for cTnT (e.g., [99]). It also showed that even among patients without CK-MB elevations, increased cTnT concentrations were associated with higher mortality, increased rates of cardiogenic shock, and congestive heart failure. The main limitations of this otherwise excellent study were: the study was a highly selected, high-risk population with acute ischemic syndromes among whom 72% had diagnosed MI. Also, patients with suspected renal failure as defined by a serum creatinine >25 mg/liter (>221 μmol/liter) had been excluded. The latter may have, therefore, increased the prognostic value of cTnT in the study. The study also used the only commercially available cTnT assay in use at the time, which was the first generation assay whose detection antibody had a 12% rate of cross-reactivity with skeletal-muscle TnT (refer to Section 4.2) [3].

The retrospective study by Antman *et al.* [103] (data was obtained from the Thrombolysis in Myocardial Ischemia Phase IIIB, TIMI IIIB) showed that in patients with the ACS, cTnI (Stratus II fluorometric enzyme immunoassay, Dade) provided prognostic information that could permit the early identification of patients with increased risk of death. The mortality rate at 42 days was found to be significantly higher in the 573 patients with cTnI levels of >0.4 μg/liter (21 deaths, or 3.7%) compared to the 831 patients with levels <0.4 μg/liter (8 deaths, or 1.0%; $p < 0.001$). A statistically significant increase in mortality was noted with increasing levels of cTnI ($p < 0.001$). However, the prognostic value of cTnI was greater among patients who presented themselves more than 6 h after the onset of chest discomfort compared to the entire study population ($\chi^2 = 5.47$, $p = 0.02$ versus $\chi^2 = 4.73$, $p = 0.03$). In addition, the prognostic potential of cTnI persisted even after adjustment for independent variables, such as an age of 65 years or older and ST-segment depression on ECG, that are known to be significantly associated with an increased risk of cardiac events. Importantly, it was also found that patients with a CK-MB concentration in the normal range but with elevated cTnI levels had an increased risk of mortality. Although a discussion of CK and CK-MB is beyond the scope of this review, it will suffice to note in passing that CK-MB has been shown to have a reduced sensitivity and specificity in detecting MI and certainly micro-infarctions [90, 105, 106]. The major limitations of this study were: no information was provided regarding the inclusion or exclusion of patients with renal insufficiency, although as discussed in Section 5.1 below, false-positivity with renal failure is less of a problem with cTnI assays. Furthermore, the authors did not make it clear in their paper what the relative risk ratio for mortality at 42 days in patients with both an elevated cTnI and CK-MB was. Only data for all patients and patients with no increased CK-MB levels but elevated cTnI was provided.

In another study by Lindahl *et al.* [107] (also known as the Fragmin during Instability in Coronary Artery Disease, FRISC), a relationship between cTnT levels and long-term clinical outcome was found in patients with unstable angina or non-Q wave MI. In that study, the risk of death or myocardial infarction after 5 months of follow-up increased significantly with increasing cTnT levels measured within the first 24 h. This study suggested that the early measurement of cardiac troponins (TnT in their case) could identify high-risk patients who would benefit from antithrombotic treatment (low molecular weight heparin in this case).

Many investigators have duplicated these studies for both cardiac TnT and TnI in different settings and circumstances. One meta-analysis that included 2847 unstable angina patients, with a median follow-up duration of 30 days, showed that a positive cTnT was associated with a cumulative odds ratio (OR) of 2.7 (95% confidence interval, CI = 2.1–3.4, $\chi^2 = 66$) for the risk of AMI and death, whereas for positive cTnI results the cumulative OR was found to be 4.2 (95% CI = 2.7–6.4, $\chi^2 = 42$) [108]. Another meta-analysis by Heidenreich *et al.* [109], also concluded that in patients with non-ST elevation ACS, the short-term odds of death are increased three- to eightfold for patients with an abnormal cardiac troponin level. In this meta-analysis, it was reported that there was no significant difference between the overall OR for mortality with cTnT (5.2, CI = 3.1–8.5) and TnI (3.9, CI = 2.3–6.6; $p = 0.8$), both having equal prognostic capability.

It has been suggested that physicians do not regard patients with marginally elevated cardiac troponins as being at any risk or indeed different from those with no cardiac troponin elevation [110]. A recent study by Henrikson *et al.* [111] has prospectively demonstrated the prognostic utility of a marginal elevation of cTnT. In this study, after adjusting for possible confounders, a marginal elevation of cTnT was significantly associated with an increased rate of death/MI/revascularization compared with the undetectable cTnT group. Marginal elevation in the study was defined as the range between 0.01 (the cTnT assay detection limit and 99th percentile reference limit) and 0.09 μg/liter (the ROC level for overt MI). In this study, patients with marginal cTnT elevations referred for further testing were no more likely to have a positive test compared to those with undetectable troponin levels, yet they were more likely to have adverse outcomes. Thus, the mechanism of troponin release in this group with marginal TnT elevation merits further consideration. This leads one to ask the pertinent question: ''Which level should be used?'' This will be a subject dealt with in Section 4.6.3.

Overall, some conclusions can be drawn from the foregoing sections. First, the cardiac troponins have been shown to have both diagnostic and prognostic utility. An elevated cardiac troponin level is predictive of a higher risk of adverse cardiac events, both during admission and follow-up. Second, it is

worth emphasizing that patients who rule out for AMI but who have an increased cardiac troponin level have an equally poor outcome. Third, time of sampling is important for risk stratification. Although measurements at admission provide the most information, levels collected at 8–16 h show a significant improvement in the ability to stratify risk [112]. Fourth, the cardiac troponins are more sensitive than CK-MB in the detection of minor myocardial damage. Fifth, whether the use of cardiac troponins results in more cost-effective strategies of treatment and better long-term outcomes remains to be shown. Depending on physician gestalt and the clinical biochemist's acumen it is possible to implement the usage of cardiac troponins in the most cost-effective and clinically useful manner.

4.3. Role in Guidance of Therapy and Interventions

Having demonstrated that the determination of cardiac troponins provides an important prognostic tool for risk stratification, it stands to reason that treatments and intervention decisions should be based on some measure of efficacy, such as a cardiac troponin level. The translation of increased troponin levels to increased risk has been simultaneous with the development of new treatments. Because these drugs can be relatively expensive and cost-benefit analyses are therefore difficult to undertake, the identification of high-risk patients with biochemical markers is of great advantage. Testing for cardiac troponins have been shown to be appropriate for triage and decision making in regards to hospital admission and interventional procedures, such as percutaneous coronary intervention (PCI) [113]. The use of biochemical markers not only for diagnosis and prognostication but also clinical management and guidance of therapy "is an idea whose time has come" [114].

In the FRISC trial of Dalteparin (low molecular weight heparin), unstable angina patients were classified into low- or high-risk groups on the basis of cTnT levels [115]. In that study it was found that patients with high cTnT levels had a higher incidence of cardiovascular events compared to those in the low-risk group. Similar results have now been found for placebo-controlled trials using platelet glycoprotein IIb/IIIa receptor inhibitors (PGRI). These agents inhibit conformational changes in the glycoprotein IIb/IIIa receptor, which is the final step in platelet activation thereby preventing thrombosis from occurring. The first trial demonstrating that cTnT was effective in predicting which patients would benefit from PGRI therapy was the c7E3 Fab Antiplatelet Therapy in Unstable Refractory Angina (CAPTURE) trial, which used Abciximab [116]. Thereafter, two other trials namely Platelet Receptor Inhibition in Ischemic Syndrome Management (PRISM) [117] and Platelet IIb/IIIa Antagonism for the Reduction of Acute Coronary Syndrome events in a Global Organization Network (PARAGON B) [118] have

demonstrated the utility of cardiac TnT and TnI using other formulations for PGRI. In contrast, the GUSTO IV trial of 7800 patients failed to show benefit of a PGRI in patients who were cardiac troponin positive or had ST depression on ECG [119]. However, this trial included patients with a relatively lower risk profile that included only 5 min of chest pain together with either ST changes or a positive cardiac troponin level.

A recent meta-analysis of all major randomized clinical trials showed that the PGRI reduces the occurrence of death or MI in patients with ACS not routinely scheduled for early revascularization [120]. Overall, the use of the glycoprotein inhibitors shows a reduced risk of death or MI (OR = 0.91, CI = 0.85–0.98) [121]. Table 2 summarizes all the major trials that have relied on cardiac troponin for selecting patients to receive treatment. These compelling data have prompted the inclusion of cardiac troponin measurements into guidelines for the identification and clinical management of high-risk unstable angina [122] and MI patients [113].

Regarding interventions, Morrow *et al.* [123] have shown that even minor elevations of cardiac TnT and TnI identify high-risk patients with unstable angina and non-ST elevation myocardial infarction (NSTEMI) who can benefit from an early invasive strategy as opposed to a conservative strategy.

Perioperative MI is the most common cause of morbidity and mortality in patients who have had noncardiac surgery and ranges from 36% to 70% [124]. Distinguishing cardiac injury due to the surgery itself from damage caused by MI is very difficult. A study by Adams *et al.* [124] demonstrated that the measurement of cTnI was a sensitive and specific marker for the diagnosis of perioperative MI. Another study by Simeone *et al.* [125] divided 43 open-heart surgery patients into three groups based on their cTnT concentrations immediately post-surgery (<1, 1–3, and >3 μg/liter). Six months later an exercise stress test was conducted, and it was observed that the patients with the intermediate and highest troponin levels had a higher

TABLE 2
THIRTY DAY INCIDENCE OF DEATH AND ACUTE MYOCARDIAL INFARCTION (% OF TOTAL) IN CLINICAL TRIALS OF LOW-MOLECULAR WEIGHT HEPARIN AND PLATELET GLYCOPROTEIN IIb/IIIa RECEPTOR INHIBITOR USED IN PATIENTS DEPENDENT ON CARDIAC TROPONIN LEVELS

Trial	Agent used	Cardiac marker used	Placebo (%)	Agent (%)
FRISC [115]	Dalteparin	cTnT	14.2	7.4
CAPTURE [116]	Abciximab	cTnT	19.6	5.8
PRISM [117]	Tirofiban	cTnT and cTnI	13.0	4.3
PARAGON-B [118]	Lamifiban	cTnT	19.0	11.4

cTnT = cardiac troponin T; cTnI = cardiac troponin I.

incidence of ischemia. A very recent study has investigated whether cTnI concentrations can predict extended hospital stay after coronary artery bypass graft surgery (CABG) [126]. In 300 patients having routine CABG surgery, they compared cTnI concentrations 6 and 24-h post-surgery with ECG findings as predictors of an extended post-operative hospital stay or in the intensive care unit (ICU). They discovered that the use of a single 24-h cTnI value could identify patients at greater risk of extended ICU and hospital stays. A study by Baggish *et al.* [127] showed that post-operative cTnT could predict prolonged ICU stay following cardiac surgery. Lehrke *et al.* [128] have recently shown that a single post-operative cTnT measurement can be used to estimate long-term survival in patients after an elective open-heart surgery. Rates of cardiac mortality, all-cause mortality, and nonfatal acute myocardial infarction (AMI) at 28 months were 6.9%, 8.8%, and 6.8% respectively. ROC curve analysis revealed that a cTnT >0.46 μg/liter at 48 h was the optimum discriminator for long-term cardiac mortality. Another study by Kathiresan *et al.* [129] also demonstrated that elevated cTnT after coronary artery bypass grafting is associated with increased one-year mortality. Chance *et al.* [130] have provided evidence that cTnT could serve as a marker of acute cardiac allograft rejection making biopsy unnecessary. Harris *et al.* [131] have compared cardiac TnT, TnI, and CK-MB for the detection of minor myocardial damage during interventional cardiac procedures, such as percutaneous transluminal coronary angioplasty, stenting, and rotational atherectomy and found that cTnI was the most sensitive indicator. Others have failed to corroborate these findings [132, 133]. However, this could be because of the small sample size and/or short follow-up duration in the latter studies.

In conclusion, the cardiac troponins have shown clinical utility in selecting patients for early treatment, whether therapeutic or interventional. In addition, the cardiac troponins have vital roles to play pre-, peri- and post-operatively in terms of guidance in the choice of a procedure or determining the outcome and success of an intervention.

4.4. Role in Other Conditions

The cardiac troponins have such sensitivity; it is not surprising that they have been detected in many other cardiac diseases. Table 3 lists other conditions in which they may be present. Whether these are "false-positives" is discussed in Section 5.10. Missov *et al.* [134] have shown elevated levels of both cardiac TnT and TnI in patients with congestive heart failure (CHF). Setsuta *et al.* [135] studied 58 patients with CHF and found using the cut-off level of 0.05 μg/liter that the 12-month cardiac event rate was 66% for patients with an abnormal cTnT level versus 15% for those with a normal

TABLE 3
ELEVATED TROPONIN DUE TO CAUSES OTHER THAN ACUTE CORONARY SYNDROME OR MYOCARDIAL INFARCTION (ANALYTICAL CAUSES NOT LISTED)

- Renal failure
- Sepsis
- Congestive heart failure, dilated cardiomyopathy
- Hypertension
- Cardiac surgery, percutaneous coronary intervention
- Tachyarrhythmia
- Drug toxicity: Adriamycin, 5-fluorouracil, herceptin, and so on
- Hypothyroidism
- Pulmonary embolism
- Infiltrative diseases of the myocardium: Amyloidosis and sarcoidosis
- Trauma: Cardioversion, defibrillators, pacing, contusion
- Coronary vasospasm
- Transient ischemic attack, stroke, or subarachnoid hemorrhage
- Pheochromocytoma
- Rhabdomyolysis with myocyte necrosis
- Inflammatory diseases: Myopericarditis, rheumatic fever, rheumatoid arthritis, systemic vasculitis
- Critically ill patients especially with diabetes
- High dose chemotherapy
- Severe burns
- Atrial septal defects

level (OR = 9.2, CI = 2.4–35). Together with the B-type natriuretic peptide (BNP), it is anticipated that the cardiac troponins will provide cardiologists with a formidable panel of markers for the diagnosis and prognosis of CHF [136].

In patients with acute myocarditis, 53% of patients had elevated cTnT levels [137] and 49% of patients with acute pericarditis had elevated levels of cTnI [138]. The cardiac troponins have also been found to be specific for excluding contusions and blunt traumas [139, 140].

An elevated cardiac troponin is not observed after electrical cardioversion of atrial fibrillation or flutter [141]. One study has shown that cTnT has a role in enabling early discharge from a district general hospital [142]. Another study has shown that elevated levels of cTnI was a frequently unrecognised complication in critically ill patients [143] and is increasingly being documented in patients with sepsis [144] and septic shock [145].

In conclusion, the pathophysiological mechanisms and prognostic significance, if any, of the elevated cardiac troponins in these other conditions remain poorly understood. Further work is required to decipher the reasons for this increase. To use the words of Jaffe *et al.*, "Why we don't know the answer may be more important than the specific question" [146].

5. Measurement of Cardiac Troponins

In response to the endorsement of the use of cardiac troponins by the ESC/ACC for the detection of MI in ACS [71], there was a huge increase in cardiac troponin testing both internationally and in the United States. According to one survey, between 1995 and 1999, the use of cTnI assays increased from 3 to 54 laboratories internationally and from 5 to 57 laboratories in the United States. The use of cTnT increased from 2 to 5 laboratories in the United States and from 6 to 28 laboratories internationally. The survey had a response rate of 5% internationally, 25% in the United States and 100% from telephone surveys in Minneapolis-St. Paul, MN, US [147].

The consensus document of the ESC/ACC has redefined an MI as having occurred when the maximum concentration of either troponin T or I exceeds the 99th percentile of a reference group on at least one occasion during the first 24 h after the index event [71]. The 99th percentile reference limit MI diagnosis was driven by the demonstration that any amount of detectable cardiac troponin release is associated with an increased risk of adverse cardiac events. In view of this, it has been necessary to establish the 99th percentiles and the total 10% imprecision (CV) for the main cardiac troponin assays available commercially. Although this information might be available in most product inserts, several assays have not been evaluated and published in peer-reviewed literature. It has therefore been necessary to objectively evaluate this independent of the manufacturers' claims.

Apple *et al.* [148] have recently determined the 99th percentile reference limits for most of the widely used commercial assays for cardiac troponins. This is the first study to use a large common reference population to evaluate all the leading *in vitro* cardiac troponin assays. Despite its merits, this study has one major limitation. The authors failed to use the manufacturer's recommended sample type for TnT, which is serum. In this study, heparin-plasma was used despite reports of the decreased concentration of troponin T and I in heparinized samples [149, 150]. Troponins bind to heparin, which decreases their immunoreactivity especially in the early phases of myocardial injury. Due to this heparin-induced decrease, the 99th percentiles reported by this study might have been underestimated. Fortunately, Apple and Murakami [151] have more recently repeated the above study using serum and found that the 99th percentile reference limits were similar to that previously reported. It is difficult to reconcile why a significant difference was not observed between serum and heparin-plasma, as reported previously by Gerhardt *et al.* [149] and Steigler *et al.* [150].

Similarly, Panteghini *et al.* [152] have determined the 10% total imprecision for most of the cardiac troponin assays. It is important to note that at present no cardiac troponin is able to achieve a precision of 10% (total CV) at the recommended 99th percentile reference limit (Fig. 6). For some assays,

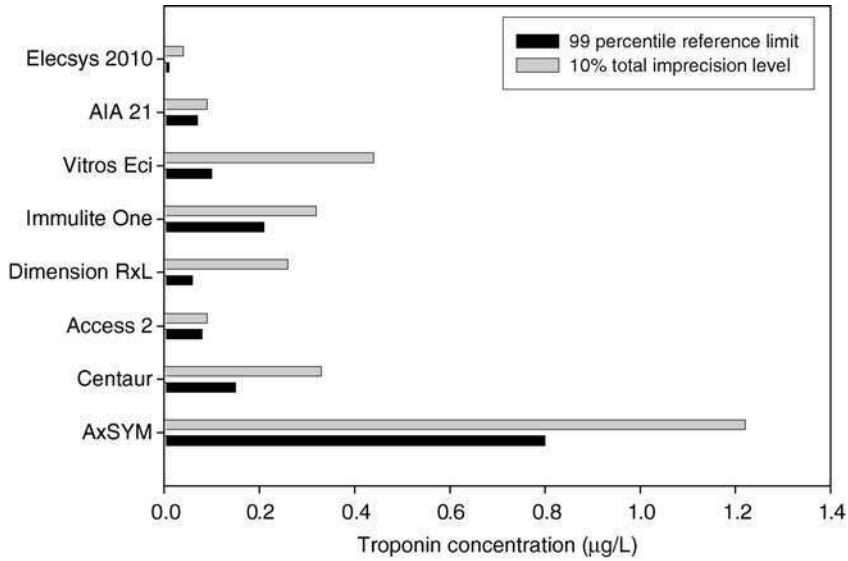

FIG. 6. Lack of concordance between 99th percentile limits and the concentration with a 10% total imprecision. The 99th percentile levels are based on the findings by Apple *et al*. [148] and the 10% total imprecision levels are obtained from the findings of Panteghini *et al*. [152].

for example the Dimension RxL, the 10% total CV (0.26 μg/liter) was approximately fourfold greater than the 99th percentile level (0.06 μg/liter). This disparity between the level at which a total CV of 10% is achievable and the recommended 99th percentile reference limit might cast into doubt the applicability of the new definition for MI [71]. It has been suggested that in the meantime, while manufacturers try to improve their respective assays, the value with a total imprecision of $<10\%$ should be used [153]. The use of this value will raise the cut-off values for assays with a poor precision, especially at the lower end, but decrease the values for those with good analytic precision.

This section will briefly describe the cardiac troponin assays available and pertinent issues in their analyses. However, to start with it is important to reflect on what constitutes an ideal cardiac biomarker.

5.1. THE IDEAL CARDIAC BIOMARKER

The Committee on Standardization of Markers of Cardiac Damage of the International Federation of Clinical Chemistry (IFCC) has published guidelines [154] for use by manufacturers of commercial assays and by clinical laboratories performing cardiac troponin analyses, addressing the

TABLE 4
THE IDEAL CARDIAC BIOMARKER AND QUALITY SPECIFICATIONS FOR CARDIAC TROPONINS

1. *Specificity*: In as much as is possible, the marker should have absolute cardio-specificity without cross-reactivity with other tissues
2. *Sensitivity*: The marker should be able to detect very minute quantities of the marker released into serum from the damaged myocardium
3. *Analytical precision*: A total imprecision (CV) of $\leq$10% at the 99th percentile reference limit has been recommended
4. *Stability*: The antibodies used should recognize epitopes located on the stable part of the molecule and must not be affected by *in vivo* and *in vitro* modifications. This implies stability during collection and storage at 20, 4, -20, and -70°C.
5. *Calibrator commutability*: The calibrator used must resemble the natural and major form of the biomarker present in blood after release from the cardiocytes
6. *Nonparallelism*: The dilution curves for equal concentrations of troponin in serum should be superimposable irrespective of the specimen's origin
7. *Structural determinants*: The size and cellular locality of the biomarker should be known. Smaller or cytoplasmic (as opposed to structural) biomarkers are released faster from ischemic tissues
8. *Biomarker kinetics*: The release from the injured tissue should be in direct proportion to the size of the infarct and should be cleared rapidly to allow the diagnosis of reinfarctions
9. *Adequate diagnostic window*: The biomarker should persist in circulation for an adequate period of time to allow for a suitable diagnostic window
10. *Versatility*: The biomarker should permit the diagnosis of infarction, reinfarction, and provide information on prognosis
11. *The absence of interference*: From autoantibodies, heterophilic antibodies, and so on

quality specifications that should be implemented in order to guarantee a satisfactory performance for clinical applications. In essence, the specifications recommended encompass the attributes of an ideal cardiac marker. However, as noted by others, a single marker that satisfies all these characteristics does not yet exist [87]. Table 4 lists the characteristics of an ideal cardiac biomarker.

5.2. CARDIAC TROPONIN T

At present, there is only one commercially available cTnT assay manufactured by Roche Diagnostics (Boehringer-Mannheim developed the assay and was later bought out by Roche Diagnostics). The first-generation cTnT assay developed in 1989 used a polyclonal capture antibody, conjugated to biotin and a detection antibody (1B10), conjugated to horseradish peroxidase [3]. This was an immunometric, one step sandwich immunoassay where the cardio-specific polyclonal antibodies were immobilized on polyvinyl chloride test tubes. A modification of the assay was later developed using a monoclonal capture antibody (M7) [155]. This assay suffered from two major

problems. First, the detection antibody cross-reacted with skeletal muscle TnT. Apparently in rhabdomyolysis there is adherence of skeletal TnT to the test tube wall, which was incompletely removed by the washing step and therefore detected by the assay. Second, the length of the assay was too long for clinical use (the initial ES300 system used was a batch analyzer with a 90-min cycle) [41].

To resolve these problems, a second-generation assay was developed with cardio-specific antibodies. The M7 capture antibody labeled with ruthenium was now used for detection and a new capture antibody (M11.7) was developed, which was biotinylated [156]. This assay was completely cardio-specific and the assay turnaround time (TAT) was reduced to 45 min, although it was still a batch assay. This assay has subsequently been transferred to a random access analyzer using electrochemiluminescence immunoassay (ECLIA) technology (Elecsys 1010 and 2010), providing a 9- or 18-min TAT respectively, with improved sensitivity [157].

Currently, there is a third-generation assay on the market that has been standardized using recombinant human cTnT to improve assay linearity, although the characteristics are similar to the latest second-generation assay [158]. The detection limit is 0.01 μg/liter, the 99th percentile reference limit is 0.01 μg/liter [148], the value with a total CV of 10% is 0.04 μg/liter [152], although the manufacturers state 0.03 μg/liter [159] and the assay has a range of 0.01–25.00 μg/liter (lower detection limit and maximum of the master curve) [159]. A fourth-generation assay has recently been developed, which incorporates a heparin blocking agent and protease inhibitors. This assay therefore allows the use of heparin plasma as a sample type.

5.3. Cardiac Troponin I

5.3.1. *Assay Platforms*

There are numerous cTnI assay platforms available commercially for clinical use. All utilize antibodies that recognize different epitopes of the same molecule. They also recognize troponin as different complexes in serum (e.g., T-I-C or C-I) [160, 161, 162, 163]. Thus, the absolute value of one assay is not necessarily equivalent to that of another and therefore the different assay platforms have different reference ranges. Table 5 lists details of some of the commonly used cTnI assay formats. It must be appreciated that the 99th percentile limits, the level with a 10% CV and the ROC cut-off stated, have been extracted from the most current peer-reviewed literature and may or may not be in concordance with the manufacturers' latest recommendation. It is crucial that physicians are aware of reference ranges of the assays in use in their local institutions. The local institutional laboratories will have this information.

TABLE 5
COMMON CARDIAC TROPONIN I (cTnI) ASSAY PLATFORMS AVAILABLE FOR CLINICAL USAGE

Platform	Manufacturer	99th percentile limit (μg/liter)[a]	10% total CV concentration (μg/liter)[a]	ROC cut-off for AMI (μg/liter) – based on serum[a]	Method	Sample type
AxSYM	Abbott Diagnostics	0.80	1.22	2.0	Microparticle enzyme immunoassay (MEIA)	S, HP
ACS:180	Bayer Diagnostics	0.1*	0.37	1.0	2-site chemiluminescence immunoassay. Immuno 1 is a heterogeneous sandwich magnetic separation assay	S, HP
Centaur		0.15	0.33	1.0		
Immuno 1		0.1	0.34	0.9		
Access	Beckman Coulter, Inc	0.04*	0.06	0.15	Enzyme immunoassay	S, EP
Access 2		0.08	0.09	0.5		S, HP
Dimension RxL	Dade Behring, Inc	0.06	0.26	1.5	One step enzyme immunoassay, two-site immunoassay, solid radial partition immunoassay, and two-site immunoassay/ immunofluorescence	S, HP
Opus		0.1*	0.90	1.5		S, HP

Stratus CS		0.07*	0.10	1.5		S, HP, WB
Stratus II		ND	ND	1.5		S, HP
Immulite One	Diagnostic Products Corporation	0.21	0.32	1.0	Solid-phase, two-site chemiluminescence enzyme immunoassay	S, HP, EP
Alpha Dx	First Medical Inc	0.15*	0.3*	0.4	Sandwich immunometric assay	S, HP, WB
AIA 21	Tosoh Corp	0.07	0.13[b]	1.4	Two-site enzyme immunoassay	S, HP
Vitros ECi	Ortho Clinical Diagnostics	0.10	0.44	1.0	Immunometric, luminescence	S, HP, EP
Aio!	Innotrac Diagnostics	0.015	0.036	ND	One-step all-in-one dry reagent immunoassays/time-resolved fluorometry	S

[a] The 99th percentile reference limits were extracted from reference [148] and the 10% imprecision from reference [152] except those marked *, which were not determined and therefore obtained from manufacturers. The ROC cut-offs were from reference [164] and methodology from reference [41]. Information regarding the Aio! assay was obtained from reference [165].

[b] The latest data for the AIA assay was obtained from reference [166]. It should be noted that some of these assays may no longer be in clinical use but are listed here for completion. ND = not determined; S = serum; HP = heparin plasma; EP = EDTA plasma; WB = whole blood.

5.3.2. *Issue of Assay Standardization*

Whereas the between-manufacturer biases for CK-MB were small (<2-fold), there is up to more than a 20-fold concentration difference between cTnI assays [167]. Thus, the standardization of cTnI assays between manufacturers will not be an easy undertaking. This is compounded by the demonstration by Katrukha *et al.* [168] that the epitopes, recognized by antibody pairs, used in the different assays will greatly determine their immunogenicity and therefore the signal generated. Furthermore, the effects of complexes, phosphorylation, and proteolytic cleavage will impact upon the interpretation of cTnI concentration in the contexts of onset of AMI, infarct sizing, reperfusion assessment, and in risk stratification. Due to the myriad cTnI assays available, one must therefore be cautious when interpreting cTnI levels, especially when comparing different cTnI assays. However, as noted by a previous author [167] there is an ever increasing volume of literature that supports the use of cTnI for the detection of AMI, as a tool for risk stratification in patients presenting with unstable angina and in monitoring treatment/reperfusion.

The need to standardize cTnI assays cannot be overemphasized and committees have been enacted to oversee it [169]. Due credit ought to be given to the American Association of Clinical Chemistry (AACC) that initiated and sponsored the cardiac marker standardization undertaking that has now resulted in the development of CK-MB and cTnI standards that are both commercially available. Standardization aims at improving trueness by minimizing bias. It requires the availability of standard material that should be homogeneous, pure, and identical to the analyte of interest as it appears in blood. It also requires a reference method, such as isotope dilution mass spectrometry. To ensure traceability, it is vital that the clinical laboratories use validated calibrators that give values that can be traced back to the primary reference material. Also, to ensure commutability the primary reference material should be identical to the native material and this must be extensively tested. The principles of immunoassay standardization have been described elsewhere [170, 171, 172].

For cTnT, because there is only one commercial assay for clinical use, standardization is not an issue. However, for the cTnI there are well over 10 assays now (refer to Table 5) and as already stated, this is compounded by the fact that the cTnI forms present in serum are a heterogeneous mixture of different complexes (T-I-C, I-C) and classes (oxidized, phosphorylated). Thus, it had been difficult to identify and agree on a reference material. Regarding a reference method, mass spectrometry on its own is certainly not sensitive enough for cardiac troponins that are present in circulation in very minute quantities [172]. This therefore complicates matters even further.

Opinion pieces by Stenman [171], Apple [167], and Panteghini [172] have addressed these issues. Until recently interim solutions had been suggested [173]. The subcommittee for cTnI standardization of the AACC together with the National Institute for Standard and Technology (NIST) and IFCC have recently identified a candidate reference material that is a complex of troponin C, I, and T purified from human cardiac tissue [174]. Furthermore, studies by Katrukha *et al*. [168] have identified that the epitopes in the central part of the cTnI molecule are not affected by the complexes formed, or by *in vivo* modifications although this has been recently challenged [175, 176]. Manufacturers are advised to use antibodies that recognize epitopes in the stable part of the cTnI molecule. The use of antibodies against epitopes in this region of the cTnI molecule will consequently increase the similarity of the antibodies used by different manufacturers and hence reduce between-manufacturer variability. It has also been suggested that the incubation of a recombinant human cardiac T-I-C complex in human serum might generate a material that closely resembles the cTnI found in blood [177]. This material can then be used to calibrate commercial cTnI assays. Others have suggested that if standardization is not possible, harmonization can be used to calibrate the various assays to give the same results [171], although it is noted that perfectly harmonized assays can all be biased.

It would be remiss not to mention that recently an attempt has been made to use affinity chromatography to isolate cardiac troponins from human myocardial tissue and from serum of AMI patients, which was then subsequently analyzed using mass spectrometry [178]. This approach could assist in the search for more suitable reference materials for cardiac troponins in the near future.

In conclusion, it is obvious that this is an area fraught with difficulties owing to a very complex situation. However, it is reassuring to know that progress in standardization is ongoing and will no doubt take some time. However, like Stenman we ought to ask ourselves, "Is it possible, who is responsible, who is capable?" [171].

5.4. Point-of-Care Testing/Near-Patient Testing

The main aim of point-of-care testing (POCT), also known as near patient or bedside testing, is to provide a rapid, accurate, and precise result on site so that appropriate decisions and treatments can be instituted immediately, leading to improved outcomes. POCT also aims to reduce turnaround time, some laboratory costs, such as transportation, and improve triage efficiency all the while, trying to maintain the provision of quality results. As cardiac markers are gaining widespread acceptance for diagnosis and risk stratification, there is need to provide results in a timely fashion. This will

inevitably result in the increased utility of cardiac troponins and enable the appropriate use of therapies and interventions that ultimately will result in shorter hospital stays, reduced complications, and better prognoses.

The technology required to provide POCT systems have improved vastly over the years and continues to do so. At a colloquium on POCT held in 1999, it was recommended that cardiac markers be offered as a POCT in the emergency department [179]. A study was cited in which it was demonstrated that the implementation of POCT for cardiac markers resulted in a 60% decrease in the admission rate of patients with AMI or unstable angina, a 30% decrease in emergency department evaluation time, and an institutional savings of Canadian $500,000 per year. Similarly, in a recent prospective randomized controlled trial of POCT in a six-bed coronary care unit of a British district general hospital, there was a significant reduction in the median length of noncoronary care-unit stay and overall hospital stay in those randomized to measurement of cTnT by POCT [180]. In another study of five hospitals in Germany, it was concluded that POCT for cTnT showed agreement with central laboratory testing and positively impacted upon the initial triage, resulting in better decisions regarding interventions [181]. Earlier, it had been shown that bedside tests of cardiac TnT and TnI were an independent 30-day predictor of death or MI in an emergency department [182].

Thus, with these few studies in mind an appraisal can be made for the use of POCT for cardiac troponins. However, resources in terms of finances and manpower are required together with a firm commitment from the relevant authorities and institutions.

Broadly speaking, there are two types of technology that support POCT: small, bench-top analyzers and hand-held devices [87]. The small, bench-top analyzers are smaller versions of the central laboratory equipment in which operator-dependent steps have been automated, whereas the hand-held devices incorporate technologies that have miniaturized all the relevant procedures into one device. The suitability of the type of POCT depends on the intended application. Screening tests require sensitivity and a very high negative predictive value to be able to be used in a ''rule-out'' protocol. Diagnostic tests require specificity so as to be applicable for a ''rule-in'' protocol.

A review of the cardiac troponin POCT devices available has been published previously in detail [41, 87]. The characteristics of the currently available POCT for cardiac markers are listed in Table 6.

POCT are not without their disadvantages. POCT-cardiac-marker testing has not yet been proven to improve patient care or outcome. The accelerated TAT for cardiac-marker testing may or may not translate into improved patient care. The existing literature provides no direct evidence that the

TABLE 6
POINT-OF-CARE TESTS FOR CARDIAC TROPONINS

Device (manufacturer)	Markers on the device	Methodology	Test duration (min)	Suggested cut-off for cardiac troponins (μg/liter)
Cardiac T rapid assay (Roche Diagnostics)	cTnT	Gold-labelled optically read immunoassay (GLORIA). Single-step sandwich assay with biotinylated capture antibody and gold-labeled detection antibody	12	0.1
Cardiac STATus device (Spectral Diagnostics)	cTnI, CK-MB, myoglobin	Solid-phase chromatographic immunoassay	15	1.5
Alpha Dx POINT-OF-NEED system (First Medical)	cTnI, total CK mass, CK-MB, myoglobin	Sandwich immunometric assay	18	0.40
Triage cardiac panel (Biosite Diagnostics)	cTnI, CK-MB, myoglobin	One-step sandwich immunoassay using immunochromatography with fluorescence detection	~15	1.0
Stratus CS STAT (Dade Behring Inc.)	cTnI, CK-MB, myoglobin	Two-site sandwich immunoassay with fluorometric detection in a solid-phase radial partition chromatography	14 to first result; 4 for each additional	0.06

cTnT = cardiac troponin T; cTnI = cardiac troponin I; CK = creatine kinase; CK-MB = creatine kinase MB isoenzyme.

increased TAT of POCT is truly advantageous to the patient's overall care other than to the resources [180, 181]. In addition, the current cardiac marker POCT devices have not been fully validated and evaluated for the 99th percentile cut-off, and their assay imprecision at this cut-off is unknown (with the exception of Dade–Behring Stratus CS). The situation gets even more compounded with the qualitative devices where no data from well-designed and sufficiently powered studies exists regarding the diagnostic cut-off of the assays in question.

The issue of quality assurance and quality control merits special mention. All sites that provide or intend to provide POCT for cardiac troponin must enlist into an external quality assurance scheme. Training and continual training must be provided to all users of the devices. To whom the responsibility of the instrument's maintenance lies is a debatable issue. The author contends that this responsibility should lie with the central laboratory, but extra funding should be provided so that a dedicated POCT officer can be employed to oversee the running of these devices and their maintenance. Regarding analyses, there is great variability observed for some devices especially at the lower limits of detection. Some devices do not provide a quantitative result (''stick'' testing), which for the case of cardiac troponins can hamper prognostication. Another important issue that needs to be highlighted is the lack of or poor record keeping. If at all possible, all results obtained must be recorded so that a serial report is available for long-term monitoring. It would be preferable if the devices were interfaced to the central laboratory so that the results are archived in the patient's records together with other results. Records like this can help establish a baseline to which future tests can be compared.

Despite these disadvantages, the use of POCT will continue to increase because there is a growing body of evidence suggesting that the early identification of patients with ACS can improve outcomes both in the short- and long-term [87].

5.5. Quality Assurance

With the recommendation by the joint ESC/ACC to measure cardiac troponins in patients with ACS [71] and with the consequent increase in the provision of cardiac troponin assays by clinical laboratories, there is now a requirement to ensure that accurate assays are being provided that show good inter-laboratory agreement. This is especially important with the demonstration that cardiac troponins are an indicator of minor myocardial damage [123, 183]. The clinical laboratory must monitor assays at the lower concentration range to ensure that accurate and precise measurements are being generated and reported to clinicians. Although not practicable at

the moment, it has been recommended that a total imprecision of <10% at the 99th percentile reference limit is required [71, 152]. Both manufacturers and clinical laboratories should strive to achieve this.

Internal quality control (IQC) is undertaken by the laboratory to ensure accuracy and precision of analyses on a day-to-day basis. IQC material is available from providers, such as Biorad Limited, in the form of stable liquid reagents for both cardiac TnT and TnI. These are used to ensure acceptable performance within the laboratory as stipulated by the Westgard rules [184].

External quality assurance schemes (EQAS) are currently provided by the College of American Pathologists (CAP), United Kingdom National External Quality Assurance Schemes for Cardiac Markers (UKNEQAS-CM), the Wales External Quality Assessment Schemes (WEQAS), and so on. The distribution frequency, number of samples per distribution, number of cycles, mode of result return, and reporting may vary between the schemes. However, all aim to ensure that there is inter-laboratory agreement, identify random and systematic errors, assess accuracy and precision, and provide a platform to highlight problems that may be common to more than one laboratory. External quality assurance and IQC can be looked upon as management tools used to define and set standards of excellence. They are in any case a prerequisite for laboratory accreditation. It has recently been demonstrated that participation in EQAS provides an opportunity to participants to continually evaluate and improve the quality of their services [185]. It is inconceivable that in the modern era, when laboratory accreditation is almost mandatory and consumer sovereignty is the order of the day that there would be any clinical laboratory offering cardiac troponin assays or indeed any other assay without enrolment in an EQAS.

5.6. How to Use the Currently Available Cardiac Troponin Assays

Clinical biochemistry begins at the bench but should not end there. A word on how to make the best use of the cardiac troponin assays might be appropriate here but this is by no means meant to be prescriptive nor authoritative.

Chest pain accounts for approximately 2–4% of all emergency department presentation in the United Kingdom. Of these presentations, 30% will be admitted and 70% discharged compared to the United States (US) where 60% are admitted [186]. In the United Kingdom and the United States about 6% and 3.5% respectively will have a missed diagnosis of AMI [186, 187]. Mortality in patients who are discharged with missed AMI is four times greater than in hospitalized patients [188]. Bearing these facts in mind, the need to accurately, reliably, and effectively identify patients with AMI

should be the goal of every emergency, cardiology, and clinical biochemistry departments.

The National Academy of Clinical Biochemists (NACB) and the Committee on Standardization of Markers of Cardiac Damage of the IFCC have published guidelines for the use of cardiac markers in patients with ACS [84, 189]. The most recent guidelines by NACB has been posted on their website although it is still a preliminary draft [190]. Recommendations have been made regarding the use of biochemical markers in the initial evaluation of patients with ACS, which all parties concerned should aim to adhere to. The class number above each list of recommendations depicts the strength or consensus level of the recommendation.

Class I

1. Biomarkers of myocardial necrosis should be measured in all patients who present with symptoms consistent with ACS.
2. The patient's clinical presentation (history, physical examination) and ECG should be used in conjunction with biomarkers in the diagnostic evaluation of suspected MI.
3. Cardiac troponin is the preferred marker for the diagnosis of MI. CK-MB by mass assay is an acceptable alternative when cardiac troponin is not available.
4. Blood should be obtained for testing at hospital presentation followed by serial sampling based on the clinical circumstances. For most patients, blood should be obtained for testing at hospital presentation, at 6– 9 h, and again at 12–24 h if the earlier samples are negative and the clinical index of suspicion is high.
5. In the presence of a clinical history suggestive of ACS, the following are considered indicative of myocardial necrosis consistent with MI:

a. Maximal concentration of cardiac troponin exceeding the 99th percentile of values (with acceptable precision) for a reference control group on at least one occasion during the first 24 h after the clinical event.
b. Maximal concentration of CK-MB exceeding the 99th percentile of values for a gender-specific reference control group on two successive samples (values for CK-MB should rise and fall).
c. In the absence of availability of a troponin or CK-MB assay, total CK greater than two times the gender-specific upper reference limit may be used. However, the assay's poor specificity should be noted.

Class IIa

1. For patients who present within 6 h of the onset of symptoms, an early marker of myocardial necrosis may be considered, such as myoglobin, in addition to a cardiac troponin.

Class IIb

1. A rapid "rule-in" protocol with frequent early sampling of markers of myocardial necrosis may be appropriate if tied to therapeutic strategies.
2. The clinical laboratory should perform cardiac marker testing on a continuous, 24-h basis with a TAT of <1 h (time from blood collection to clinical awareness).

Class IIc

1. Assays for cardiac biomarkers should have a total imprecision (% CV) of <10% at the 99th percentile reference limit. Assays should also be characterized for potential interferents.
2. Laboratories that cannot deliver a TAT <1 h should consider implementing POCT systems. There should be acceptable harmonization between POCT and central laboratory results.

Class III

1. Total CK, aspartate aminotransferase (AST, SGOT), beta-hydroxybutyric dehydrogenase, and/or lactate dehydrogenase (LDH) should not be used as biomarkers for the diagnosis of MI.
2. For patients with diagnostic ECG abnormalities on presentation (ST-segment elevation), diagnosis and treatment should not be delayed while awaiting biomarker results.

The draft document has also made recommendations regarding the use of biochemical markers in early risk stratification, in the management of NSTEMI, and in the management of STEMI [190].

The two main uses that the cardiac troponins are not recommended for at present are screening asymptomatic patients for the presence of ACS and infarct sizing [84].

5.6.1. *Which Test is Best? Cardiac Troponin T versus I*

It is difficult to categorically state, which of cardiac TnT or TnI is better than the other. It should also be noted that most of the earlier studies comparing the two used the earlier generation of the assays, which had lower analytical sensitivities. Wherever possible, the assay name and/or assay generation is cited. Free cytosolic proteins are released earlier than those bound to the sarcomere. Approximately, 6–8% [42] and 3–8% [11, 43] of cardiac TnT and TnI exist freely in the myocytic cytoplasm. This cellular distribution will most certainly affect the release kinetics. Additionally, per gram of wet cardiac tissue, cardiac TnT, TnI, and TnC content are 11 mg, 5 mg, and 1.4 mg respectively. As already alluded to and worth restating here, cTnT is released in free form and as a T-I-C complex, whereas cTnI is

only released in complexed forms. Cardiac TnI is also prone to proteolysis [66], phosphorylation [63], and oxidation [65]. In the final analysis, these may all impact on the assay release kinetics, stability in blood, and their clearance rate, which in turn may affect how the assays perform in a clinical setting to diagnose ACS and indeed MI. The sensitivities in the diagnosis of MI and unstable angina for both cardiac TnT (Roche first generation) and TnI (Sanofi Diagnostics Pasteur) have been shown to be similar in one study [191, 192]. Other studies have shown that cTnI (Stratus II analyzer, Dade International) demonstrated lower sensitivity compared to cTnT (Roche second generation) in the early detection of MI [183, 193]. A study by Ooi and colleagues also showed that the cTnT (Roche second generation) was less sensitive than cTnI (AxSYM, Abbott Diagnostics) in detecting minor cardiac damage [194]. In another head-to-head comparison, the absolute sensitivity for AMI using cTnT (Roche second generation) was 98% and using cTnI (Access analyzer, Beckman Coulter) it was found to be 100% [195]. This lack of concordance may be attributable to the different sample sizes used and the different populations sampled. Importantly, it may in part also be due to the different generations of assays used, with earlier versions having lower analytical sensitivities.

Regarding specificity, cTnI appears to be more specific than TnT as the former does not suffer from interference in renal failure [191, 196] (refer to Section 5.1 for further elaboration on the effect of renal failure on cardiac troponins). One study has demonstrated that cTnT (Roche second generation) was more frequently increased in patients with unstable angina compared to cTnI (Access system, Sanofi Pastuer); cTnT also increased to higher relative values compared to cTnI in the same study [193]. Another study showed that the specificity was higher for cTnI at 78.7% (Access analyzer, Beckman Coulter) compared to cTnT at 68.1% (Roche second generation) [195].

However, both give equal prognostic information [191, 197]. As mentioned in Section 3.2 , two meta-analyses have concluded that both cardiac TnT and TnI can equally predict AMI and cardiac death [108, 109]. It is worth highlighting that among patients with ACS in the GUSTO-IIa trial, a cTnT concentration measured at presentation appeared to provide more prognostic information than cTnI in the prediction of 30-day mortality [198]. This conflicting finding could be due to the 12–18 month delay in measuring cTnI compared to cTnT, which was performed within 60 days after collection. Whether the cTnI is stable for this period of time even when frozen has been questioned [199]. Further, as noted by Apple [199], an inappropriate upper reference limit for AMI was used in the study, that is, 1.5 μg/liter for the Dade Stratus II system as suggested by the manufacturer package insert instead of 0.6 μg/liter, which had been found to be the 97.5 percentile of 150 individuals presenting with chest pain but subsequently found to have had a nonAMI [199].

In conclusion, it is surmised that either cardiac TnT or TnI can be used to predict outcomes in patients with ACS and to diagnose MI. It is, however, important to ascertain the reference limits for each assay and to question how they were determined especially if not validated in the peer-reviewed literature. Whether an institution chooses cardiac TnT or TnI will depend on local pragmatic issues and financial considerations; either test is suitable.

5.6.2. *When to Sample?*

There is an apparent lack of consensus internationally regarding when to sample blood for cardiac troponin testing. This is driven by different health care delivery structures in different countries, some observing a more interventional philosophy, while others embracing more conservative approaches. If we use the transatlantic divide as an example, the US approach is best encapsulated in the NACB consensus document [190]. This document advocates for the sampling of blood at admission, after 6–9 h, and an optional measurement at 12–24 h, if the previous results were negative. The IFCC had previously recommended sampling blood at admission, 4, 8, and 12 h (or the next morning) [189].

The UK approach is more conservative. Patients are stratified into high- and low-risk groups based on clinical and ECG findings, which is then followed up by biochemical testing for cardiac markers. Patients judged to be at high risk have their cardiac troponin levels measured at least 12 h post-onset of chest pain before undergoing further investigations by stress testing, radionuclide ventriculography, or angiography. Patients deemed to be low risk are discharged after emergency triage. In the United Kingdom, only patients with AMI and unstable angina meeting Braunwald IIIb criteria are admitted into hospital [200].

With the introduction of immunoassays for cardiac troponins and the availability of POCTs, these assays can now be provided rapidly, 24 h a day, 7 days a week. For some countries, this might be contingent on the availability of funding. In one study, the qualitative measurement of cardiac TnT or TnI, using POCT, was able to rule out AMI at 6 h after the onset of symptoms [182]. However, in keeping with the release patterns of the cardiac troponins (Fig. 4), it would be cogent to measure the cardiac troponins at admission and at least one other time point, 6–16 h post-admission. The study by Newby *et al.* [112] has clearly demonstrated that levels collected at 8- and 16-h added to the strength of the baseline result in predicting 30-day mortality (baseline + 8-h, $\chi^2 = 12.04$, $p = 0.0072$; baseline + 16-h, $\chi^2 = 13.52$, $p = 0.0036$), whereas the study by Christenson *et al.* [198] showed that a cTnT measured at presentation provided more prognostic information than cTnI in the prediction of 30-day mortality.

The question of whether the basal time should be assigned to the time of onset of chest pain or presentation to the emergency department is at best a

semantical debate. There is no doubt in initiating treatment and admission in patients presenting with overt signs and/or ECG changes consistent with MI. However, in patients with no ECG changes or a small infarct, due to the waxing and waning time course of myocardial ischemia, it may be difficult to discern whether a patient has suffered an MI or not. The NACB document recommends that all blood collections should be referenced relative to the time of presentation at the emergency department/hospital or when available with certainty at the time of onset of chest pain [84, 189].

5.6.3. *Which Cut-off Value to Use? Impact of the 99th Percentile Reference Limit for Cardiac Troponins*

With the redefinition of MI by the ESC/ACC, it has been necessary to evaluate the impact of this on clinical practice. One obvious outcome is that the adoption of the 99th percentile will substantially increase the incidence of positive tests for MI. In one recent study, the analysis of cTnI in 1719 ACS patients demonstrated an 85% increase in MI diagnosis when the 99th percentile reference limit (0.06 μg/liter) for the Dimension RxL cTnI assay (Dade-Behring,) was used compared to the ROC curve cut-off (0.6 μg/liter) [106]. In the same study, using the concentration with a 10% total CV (0.26 μg/liter) instead of the ROC cut-off produced a 26% increase in all cTnI positive cases. The increase in positive cardiac troponin cases, whether true or false, will result in increased costs for laboratories offering the test and will prompt further investigations that will also incur increased expenses for other hospital departments.

Additional work is therefore required to establish whether the 99th percentile reference limit for assays, such as cTnT, with poor assay precision at the lower end of the analytical range is the best diagnostic criteria. Panteghini and colleagues have clearly demonstrated (see Fig. 6) that no cardiac troponin assays was able to achieve the 10% CV recommendation at the 99th percentile reference limit [152]. In assays, such as these it might be more practical to use the traditional ROC curve cut-off values or the concentration with a total imprecision of 10%. For example, for the Roche TnT assay, the 99th percentile reference limit is 0.01 μg/liter, where according to unpublished data in our laboratory the total imprecision was more than 25%. A 10% total imprecision is only achievable at a concentration of 0.04 μg/liter [152]. Thus, it might be more practicable to recommend the use of 0.04 μg/liter rather than the 0.01 μg/liter until a more sensitive and precise generation or version of the assay becomes available. Koerbin *et al.* [201] have recommended the use of the concentration that corresponds to the 20% CV, which is the functional sensitivity of an immunoassay [202]. This latter approach might be more pragmatic given that the 99th percentile is currently difficult to achieve, whereas the 10% CV might not be sensitive enough

and will most certainly miss patients with ACS who have a prognostically important increase in the cardiac troponin levels.

Apple *et al.* [148] have also demonstrated that selective gender and ethnic differences are present in the 99th percentile reference limits. For example, the Beckman Access showed a statistical significance between male and female 99th reference percentile limits, whereas the Tosoh AlA showed a significant difference between the 99th percentiles for Caucasians and Blacks. Depending on the choice of cardiac troponin assay, gender and ethnic specific levels might also have to be taken into consideration, further compounding the issue.

Overall, the adoption of the 99th percentile will impact on the resources of the hospital in terms of cost and manpower. A well-powered, prospective study is required to unequivocally demonstrate the utility of the 99th percentile reference limit of cardiac troponins in diagnosing MI. Since there is no "true gold standard" test for MI, it is impossible to determine the prevalence of true and false positives in patients with indeterminate chest pain. A prospective study might therefore have to document the evidence of myocardial ischemia in a post-mortem follow-up.

The British Cardiac Society Working Group is seeking to address the inconsistencies in the nomenclature for ACS to meet treatment and prognostic needs of patients. They are also recommending a diagnostic threshold to distinguish patients with MI from those with ACS that might also have prognostically important increases in cardiac troponin concentration [203, 204].

In conclusion, the shortfalls and pitfalls of cardiac troponin tests in the diagnosis of MI or indeed the spectrum of the ACS must be recognized. While studies have shown the diagnostic and prognostic utility of the cardiac troponins, the 99th percentile reference limit definition needs further validation. The clarification of the nomenclature, the continued standardization and/or harmonization of cTnI assays, the improvement of the analytical sensitivity of the current cardiac troponin assays together with the validation of the 99th percentile reference limit will hopefully result in a unified approach to the diagnosis of MI/ACS.

6. Factors Affecting the Measurement of Cardiac Troponins

Numerous analytical and biological interferents have been implicated in causing a false positive or false negative result in cardiac troponins measurements. In patients without ACS, the causes of increased cardiac troponins remain controversial and open to debate as to the likely mechanisms. Because it is unethical to perform an endomyocardial biopsy in patients, it is almost impossible to clarify the basis for an increased cardiac

troponin level. However, based on one classic study by Ooi *et al.* [194], cardiac pathologies determined at postmortem were highly correlated with the antemortem cardiac markers including cardiac troponins. In this study, 78 autopsied patients without clinical MI were examined. In all cases with increased serum CK-MB, cardiac TnT and TnI, there were a significant number of cardiac histological changes observed. In addition, 72% and 64% of patients with increased cardiac TnI and TnT respectively, showed histological signs of a recent or healing MI. Based on this report, it can be argued that these increases in cardiac troponin rather than being spurious are indicative of underlying cardiac disease. As noted by Jaffe and colleagues, ''Detectable increases in biomarkers of cardiac injury are indicative of injury to the myocardium, but elevations are not synonymous with an ischemic mechanism of injury. Therefore, increases do not...mandate a diagnosis of myocardial injury'' [205].

Furthermore, it should be noted that any increase in the cardiac troponin concentration is associated with a poor prognosis [206]. However, this is not to imply that false positive and false negative results do not occur. Interferences in immunoassays are a recognized problem affecting many assays [207, 208, 209].

This section will review the factors that have been reported to affect the accurate measurement of cardiac troponins.

6.1. Renal Failure

Among patients with end stage renal failure (ESRF), cardiac disease is the single, most common cause of mortality, accounting for nearly 50% of all deaths [210]. It has been well established that the traditional markers of myocardial necrosis, such as CK, CK-MB, and myoglobin, as well as the cardiac troponins are commonly increased in renal failure even in the absence of clinically suspected myocardial infarction [26, 211, 212]. The significance of this observation has been the subject of recent debates regarding the universal acceptance of cardiac troponins as specific markers of myocardial infarctions. Furthermore, studies have shown the importance of troponin elevation in risk stratification, prognosis, and therapeutic interventions [102, 103, 116]. However, it is important to point out that most of these studies excluded patients with renal failure. Recently, two landmark studies have shown that even mild renal disease, as assessed by the estimation of the glomerular filtration rate (GFR), should be considered a major risk factor for death, cardiovascular events, and the use of specialized health care [213, 214]. Thus, considerable resources are being allocated to treat and prevent cardiovascular disease in this group of patients with renal failure.

It was initially believed that the commonly elevated cTnT in renal failure was a false-positive limitation of the test, especially with the demonstration that cTnI was less commonly increased in ESRF [215, 216]. However, a more recent study has shown the prognostic significance of a raised cTnT concentration in patients with a suspected ACS, irrespective of the degree of renal impairment [217]. In this study, it was reported that an abnormal cTnT concentration at baseline was associated with an increased risk of death within 30 days in all patients (adjusted OR = 3.4). One critique that can be leveled at this study was the relatively few patients receiving dialysis with only 11 patients with a creatine clearance of less than 10 ml/min.

Another study has demonstrated that 1 in 3 patients with ACS has renal insufficiency [218]. After adjusting for potential confounders, the decreasing renal function was independently associated with in-hospital death, bleeding, and contrast-induced nephropathy. The authors surmised that in general, the increased risk of adverse outcomes in patients with ACS with renal insufficiency was due to: excess of comorbidities, therapeutic nihilism, toxicity of therapy, and abnormal vascular pathobiology of renal failure [218]. The most recent TACTICS-TIMI sub-study [219] has clearly shown that an elevated troponin in ACS is associated with a higher risk for death or reinfarction, even among patients who do not have significant angiographic coronary artery disease. Thus, mounting evidence suggests that a raised troponin concentration in uremic patients is reflective of myocardial injury.

An important issue that is worth highlighting is how ACS should be diagnosed in the face of renal impairment. In patients without renal impairment, an increased serum cardiac troponin concentration in the presence of clinical symptoms, with/without ECG changes, is considered sufficient to entertain a diagnosis of ACS. In the setting of ESRF, confusion prevails on how to best establish the diagnosis. Diagnosis of ACS in ESRF is further complicated by the observation that such patients are at a higher risk of silent myocardial ischemia, have atypical clinical symptoms, have ECG abnormalities, such as left bundle branch block, and often do not have a history of unstable angina [220, 221]. It has been suggested that a higher threshold of cTnT (0.5 μg/liter) could be used [222]; however, this would lead to an increased incidence of false negatives. Alternatively, in view of the relative lack of false positivity of cTnI in uremic patients, this test could be a better marker of ACS in ESRF [26, 223]. It may also be useful to document changes in the concentration of cTnT over time [196]. A study by Ooi *et al.* [224] noted a marked increase in mortality in patients with an increased cTnT concentration after 1 year. One problem with the latter strategy of noting changes over time is the delay in instigating vital treatments. A very recent study by Apple *et al.* [225] showed that mechanisms notwithstanding,

TABLE 7
WHY ARE CARDIAC TROPONINS (ESPECIALLY cTnT) RAISED IN RENAL FAILURE?

Causes
1. Lack of assay specificity especially first generation assay
2. Chemical modifications: phosphorylation, oxidation, and proteolysis
3. Reexpression of cTnT isoforms in injured or regenerating skeletal muscle
4. *In vivo* fragmentation of cardiac troponins followed by accumulation of immunoreactive fragments due to impaired renal clearance
5. Microinfarctions/silent myocardial necrosis
6. Comorbidities: patients with renal failure tend to have left ventricular hypertrophy, which is correlated with increased cTnT
7. Increased membrane permeability and apoptosis
8. Unknown highly homologous entity to cTnT!

increased plasma levels of both cardiac TnT and TnI above the cut-offs for the reference population were independently predictive of subsequent death in ESRF patients.

What is the origin of cardiac troponin in patients with renal impairment? For a long time it was hypothesized that uremic-induced skeletal myopathy was the source of the increased troponins in renal failure [28]. The uremia promoted the reexpression of cTnT from injured or regenerating skeletal muscle. Table 7 summarizes the causes of increased cardiac troponin concentrations in patients with renal failure. The various hypotheses put forward by various groups and the evidence for or against it are discussed below.

The capture antibody of first generation cTnT assay was cardiac-specific but the detection antibody cross-reacted with skeletal muscle TnT [157]. With this assay, 71% of a group of patients with ESRF had increased TnT without clinical or electrocardiographic evidence of AMI; in these patients only 7% had an increase in TnI [196]. Development of a second-generation assay where both capture and detection antibody were cardiac-specific resulted in less false-positives [158]. However, increased TnT in up to 53% of patients with renal failure with no clinical evidence of AMI has been reported [196]. The third generation assay utilizes cardiac specific antibodies but uses recombinant human cTnT as the standard material for calibration [160]. However, false positives continue to be seen [29, 226].

Dialysis has been shown to affect cardiac troponin levels. Cardiac TnI levels decreased by 86%, but TnT increased posthemodialysis. This was explained either due to hemoconcentration or due to the absorption of TnI, which is hydrophobic onto the dialysis membrane [227, 228, 229]. In contrast, others have refuted these claims [230, 231]. In the study by Ie *et al.* [231], in 11 out of 49 asymptomatic patients on hemodialysis, cTnT, myoglobin, and creatine kinase were measured before and during

hemodialysis. No change in any of the parameters was observed. It was concluded that elevated cTnT levels in asymptomatic HD patients are not caused by acute myocardial injury or by hemodialysis itself. It may be more due to chronic myocardial damage and/or decreased renal clearance.

Biochemical modifications including phosphorylation [63, 64], oxidation [65], and proteolysis [66, 232] can also alter the troponin molecules released into circulation. These modifications alter the immunogenicity of troponins to monoclonal antibodies and this can potentially affect the levels measured. Whether these modifications are enhanced in renal failure remains to be ascertained.

It has been hypothesized that uremic-induced skeletal myopathy causes an increase in troponin levels by virtue of reexpression of cTnT from injured or regenerating skeletal muscle. Early reports using the first generation TnT assays, which have known cross-reactivity with skeletal TnT, describe elevated TnT levels in patients with skeletal muscle injury [233] or inflammatory myopathies [234] without any evidence of myocardial ischemia.

Several isoforms of cTnT have been described in developing and adult myocardial tissues [25, 235, 236]. A "cardiac-like" TnT isoform has been reported in human fetal skeletal muscle [237] and in some developing animals [25]. This decreases with maturation such that there is none present in nondiseased adult human and rat skeletal muscles. In contrast, neither cTnI nor its isoforms have been demonstrated in skeletal muscle [238]. The perturbations that stimulate the reexpression of cTnT isoforms in skeletal muscle in patients with renal failure remain unknown. As suggested by Freda *et al.* [196], if reexpression of cTnT isoforms takes place in skeletal muscle, can they be measured in serum of patients with renal failure by the latest generation of cardiac troponin assays? If troponins are indeed prognostic of a higher risk of death [219, 225] or reinfarction [219] in patients with renal failure, then it might be important to measure it in such patients.

Various groups have identified "cardiac-like" TnT isoforms and its mRNA from patients with ESRF [55, 61]. Using the second generation cTnT assay monoclonal antibodies, cTnT isoforms could not be detected in the patient samples [61], or in Western blots of skeletal muscle biopsies from patients with Duchenne muscular dystrophy who had increased serum cTnT without any evidence of cardiac disease [239]. These data are consistent with the findings of Bodor *et al.* [238] who demonstrated that cTnI is not expressed in fetal and healthy or diseased human skeletal muscle. Using biopsies from diseased renal tissue in 10 patients without known cardiac disease, Davis *et al.* [240] did not detect cTnT using the Western blot technique and concluded that the kidney is not the source of circulating levels of cTnT. More recently, Fredericks *et al.* [241] have shown that cTnT and CK-MB are

not increased in skeletal muscle of patients with renal failure or in the uremic rat [242]. It has also been shown that cTnI is not abnormally expressed in skeletal muscle of patients with renal failure [243]. Thus, the hypothesis of reexpression of developmental cardiac troponin isoforms of cTnT lacks irrefutable evidence and warrants reevaluation.

Another cause of the presence of cardiac troponins in patients with renal failure suggested is decreased renal clearance of troponins in circulation. Ziebig and coworkers [244] measured both cardiac troponin T and I in the plasma and urine of selected patients with differing renal function. Renal function was assessed by the determination of GFR. Urinary albumin was measured as a marker of glomerular disorder, whereas α1-microglobulin as a marker of tubular disorder. The protein ratio in urine and plasma was also determined to demonstrate that the renal elimination of the troponins was comparable to that of other known proteins. This study concluded that kidney function contributed to the elimination of cTnT.

Based on this study, Diris and colleagues [29] sought to determine whether *in vivo* fragmentation of cTnT could explain the increase of serum cTnT in 63 patients with renal failure undergoing dialysis. They used a highly sensitive immunoprecipitation assay to isolate and concentrate cTnT fragments separated by gel-electrophoresis and visualized by Western blotting. They showed that cTnT is fragmented into molecules small enough (ranging in size from 8 to 25 kDa) to be cleared by the kidneys of healthy subjects. Impaired renal function causes accumulation of these fragments and contributes to the unexplained elevation of serum cTnT. While this is the best explanation to date of the cause of raised cTnT in patients with renal failure, this study has major limitations.

First, antibodies from the only commercially available cTnT assay (Roche Diagnostics Inc.) were not used. Therefore, it is impossible to compare the intensity of the detected fragments and the measured cTnT concentrations in the patients. This study needs to be repeated using the Roche antibodies M7 and M11–7. Second, the authors suggest that they were able to visualize cTnT fragments even in serum with cTnT concentrations below the limit of detection of the current assay, which is 0.01 μg/liter. This might have been possible because a mixture of five anti-cTnT antibodies, directed at different epitopes throughout the cTnT molecule were used. Since these antibodies and their respective epitopes have not been fully characterized, it remains uncertain how specific they are for cardiac troponins. Is it possible that these other antibodies are cross-reacting with skeletal TnT or indeed other clinically irrelevant cTnT isoforms? Finally, in a recent editorial, it was stated that the inventor and patent holder of the cTnT assay, Hugo Katus, disputes this latest theory and argues that the so-called ''false positives'' are in fact patients with clinically significant but asymptomatic myocardial ischemia

[245]. We wait with anticipation for further developments in this arena, but there is no doubt that the "fog is slowly lifting" [245].

Other possible causes of increased cTnT include the presence of micro-infarctions/silent myocardial necrosis in patients with elevated troponins [194, 246]. Such patients may not present with overt clinical symptoms. It is also plausible that patients with ESRF are more likely to incur episodes of silent micro-infarctions due to the high incidence of coronary artery disease. In clinical studies, a positive correlation between elevated concentrations of cTnT and an increase in left ventricular mass has been reported [247, 248, 249]. However, it is important to note that the relative amounts of cardiac troponin isoforms also alter in hypertrophy [250]. Whether a different mechanism of release of troponin into serum is present in hypertrophied hearts remains to be determined. There is a possibility that apoptosis may explain the modest elevation of serum troponin in patients with heart failure [196, 251, 252]. In conditions such as sepsis, coagulation in the capillary bed may lead to reversible myocardial hypoxia causing apoptosis, which is associated with the leakage of cardiac troponins into the serum. However, experimental evidence to prove this conjecture is lacking. Finally, it is possible that a hitherto unknown entity that is highly homologous to the cardiac troponin (especially cTnT) is released during renal failure and is therefore causing a false positive using current assays. Further research is required in this area.

In summary, controversy reigns regarding the cause of the elevated troponins in renal failure. However, one thing remains clear, that is, elevated cardiac troponins are highly prognostic of future cardiac events, complications, and even death. Renal failure patients with elevated cardiac troponins might benefit from a more aggressive cardiac intervention strategy. Accordingly, there is an urgent need for the clinical laboratory to make available reliable and validated assays for biomarkers that can predict adverse events, especially among patients with renal impairment. The use of a multi-biomarker panel consisting of troponins and natriuretic peptides, namely BNP or Pro-NT-BNP, for risk stratification in patients with ESRF has recently been published [253, 254]. This leaves an open field as to what the future holds in terms of choice of markers and their combinations.

6.2. Sepsis

Sepsis is the leading cause of death in the noncardiac ICU [255] and affects approximately 750,000 people with over 200,000 deaths each year in the United States alone [256]. The interruption in blood flow and therefore oxygen delivery leads to organ dysfunction, including myocardial dysfunction and finally to multiple organ failure. Causes of myocardial dysfunction in patients with sepsis are immunomodulatory intracellular

mediators and myocardial ischemia [144]. The latter is accompanied by the leakage of cardiac troponins from myocytes. The leakage is due to the increased permeability of cell membranes caused by tumor necrosis factor α [257, 258]. In rat cardiomyocytes, only 15 min of mild ischemia has been shown to be sufficient to allow the release of TnI, an interval that is deemed too short to induce cell death [52].

Thus, in a study by Spies *et al.* [144] the prognostic utility of cTnT was evaluated. As many as 18 out of 26 patients had high cTnT levels, which was associated with an increased mortality rate. It was concluded that cTnT was a prognostic marker in early sepsis. In another study by Ammann *et al.* [259], it was found that cTnI was raised in up to 85% of patients with sepsis without any evidence of ACS. In our own hospital, out of 58 patients with elevated cTnT without evidence of ACS, 14 patients had sepsis and the cTnT ranged from 0.11 to 0.70 μg/liter [226]. Thus, it is important to bear in mind that sepsis can serve as a false positive and lead to unnecessary investigations for ACS, resulting in increased medical costs.

6.3. Heterophilic Antibodies

Heterophilic antibodies (HA) are typically of the IgG class and recognize epitopes on the constant (Fc) portion (rarely Fab region) of the foreign immunoglobulin. The use of monoclonal antibodies for radio-imaging tumors and treatment of certain cancers, incidental or occupational exposure to foreign proteins, for example, by farm workers or due to pets in the home, can all give rise to HAs in humans [260]. Exposure to an antibody deemed foreign by a person's immune system triggers an immune response. In fact, the use of monoclonal mouse antibodies in the treatment of cancer patients has given rise to a subset of HAs specific for mouse proteins referred to as "human anti-mouse antibodies" (HAMA) [261]. It has been estimated that it is present in $<$1–80% of patients being treated. The prevalence of HA itself in the general population has been estimated to be as high as 40% [262]. They usually cause interference by binding simultaneously to the capture and detection antibodies in the assay reagent, thereby simulating the presence of an analyte and causing a false positive result.

There have been reports documenting the false increase in cTnI with HAs when using the Abbott AxSYM [260, 263]. Yeo *et al.* [260] estimated that the interference due to HAs, when using the Abbott AxSYM, had a prevalence of 0.19% in their overall study population. Other studies using the Beckman Access immunoassay have also reported false positive results due to interference by HA [264, 265].

Potential solutions to this interference include the use of nonspecific, "blocker" immunoglobulins in the reagent that originate from the same

species as the analyte-specific antibodies in an attempt to "mop up" any HAs present in serum. In a recent opinion piece, Ismail highlighted the reports of serious consequences incurred due to interferences in immunoassays and has called for a radical approach to eliminate this interference using polyethyleneglycol (PEG) precipitation as a preanalytic step before analyses [208].

6.4. Rheumatoid Factor

Rheumatoid factors (RF) are a group of heterogeneous immunoglobulins whose common property is the reaction with the Fc portion of other immunoglobulins [266]. This heterogeneity and the use of different monoclonal antibodies in different manufacturer's assay may account for the lack of uniform interferences seen in various immunoassays. It has been estimated that 5% of the healthy population have positive RF levels [267]. Patients with rheumatoid arthritis and other connective tissue disorders have an even higher rate of positive RF [266]. Thus, it is not inconceivable that up to 10% of patients presenting with chest pain to an emergency department will have positive RF levels. This increases the likelihood that RF is a potential source of false positive interferences in immunoassays.

In a study by Krahn *et al*. [268], 100 outpatients with positive levels of RF (>100 IU/mL; reference range <20 IU/ml) had cTnI measured by two different assays (Abbott AxSYM, Abbott Diagnostics and Bayer Immuno 1, Bayer Corporation). It was presumed that since these were outpatients, none of them had an acute coronary event, although no clinical evaluation was undertaken to rule out any cardiac pathology. This was the main limitation of this study. However, they found that the AxSYM assay had 15% of values elevated into the range consistent with a diagnosis of AMI, whereas the Immuno 1 had none. Furthermore, this group of patients had both negative myoglobin and CK-MB levels as measured on the Immuno 1. This was taken as evidence that this group did not have an AMI. No correlation was found between the RF levels and the degree of interference. In the range where minor myocardial damage could not be ruled out (0.5–2.0 μg/liter for AxSYM and 0.2–0.8 μg/liter for Immuno 1), 18% and 10% of the values on the AxSYM and Immuno 1 respectively fell into these ranges. It was estimated that the false positive rate due to RF was between 0.9–1.8% for AxSYM and 0.5–1.0% for Immuno 1. The authors suggested that banks of sera that include RF among other interferents should be used in clinical validations of commercial cardiac troponin immunoassays.

Another study reported false positive cTnI (Abbott AxSYM) in 12 patients who had positive levels of RF but no indication of myocardial infarction [269]. None of these patients had detectable cTnT levels. Use of a polyclonal antisera against RF eliminated this interference.

6.5. Autoantibodies

Autoantibodies are antibodies produced by the immune system against self [270] and are usually the basis of autoimmune diseases. Although heterophilic antibodies can be autoantibodies, it has been argued that natural antibodies are the major source of heterophilic antibody interference in normal people [270]. There is one early report in the literature where a false-negative immunoassay result was documented due to the interference of circulating IgG-class autoantibodies showing high affinity for cTnI (Stratus Cardiac Troponin-I Fluorometric Enzyme Immunoassay) [271].

An even more recent publication has shown the presence of circulating autoantibodies in 10 patients with low recovery of added cTnI to their serum [272]. In this study two cTnI assays were studied: the commercially available Aio-cTnI, Innotrac Diagnostics that uses antibodies that recognize the stable central portion of cTnI (amino acid residues from 30 to 110), which as it turns out is the favored analytical approach for assay design [273] and a novel assay design which incorporates antibodies with epitopes outside the stable mid-fragment region. For the novel assay, samples taken from a patient with ACS at admission, at 6–12 h, and at 24 h were all clearly positive while with the commercially available Aio-cTnI assay, only the 24-h sample had detectable cTnI. The study concluded that autoantibodies to cTnI could hamper the triage of patients with ACS by causing delays due to the inaccurate detection of the correct cTnI level. The authors of this study [272] have recently undertaken further investigations into the negative interference in cTnI assays by circulating troponin autoantibodies and recommended a novel antibody combination, different from the conventional mid-fragment region antibody approach, which offers improved detection of cTnI in samples containing troponin autoantibodies [175, 176].

It is difficult to study the extent of the interference of autoantibodies because a large representative sample is not easy to obtain. Apart from thyroglobulin [274], no immunoassay has been systematically studied to ascertain the magnitude of the problem posed by interference with autoantibodies.

6.6. Antiphospholipid Antibodies

The antiphospholipid syndrome (APS) is a disorder characterized by recurrent thrombosis and/or pregnancy complications due to the binding of antibodies to negatively charged membrane phospholipids (e.g., anticardiolipin antibody, antiphosphatidylserine) or against associated plasma proteins (mainly β2-glycoprotein 1) leading to hypercoagulability that can affect any organ system [275]. It usually occurs in patients with systematic lupus erythematosus (SLE), rheumatic diseases, and some infections. Antiphospholipid antibodies are present in as many as 50% of individuals with

SLE and in 1–5% of the healthy population [276]. Catastrophic APS is a more serious variation and has a mortality rate of about 50%. It is usually characterized by multiple organ infarctions over a period of days to weeks [276].

There is one case in the literature reporting very high levels of both cTnI (AccuTnI, Beckman Access) and cTnT (Roche Elecsys 2010, Roche Diagnostics) in a 34-year-old woman with the catastrophic antiphospholipid antibody syndrome with MI [276]. At admission, the patient's cTnI value was 47 μg/liter that rose to 2121 μg/liter 8 h later, after the patient had undergone catherization and stent placement. The following morning the level had risen to 2847 μg/liter. Serial dilutions performed were all linear. These levels decreased to 161 on day 3, 125 on day 4, and 48 μg/liter on day 14. Cardiac TnT measured post catherization was 885 μg/liter and decreased to 264 on day 3 and 10 μg/liter by day 14. To the best of my knowledge, these are the highest values reported in the literature for both cardiac troponins in a patient who suffered an MI. A merit of this study is that it ruled out heterophilic antibody and rheumatoid factor interferences such that any interference present was due to antiphospholipid antibodies alone. However, no mention is made of the concentration of antiphospholipid antibodies measured. In addition, based on one case report with a person who had an MI anyway, it is difficult to offer any generalizations about the interference of antiphospholipid in cardiac troponin assays. Further reports are required to confirm these findings.

6.7. Proteolysis

It has been shown that cTnI is very susceptible to proteolysis and is therefore less stable in serum/plasma where it is rapidly degraded [51, 52, 168, 277]. The degradation of cTnI produces a wide array of fragments. N- and C-terminal regions are rapidly cleaved by proteases, whereas the fragment located between residues 30 and 110 are more stable, presumably because they bind to TnC, which confers some protection [168]. Cardiac TnT on the other hand has better stability but has been shown to be liable to proteolytic degradation [51].

Recently, it has been suggested that intracellular proteases, such as cathepsins, might be released into circulation from erythrocytes, which might cause the degradation of cardiac troponins [232]. However, to date evidence for this hypothesis has not been provided. If shown to be correct, it might explain the degradation of cTnT noted *in vitro* upon hemolysis [278, 279].

For reliable and reproducible results, it is important to take into account the role of proteolysis when selecting antibodies for cTnI immunoassays. Those used should preferentially recognize epitopes located in the regions

more resistant to proteolysis. Proponents in this field have indeed been advocating for this to occur [172].

6.8. Hemolysis

It has been observed that hemolysis causes interference in both the cardiac TnT and TnI assays [278, 279, 280]. Whether this interference in the assay is attributable to hemolysis per se or to proteolytic cleavage due to proteases released form erythrocytes when hemolyzed is not yet known [232]. It has been shown that a concentration-dependent negative effect of hemolysis on the measurement of cTnT is mediated by increasing hemoglobin concentration [279]. Another study has published that it is not hemoglobin that is the interferent in one cTnI assay but hemolysis itself [280]. This raises an important reminder that "hemoglobin is not equivalent to hemolyzate" [280]. Other factors present in, or released from the ruptured cells might interfere with the respective assay. One report has shown that with increasing hemolysis, there was a positive bias for cTnI and a negative bias for cTnT [278]. A mechanism to account for this divergent action is at present lacking.

6.9. Heparin

The use of plasma as a sole, universal sample for most if not all tests can improve turnaround time. It can also potentially avoid the incomplete separation that can occur when using serum in subjects receiving anticoagulant therapy, which in turn may potentially produce falsely increased results [281]. In fact, heparin-plasma was originally recommended for all cardiac troponin assays, but with reports of its interference in the measurement of both cardiac TnT and TnI this view has been altered. Gerhardt *et al.* [149] have reported that the mean cTnT was 15% lower in heparinized-plasma compared to serum. They demonstrated that both cardiac TnT and TnI (IMMULITE system) decreased with increasing heparin concentrations added to sera. They also showed that heparin-induced losses were greater in early than in late phases after onset of chest pain with losses of up to 33% at 1–12 h post-onset of chest pain, 17% at 13–48 h, and 7% after 48 h. They concluded that binding of heparin to troponins decreases their immunoreactivity, hence the decreased concentrations, although they did not provide any direct evidence to support this theory. The reduced immunoreactivity may be due to either a conformational change in the troponin molecules or the direct hindrance of the analytically relevant epitopes [282].

Another study by Steigler *et al.* [150] obtained both serum and heparin-plasma from 50 patients with ACS and 50 patients after open-heart surgery and measured cardiac TnT and TnI. The latter was measured on two

independent platforms: Abbott AxSYM and Bayer ACS:Centaur. This study also concluded that both cardiac TnT and TnI were markedly lower in heparin-plasma compared to serum with differences of greater that 20% seen in 11% of results on the ACS:Centaur, 9% of results on Elecsys cTnT, and 2% of results on the AxSYM. This study even demonstrated that treatment with heparinase reversed this decrease. In addition, they investigated the influence of incomplete serum separation and found that a second centrifugation had no effect on serum results by any of the stated assays.

Katrukha *et al.* [282] have noted similar results for a cTnI assay when measured in serum although others have not [41].

A more recent study by Speth *et al.* [283] has elegantly attempted to elucidate the mechanism by which heparin apparently affects cardiac TnT and TnI in plasma. The effect of heparin was analyzed by simultaneously collecting serum and heparin-plasma samples from 32 patients after coronary bypass surgery. The cTnT was measured using the Roche Elecsys, whereas the cTnI was measured using the Dade-Behring/Opus and Bayer ACS:Centaur immunoassay systems in the absence or presence of either heparin or protamine. Affinity chromatography was used to determine the association between the cardiac troponins and the anticoagulants. This study reported that indeed heparin did produce an apparent decrease in both cardiac TnT and TnI and pretreatment with heparinase or protamine did not completely reverse the heparin-induced decrease. Thus, addition of these reagents to commercial assays might not prove worthwhile. Affinity chromatography with heparin sepharose demonstrated that whereas cTnI shows minor affinity to the immobilized heparin and was eluted at near physiological conditions, cTnT was bound and could only be quantitatively recovered by solutions of high ionic strength. It was concluded that the decrease in TnT was a result of direct molecular interaction between the negatively charged basic residues within its sequence and heparin, in contrast to TnI whose interaction with heparin is induced indirectly by changes within the matrix itself.

In conclusion, heparinized plasma samples should not be used for the measurement of cardiac troponins due to the high likelihood of a false negative result being obtained. However, a recent report failed to show significant differences between serum and heparin-plasma [151]. Recently, Roche Diagnostics have released a fourth-generation assay that permits the utilization of heparinized plasma samples.

6.10. Other Conditions and Interferences

Various other conditions and interferences have been shown to affect the accurate measurement of cardiac troponins [284]. These are listed in Table 3. It is worth specifically mentioning that fibrin (from serum clotting) or

microparticles have been shown to produce false positive results in some cTnI assays [41, 264, 285]. A yet unidentified, common interfering factor (IF) that negatively affects the measurement of cTnI has also been reported [286, 287]. Antibodies against the midfragment epitopes of cTnI are affected by this IF to a significant but variable extent. Recently, this IF has been identified as circulating autoantibodies to cTnI [271, 175, 176].

Exercise, especially ultraendurance events (marathons) can also increase both cardiac TnT and TnI levels [288, 289, 290]. In studies where rats were forced to swim for up to 5 h with a weight attached to their tail, cTnT increased for up to 3 h after exercise and was followed by microscopic signs of myocardial necrosis. When made to swim for only 3.5 h or after an 8-day period of training, only small cTnT increases were observed without histological alterations [291]. This elegant study demonstrated that plasma cTnT levels were dependent on the duration of exercise and training status/fitness. Urhausen *et al.* [290] showed that of 105 asymptomatic ultraendurance event finishers, the concentration of cardiac TnT and TnI rose to above the 99% upper reference level in 24 and 34 subjects respectively. About 3 months after the event, 21 troponin-positive participants underwent an extensive cardiac evaluation, which showed that only 1 (who had a critical coronary heart condition anyway) revealed signs of cardiac damage. The release of cardiac troponin from the unbound cytosolic pool via a transient change in membrane permeability of the myocytes is one plausible mechanism to explain the elevation of cardiac troponins postexercise. A musculo–skeletal origin is also another possibility [292].

7. Conclusions and Future Directions

For the present time, it is my opinion that the cardiac troponins are destined to stay. Only time will tell whether a better successor will emerge; when it does, numerous studies will have to validate its utility and provide the appropriate level of evidence-base. Several generations of research and assay refinements have validated the cardiac troponins as a diagnostic and prognostic marker for the ACS. For the future, it is anticipated that more sensitive assays will emerge that might be able to achieve a precision of 10% (total CV) at the 99th percentile reference limit. In addition, the current detection limits of the cardiac troponin assays do not allow the measurement of normal levels in healthy controls. Thus, it should be the goal of cardiac troponin assay manufacturers to improve the sensitivity and precision of their respective assays to allow levels in healthy controls to be determined. Perhaps only then will progress be made in finally delineating the frontiers between ischemia and infarction. The reasons for, and the mechanisms for

the increase in cardiac troponins levels in conditions other than ACS also need to be elucidated. Why is the cardiac troponin present? How is it being released? And what does it mean? It is likely that the cardiac troponins detect the presence of malfunction rather than a single specific disease [293].

With the demonstration that the cardiac troponins can be separated from serum and analyzed by mass spectrometry [178], it is envisioned that this will aid in the development of better reference materials and possibly enable more easily the characterization of the cardiac troponin "fragments" shown in some studies [29, 51]. It is hoped that with the steps already taken by the AACC, NACB, and IFCC, the cTnI assays will be standardized so that there is more comparability across different trials and among studies being carried out. At the molecular level, the partial crystallization of the core domain of the human cardiac troponin has yielded vital information regarding its overall architecture [36]. The correlation of phosphorylated cardiac troponins with cardiac pathologies is an interesting avenue to venture into. Further work is already underway to add to the growing body of literature. Perhaps it is not long before we have the "unified theory of the interaction of troponin-tropomyosin-actin in muscle contraction and its role in health and disease."

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LEPTIN PHYSIOLOGY AND PATHOPHYSIOLOGY IN THE ELDERLY

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1. Abstract

Leptin is not only considered an adipocytokine, important in the regulation of food intake and energy balance but also a hormone with an important systemic, metabolic, and endocrine functions. Leptin appears to have an

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important role in human physiology and in the pathophysiology of different disease states. However, only a minority of studies has been specifically designed to address topics related to leptin physiology and pathophysiology in the elderly. This review focuses on the physiology of leptin production in the elderly. Age-related changes in body composition and decline in function of different hormonal axes may alter the physiology of leptin secretion in the elderly. It is unclear if aging has an independent effect on leptin levels, or if the association between leptin and fat content and distribution changes with age, or if a sexual dimorphism in leptin levels may be present in old age. Growing evidence, however, appears to support a role for leptin in the pathophysiology of different disease states in the elderly. Alterations in glucose and lipid metabolism as well as bone turnover, widely prevalent in old age, have been related to leptin.

2. Introduction

Since the discovery of leptin in 1994 [1], a large amount of scientific data has provided evidence that leptin is not only an adipocyte product important in the regulation of food intake and energy balance but also a hormone with an important systemic, metabolic, and endocrine roles [2–4].

Leptin is synthesized mainly by white adipose tissue and secreted into the blood stream. As such, circulating leptin levels are strongly related to the amount of fat [2–5]. In the central nervous system (CNS) leptin acts as a part of a feedback mechanism, signaling to the brain the amount of fat stored and regulating food intake and energy expenditure [2–4]. Leptin appears also to have several peripheral effects. Leptin plays an important role in normal sexual maturation and reproduction [2–4]. Interactions between leptin and the corticotropic thyroid, and growth hormone (GH) axes have been extensively described [2–4]. Moreover, it is now well established that leptin is an important regulator of glucose metabolism, directly influencing insulin secretion from pancreatic β-cells as well as insulin sensitivity of adipose tissue and skeletal muscle [2–4]. The immune system is another target of leptin affecting cytokine production, monocytes/macrophages activation, and proliferation of a variety of immune cells and hematopoietic progenitors [6]. Thus, leptin appears to have an important role in human physiology and pathophysiology of different diseases [2–4].

This review focuses, in the first part, on the physiology of leptin production in the elderly. Several nutritional and hormonal factors have been shown to regulate leptin production in humans [7]. However, it is not completely clear if age-related changes in body composition as well as the decline in function of different hormonal axes with aging may alter the physiology of leptin secretion

in the elderly. Thus, different hormonal, nutritional, and lifestyle factors involved in the regulation of leptin production and their interaction with aging are reviewed. Moreover, we have tried to establish if aging has an independent effect on leptin levels, if the association between leptin and fat content and distribution change with age, and if a sexual dimorphism in leptin levels is still present in old age. The second part of this review focuses on the role of leptin in different disease characteristics of elderly populations. A growing amount of evidence supports a role of leptin in the pathophysiology of anorexia in the elderly. Alterations in glucose and lipid metabolism as well as in bone turnover, widely prevalent in old age, have been partly ascribed to leptin.

3. Background

Leptin is mainly produced in white adipose tissue. Leptin mRNA expression has also been demonstrated in brown adipose tissue, a number of nonadipocyte tissues such as the gastric mucosa, mammary epithelial cells, myocytes, and the placenta [2–4].

At least six different splice variants of leptin receptors have been characterized differing in the extent of transmembrane and intracellular domains [8, 9]. These forms may transduce the leptin signal and may also be involved in transport/uptake of circulating leptin. A long isoform of the leptin receptor is present in hypothalamic centers and also in other tissues at low levels [8, 9]. Only the long form of this receptor appears to mediate intracellular signaling. The molecular mechanisms underlying leptin receptor activation have been widely investigated and seem to involve the Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) signaling pathway [8, 9].

Leptin is present in plasma in several forms, including free monomeric hormone and as bound to several different proteins [3, 10]. Leptin is partly bound to a soluble receptor, but the identity of other binding proteins is unknown. The free hormone is thought to be the biologically active form because only free hormone is detectable in cerebrospinal fluid [3, 10]. The relative proportions of free and bound leptin differ with degree of adiposity, with higher proportion of free leptin in obese subjects [10]. Despite this finding, elevated circulating leptin levels fail to mediate weight loss, suggesting that most human obesity represents a form of leptin resistance [2, 8, 9]. Leptin resistance is likely due to desensitization for leptin signaling with saturation of leptin transport across the blood–brain barrier or abnormalities at the level of leptin receptor activation and/or signal transduction [2, 8, 9].

Several studies have shown a central role for the kidney in leptin elimination [11]. However, it remains unclear whether leptin is removed from the plasma by glomerular filtration or by active uptake.

Serum leptin displays a diurnal rhythm with the highest levels between midnight and the early morning hours, after which leptin declines until early afternoon [12]. Little information is available about age-related changes in the circadian rhythm of leptin in humans. In the study of Franceschini *et al.* [13], a significant diurnal variation in leptin levels was generally preserved in a small group of elderly men. This study found an earlier acrophase and decreased amplitude for leptin when compared to 24-h variation observed in middle-aged men matched for body mass index (BMI). The mechanisms through which age may affect the diurnal variation in leptin levels are not understood even though a decrease of several factors stimulating leptin secretion has been described in elderly subjects [13].

4. Regulation of Leptin Levels in the Elderly

4.1. Leptin and Adiposity: Does Aging Change the Association Between Leptin and Fat Content and Distribution?

The main mechanism regulating circulating leptin levels is represented by the amount of adipose tissue itself. As leptin is synthesized mainly by white adipose tissue and then secreted into the bloodstream, its circulating levels are strongly associated with amount of body fat [2–5]. Several studies have described this significant association from young to middle age, between leptin and different surrogates of adiposity as body weight, BMI, waist and hip circumferences, and fat mass directly evaluated by computed tomography (CT) and dual energy X-ray absorptiometry (DXA) or indirectly measured by skin-fold thickness or bioelectrical impedance analysis (BIA) [2–5].

Aging is associated with substantial body composition changes in both sexes with a progressive decrease in muscle mass and increase in body fat, with central redistribution of adipose tissue [14–16]. Irrespective of body weight change, the actual amount of fat significantly increases with age [17]. It remains unclear, however, if aging is associated with changes in leptin secretion itself from adipose tissue. This is discussed later.

Does aging change the association between leptin and fat mass? In Table 1 we have summarized the main findings of studies that focused on the relationships between leptin and age, and between leptin and body fat content [18–29]. The studies were selected on the following criteria: publication time from 1994 to 2004, sample size of at least 100 subjects, and upper limit of age range of at least 60 years.

One of the first studies published in 1998 described a significant correlation between leptin and relative body fat content only in young subjects but not in

TABLE 1

CROSS-SECTIONAL STUDIES ON THE RELATION BETWEEN LEPTIN, AGING, AND BODY FAT IN SAMPLES OF SUBJECTS INCLUDING MEN AND WOMEN OLDER THAN 60 YEARS

Author	Sample m/w age, BMI	Measures of adiposity	Gender effect	Relation leptin–adiposity (adjustments)	Relation leptin–age (adjustments)	Predictors of leptin levels
Gomez JM *et al.* [18]	268 (134 m, 134 w) (Spain) 15–70 years BMI 20.9–32.8 kg/m^2	Anthropometric measurements BIA	Not evaluated	Positive correlation between leptin, BMI, and FM in both sexes	Increase in leptin in the fourth decade in men, in the last decades in women	In regression models age as positive predictor of leptin besides FM only in women
Mendoza-Nunez VM *et al.* [19]	197 (59 m, 138 w) (Mexico) wide age-range; normal- and over-weight	Anthropometric measurements	Present	Strong positive correlation between leptin and BMI	No significant difference in leptin between young and old subjects in both sexes	In regression models age as a negative predictor of leptin, besides BMI, gender, waist, HDL-C, and insulin
Ruhl CE, Everhart JE [20]	2937 m, 3366 w (USA cauc, non hisp blacks, mex-am) >20 years BMI 26.8 $\pm$ 5 kg/m^2 m; 26.4 $\pm$ 6.1 kg/m^2 w	Anthropometric measurements Skinfold thickness	Present	Strong positive correlation between leptin and measures of adiposity (adjusted for age and ethnicity)	Increase of leptin with age in both sexes	In regression models age was a negative predictor of leptin, besides ethnicity, sum of skinfold thickness, and waist in both sexes
Isidori AM, *et al.* [21]	150 m, 320 w (Italy) 18–77 years BMI 18.5–61.1 kg/m^2	Anthropometric measurements	Present (adjusted for BMI, age, E, T, SHBG, DHEAS)	Positive correlation between leptin and BMI in all groups	Significant decrease of leptin with age in women (adjusted for BMI)	In regression models age was a negative predictor of leptin besides BMI, E, and DHEAS in women, besides BMI, T, and SHBG in men

(*continues*)

TABLE 1 (*Continued*)

Author	Sample m/w age, BMI	Measures of adiposity	Gender effect	Relation leptin–adiposity (adjustments)	Relation leptin–age (adjustments)	Predictors of leptin levels
Neuhäuser-Berthold M *et al.* [22]	82 m (69 ± 5 years; BMI 26 ± 2.6 kg/m^2) 122 w (69 ± 6 years; BMI 26.3 ± 3.6 kg/m^2) (Germany)	Anthropometric measurements BIA	Present (adjusted for FM)	Positive correlation between leptin, BMI, and FM in both sexes	Significant decrease of leptin with age in women (adjusted for FM)	Not evaluated
Thomas T *et al.* [23]	345 m (23–90 years); BMI 25.3 ± 3.5 kg/m^2; 166 w (21–93 years); BMI 25.2 ± 3.9 kg/m^2 (USA 96% cauc)	Anthropometric measurements DXA	Present (adjusted for FM)	Positive correlation between leptin, BMI, and FM in both sexes (adjusted for age)	Significant positive correlation between leptin and age only in men	In regression models FM, FFM, E, T, DHEAS, and insulin explain up to 83% of leptin variance (in all groups)
Baumgartner RN *et al.* [24]	106 m, 166 w (USA) 62–98 years BMI 25.3 ± 3.5 kg/m^2 m; 25.2 ± 3.9 kg/m^2 w	Anthropometric measurements DXA	Present (adjusted for FM)	Positive correlation between leptin and FM in both sexes	Significant positive correlation between leptin and age in men (adjusted for FM);	In regression models age was no more a significant negative predictor of leptin besides gender, FM and insulin resistance after adding SHBG, T, or E.

	22 m, 52 w, 14 years follow-up				After 14 years follow-up significant increase in leptin only in men (stable BMI)	After 14 years follow-up increases in leptin were associated with T in men and with changes in BMI and age in women
Van den Saffele JK *et al.* [25]	271 m (72–78 years; BMI 26.3 ± 3.7 kg/m^2) 61 m (37–46 years; BMI 26 ± 3.4 kg/m^2) 40 m (23–29.5 years; BMI 22.8 ± 2.4 kg/m^2) (Belgium)	Anthropometric measurements BIA	Not evaluated	Positive correlation between leptin, BMI, and FM in the three age groups	Increase of leptin with age, but plateau after 45 years (even after adjustment for BMI)	In regression models age was a positive predictor of leptin besides BMI, insulin, and T
Moller N *et al.* [26]	24 m, 36 w (USA) 20–79 years BMI 19–35.8 kg/m^2	Anthropometric measurements DXA	Present	No association between leptin and FM in middle-aged and elderly subjects	Trend toward an increase in leptin levels in old women but toward a decrease in old men	In regression models only gender was associated with leptin in old subjects
Koistinen HA *et al.* [27]	132 m, 136 w (Finland) 22–85 years BMI from 24 ± 1 to 28.6 ± 0.8 kg/m^2 in different groups	Anthropometric measurements	Present	Not evaluated	75-year-old men had significantly higher leptin than 30-year-old men; 75-year-old women had significantly lower leptin than 65-year-old women (BMI adjusted)	Not evaluated

(*continues*)

TABLE 1 (*Continued*)

Author	Sample m/w age, BMI	Measures of adiposity	Gender effect	Relation leptin–adiposity (adjustments)	Relation leptin–age (adjustments)	Predictors of leptin levels
Perry HM *et al.* [28]	94 w (USA: 53 afr-am; 41 cauc) 18–89 years BMI from 23.4 ± 1.1 to 32.9 ± 1.9 8 kg/m^2	Anthropometric measurements DXA	Not evaluated	Positive correlation between leptin, BMI, and FM in the whole group, as well in afr-am and cauc women	Nonlinear relation between leptin and age (increase of leptin in middle-age and decrease in old age)	Not evaluated
Ostlund RE *et al.* [29]	84 m, 120 w (USA) 18–80 years BMI 27.6 ± 78 kg/m^2	Anthropometric measurements Skinfold thickness	Present (adjusted for FM%)	Positive correlation between leptin, BMI, and FM in the whole group	Significant negative relation between leptin and age (especially after adjustments for FM and gender)	In regression models age was a significant negative predictor of leptin besides FM in the whole group, as well in both men and women separately

m = men; w = women; cauc = Caucasian; non hisp blacks = non-Hispanic blacks; mex-am = Mexican–Americans; afr-am = African–Americans; BMI = body mass index; BIA = bio-impedance analysis; DXA = dual energy X-ray absorptiometry; FM = fat mass; FFM = fat-free mass; waist = waist circumference; E = estrogen; T = testosterone; DHEAS = dehydroepiandrosterone sulphate; SHBG = sex hormone-binding globulin; HDL-C = high-density lipoprotein cholesterol.

middle-aged and elderly groups [26]. This finding led the author to hypothesize a disruption in the relationship between fat and leptin with aging [26]. Several studies [18–25, 28, 29], however, did not confirm these results. These studies, performed in populations with wide age-ranges found significant correlation between leptin and adiposity, independent of body fat by a variety of measures: BMI [18–23, 25, 28, 29], fat mass estimate by BIA [18, 22, 25] or DXA [23, 24, 28], and skin-fold thickness [20, 29]. In 3366 women and 2937 men with a wide age-range participating in the Third US National Health and Nutrition Examination Survey (NHANES III), leptin levels were strongly related to BMI and different anthropometric measurements both in univariate analysis as well as when age and ethnicity were controlled [20]. Using DXA, Thomas *et al.* [23] found a strong and consistent association between leptin and fat mass in a wide sample of subjects (23–90 years) for both genders and any age group. Cumulatively, literature data confirms the existence of a strong association between leptin and body fat at least in healthy elderly subjects.

The relation between leptin levels and body fat distribution in adults as well as older subjects, however, remains unclear. The relation of leptin concentrations with measures of body fat distribution has been explored in several studies, including subjects with wide ranges of age and BMI [2–5, 20, 29–35]. It has been hypothesized that a stronger relationship exists between leptin and measures of subcutaneous fat compared to measures of total or visceral adiposity (BMI or waist circumference) due to greater leptin production in subcutaneous fat versus visceral adipose tissue [30–32].

In the cohort studied by van Gaal *et al.* [2] leptin significantly correlated with most anthropometric parameters, such as BMI, hip circumference, fat mass and fat-mass percentage, determined by DXA as well as with total abdominal fat and subcutaneous fat measured by CT. In this study the association between leptin and subcutaneous fat was significant even after adjustment for total fat [2]. Similarly, in obese patients with a wide age-range, a very strong relationship was observed between leptin levels and subcutaneous fat thickness, but not intra-abdominal fat thickness as determined by ultrasound [33]. This association persisted when total adiposity was taken into account [33].

In the wide NHANES III study, leptin levels were related to waist and hip circumferences as well as to skin-fold thickness independent of BMI [20]. However, in a sample of 204 subjects with wide age-range and BMI, no independent relationship between plasma leptin and fat distribution indices was described [29]. In this study, the negative relationship found between waist to hip ratio and leptin level was abolished after adjustment for body fat and gender [29]. Other authors have described an association between leptin levels and waist circumference independent of BMI [34, 35].

Clinical studies have provided conflicting evidence as to the relationship between fat distribution and leptin in adults and older subjects. A likely

contributor to this uncertainty is the fact that the majority of methods used to evaluate body fat distribution indirectly estimate subcutaneous and visceral fat and, at times, cannot completely discriminate these compartments. For example, waist circumference is a measure of abdominal obesity. However, it is unable to distinguish between visceral and subcutaneous abdominal fat. It is important to note, however, that subcutaneous adipose tissue usually accounts for up to 90–95% of total body fat. As such, it is not surprising that most studies have shown that the total amount of fat is the best predictor of leptin level thus abolishing any association of leptin with anthropometric measurements.

4.2. Leptin and Gender: Is a Sexual Dimorphism in Leptin Levels Still Present in Old Age?

From the first clinical study on leptin it was evident that a clear gender difference existed [2–5]. Leptin levels were found to be two- to threefolds higher in women than men [2–5]. This sexual dimorphism is consistent in studies that also included elderly subjects [19–24, 26, 27, 29, 33, 34, 36–40] (Table 1).

Gender differences in leptin level is not surprising because leptin is secreted mainly by adipose tissue and particularly by the subcutaneous depot [30–32]. In addition, women have significantly higher amounts of total body fat with peripheral distribution during aging [14, 16]. Thus, gender differences in leptin level should be interpreted only after accounting for body fat.

Even after adjustment for total fat mass, directly measured by DXA [23, 24, 36] or indirectly estimated by bioimpedance [22, 33], a gender difference in leptin levels was present in several studies that included elderly men and women. In a sample of healthy elderly subjects, we have recently shown that elderly women had significantly higher circulating levels of leptin compared to men even after adjusting for age, fat mass, or waist circumference [36]. Similarly, after correction for body fat percentage, measured by hydrodensitometry in 67 men and women with a wide age and BMI range, leptin levels were still significantly higher in pre- and postmenopausal women compared to men [37]. However, controlling for percent body fat completely eliminated the sex difference in leptin levels described in 3000 subjects (70–79 years) who participated in the Health, Aging, and Body Composition Study [38]. Similarly, in another wide cohort of elderly men and women, percent body fat explained all gender differences in leptin concentration [39]. Differences in the study sample characteristics and body fat measurements could explain the discordant results of these studies [38, 39] compared to the findings of others [22, 24, 33, 36, 37].

On the other hand, if we assume that leptin is secreted in proportion to fat mass, but leptin circulating level depends on its distribution into blood volume (represented by lean mass), fat mass adjusted for lean mass would provide, in theory, the best adjustment to be used to interpret the gender difference in leptin level. Although Rosenbaum *et al.* [37] first hypothesized that lower fat-free mass and blood volume in women could explain higher leptin level, this association was not tested. In the San Luis Valley Diabetes Study of Marshall *et al.* [39], leptin was inversely related to lean mass. Adjustment for lean mass, in addition to fat mass, consistently reduced the gender difference in leptin level in these subjects. In this context, lean mass may be more than just a surrogate for blood volume as a strong correlation between fat-free mass and different hormones have shown.

Many of the studies conducted to explain the sexual dimorphism in leptin levels have hypothesized a role for sex hormones [40]. *In vivo* and *in vitro* studies support a primary endocrine basis for this sexual dimorphism. Estrogens increase *in vitro* leptin production in adipose tissue from women but not men [41]. In women with a wide age-range, serum leptin was related to both serum estradiol and testosterone in univariate analysis [28]. Using a multiple linear regression model, Isidori *et al.* [21] described a significant and independent association between circulating leptin level and BMI, age, and estradiol in nonobese and obese women. Androgens, on the other hand, inhibit leptin production. Testosterone has been shown to suppress leptin mRNA expression and leptin secretion in adipose cell cultures from men [42]. BMI, age, and testosterone were significant contributors to serum leptin level in a group of nonobese and obese men [21]. Similar findings were reported by different investigators who found a significant negative association between testosterone and leptin level even after adjustment for BMI [25] or fat mass [23, 24] in men with a wide age-range.

However, the study of Isidori and colleagues [21] found that gender differences in leptin level could not be fully explained by differences in sex hormones, BMI, or any other investigated variable. This report supported the hypothesis of a genetic origin of sexual dimorphism in leptin level.

Martin *et al.* [43] used full pedigree-based variance decomposition analysis to examine the contribution of both genetic and environmental factors to sexual dimorphism in leptin level. In 1147 Mexican-Americans from the San Antonio Family Heart Study, the sexual dimorphism in leptin was not simply explained by differences in fat mass between sexes, but was the result of differential expression of genes by sex as well as due to the effect of sets of sex-specific genes [43]. In the same study the author also hypothesized the existence of environment by sex interactions influencing serum leptin levels, suggesting that men and women may react differently to environmental factors [43].

4.3. Leptin and Aging: Does Aging have an Independent Effect on Leptin Levels?

To clarify the relation between leptin circulating levels and aging we limited our search on Medline to papers focused on this outcome, published from 1994 to 2004, with a sample size of at least 100 subjects and upper limit of age-range of at least 60 years. A summary of these studies [18–29] is presented (Table 1).

As can be seen, studies have reported discordant results. Some authors have described a positive relationship between leptin and age [18, 20, 23–25, 27]. In contrast, others have described a negative relationship [21, 22, 27, 29]. In some, no significant relationship was found [19, 26]. Results of these studies may be biased by characteristics of the study sample itself, by the type of statistical analysis performed, as well by the adjustments made for potential confounders.

4.3.1. *Sample Characteristics (Age, BMI, and Gender)*

Differences in the size of the study population, and in particular in the size of each age group, and therefore in statistical power of the study, could explain at least in part discrepant results between different studies.

Only a few studies [23, 24, 27, 28] included subjects older than 80 years of age. As such, it is difficult to interpret the relationship between leptin and age in the oldest age groups. Further it is not clear if the relationship between leptin levels and aging may be altered by obesity. Only Isidori *et al.* [21] investigated the association between leptin and age after stratifying the study sample into normal- and obese-weight subjects.

4.3.2. *Type of Statistical Analysis*

The use of regression models appears to be the best method to estimate adjusted leptin concentration [39]. In fact, as pointed out by Marshall *et al.* [39], the use of ratios between leptin concentration and measure of body fat assumes that a linear relationship exists between leptin and adiposity as well as an intercept of zero. If this assumption is not met, spurious results may likely occur. Finally not in every study has been performed log transformation of leptin [28] despite the observation that leptin levels are highly skewed [2–5].

4.3.3. *Adjustment for Body Composition*

As previously mentioned, because leptin is mainly produced by adipose tissue, adjustment for the amount of fat is mandatory in the interpretation of the relationship between leptin and age.

Some of the studies adjusted for BMI to assess the relationship between leptin and age [19, 21, 25, 27] (Table 1). BMI, however, does not appear to be

a reliable measure of body fat especially in elderly cohorts. Even without BMI adjustment, changes in the amount of fat significantly increase with aging [17].

Several studies (Table 1) considered the relationship between leptin and age independently of fat mass [18, 20, 22–24, 29]. However, in some of these [20, 29], percentage body fat was calculated from data on skin-fold thickness using age-specific regression equations. In others [18, 22], fat mass was derived from bioimpedance equations where age was a covariate. As expected, unreliable information on the independent relationship of age to leptin level occurs when using in the statistical analysis indirect measures of body adiposity in which age is *a priori* included in equations used to obtain these measures. Moreover, DXA and CT measures appear more accurate than skin-fold thickness or bioimpedance analysis to assess body composition in the elderly [44].

4.3.4. *Adjustment for Other Confounders*

Leptin is strictly related with several hormonal pathways [2–4, 7]. However, only a few studies reported hormonal measurements as covariates in multiple regression analyses to evaluate the independent contribution of age to leptin levels [19, 21, 23–25]. Other characteristics of the study population might further confound results. In fact, health and nutritional status, weight change, diet composition, and level of physical fitness are all factors known to regulate leptin production in humans [2–4, 7].

After considering the different characteristics of study samples, type of statistical analysis performed, and adjustments used, only three studies [21, 24, 29] appear sufficiently well designed to define the relationship between aging and leptin level. These three studies were conducted in populations with similar age ranges. One study [21] also included obese as well as normal-weight subjects. More importantly, all three studies took into account fat mass and performed multiple regression analyses to assess the independent effect of age on leptin level. Isidori *et al.* [21] found that BMI-adjusted leptin levels were progressively lower with increasing age in women, especially after menopause, whereas in men only a nonsignificant negative trend was described. In the same study multiple regression analysis showed that age was negatively correlated with leptin in both genders and was independent of BMI and sex hormones [21]. Similarly, the study of Ostlund *et al.* [29] found that age was a significant negative predictor of leptin in multiple regression models besides fat mass in the group as well as in men and women separately. Baumgartner *et al.* [24], however, showed a slight nonsignificant decrease with age in leptin in women, but a significant increase of leptin with age in men independent of body fat. In this study a multiple regression model was used. These results were further supported by longitudinal analyses in a small

group of elderly subjects after 14 years of follow-up without BMI changes [24]. In this study, leptin levels were found to be significantly increased in men but not women [24]. It might be possible to hypothesize that age-related increases in serum leptin in men could be associated with age-related decrease in serum testosterone independent of body fat. In fact, in this study, age was no more a significant predictor of leptin independent of body fat after accounting for sex hormones [24].

It is still not definitively clear if aging has an independent effect on leptin production from adipose tissue. However, if changes in serum leptin occur with age in elderly men and women, these findings can only partly be explained by changes in body fat.

4.4. Leptin and Hormonal Changes During Aging

A number of hormonal factors have been identified in experimental and human studies that influence leptin production [3, 4, 7] (Fig. 1). The influence of estradiol on circulating leptin levels is still poorly understood. Estrogens increase *in vitro* leptin production in adipose tissue from women [41]. In women with a wide age-range, serum leptin was related to estradiol levels in univariate analysis [28]. However, most studies found no difference in serum leptin levels between pre- and postmenopausal women when matched for BMI [45, 46]. Hormone replacement therapy also does not seem to affect leptin levels in postmenopausal women [45].

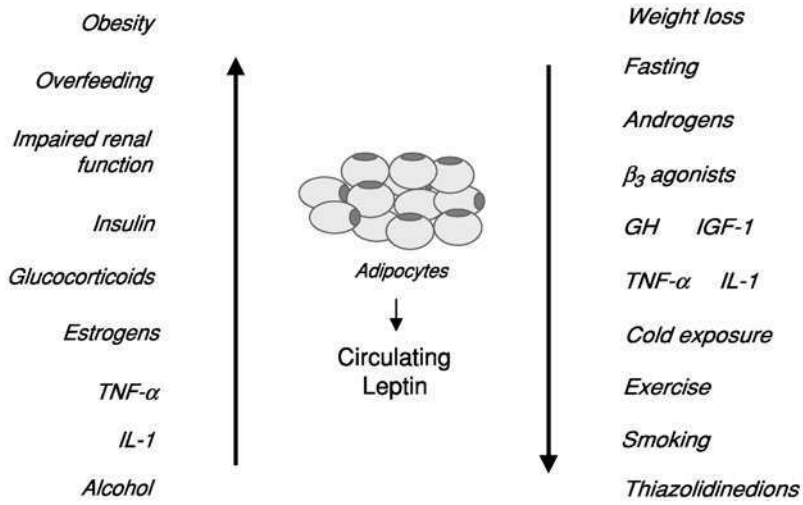

FIG. 1. Regulation of leptin production in humans. TNF-α = tumor necrosis factor alpha; IL-1 = interleukin-1; GH = growth hormone; IGF-1= insulin-like growth factor-1.

Epidemiological and experimental data support a negative effect for androgens on serum leptin levels in men. Testosterone has been shown to suppress leptin mRNA expression and secretion from adipose cell cultures from men [42]. BMI, age, and testosterone were significant contributors to serum leptin level in a group of nonobese and obese men [21]. Others, however, have described a significant negative association between testosterone and leptin level even after adjustment for BMI [25, 47] or fat mass in elderly men [23, 24]. The relationship between testosterone and leptin levels in women remains controversial. Some have reported a positive association [28] but others have not [48]. Söderberg *et al.* [49] described a strong association between testosterone and leptin in men and women from the Northern Sweden MONICA Project. This subsample study showed a negative relationship between testosterone and leptin level in men and a positive relationship in women. However, in both sexes this association was lost with increasing adiposity [49].

Insulin is one of the most important regulators of leptin production. *In vitro* insulin has been shown to stimulate mRNA expression and leptin secretion from cultured human adipocytes [50, 51]. Moreover, in clamp experiments, hyperinsulinemia led to a long-term rise in leptin level [51]. Finally, insulin treatment was reported to raise leptin level in diabetic patients [52].

Similarly to insulin, glucocorticoids have been reported to increase leptin and leptin mRNA *in vitro* and *in vivo* [50, 53]. Increased leptin secretion could be related to elevated insulin levels induced by glucocorticoids, thus, raising the possibility of an indirect effect of this group of hormones on leptin. However, Larsson *et al.* [54] found that increased plasma leptin after dexamethasone treatment was independent of insulin level or sensitivity. Data from *in vitro* studies indicated a synergistic action of both insulin and cortisol in maintaining leptin production in adipose tissue [53].

Whether leptin secretion from adipose tissue may also be influenced by GH is unclear. GH receptors are expressed on adipocytes, but chronic incubation with either GH or insulin-like growth factor-1 (IGF-1) had no effect on leptin secretion and leptin mRNA expression [50]. Several studies suggested an inhibitory role of the GH axis on leptin production not only in adult and young-old subjects but also in healthy centenarians [55, 56]. However, it is not clear if GH has a direct effect on leptin production or rather an indirect effect through suppression of insulin secretion and stimulation of lipolysis in adipocytes. Moreover, acute or chronic GH administration has been proven to alter leptin levels only in subjects with GH deficiency [55, 57, 58]. However, decreased leptin level in these patients, concomitant to chronic treatment, often paralleled the observed decrease in body fat and was not independent of body composition change related to GH replenishment [57]. In the study of Gill *et al.* [58], chronic GH treatment failed to change

leptin level despite favorable changes in body composition in GH-deficient elderly subjects [58]. However, discontinuation of GH reversed the body composition change and led to increased leptin [58]. In conclusion, the GH axis may be involved in the regulation of leptin level, but it is unclear whether this phenomenon is a direct effect or merely an indirect effect mediated through changes in body composition and insulin.

In addition to hormones, cytokines have been shown to influence leptin production in an autocrine or paracrine manner. In the study conducted by Bruun *et al.* [59], a significant effect proinflammatory cytokines interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) on leptin release was described. In this experiment the initial increase in leptin release, probably due to secretion of preformed peptide, was followed by long-term decrement in leptin release and gene expression [59]. Leptin production, however, was unaffected by IL-6 or IL-8 in this study [59].

4.5. Leptin and Nutritional Status

Leptin level is profoundly influenced by fasting. Short- and long-term energy restriction is associated with a reduction in circulating leptin level in young to middle-aged subjects [60–62]. Plasma leptin was shown to decline progressively to 40–70% of baseline values during 4–36 h of fasting, despite no significant change in fat mass for obese and lean subjects [61]. Leptin concentration, however, rapidly returned to baseline values when calorie restriction is terminated. Acute overfeeding increased plasma leptin by 40% after 5 h [62]. The mechanism for these findings is unclear. To our knowledge no data are available in old age subjects.

Leptin level appears to be related to alterations in macronutrient diet composition [7]. In animal models, a high-fat diet increased leptin mRNA expression and circulating leptin level [63]. In humans, the relationship between diet composition and leptin level is still not well understood. This relationship appears complicated by insulin change and other factors that influence energy metabolism and as such may be different in each gender. Total fat and monounsaturated fat intake were positively associated with plasma leptin level, even after adjusting for BMI and other confounding factors, only in normal-weight men aged 47–83 years [64]. In another study, a diet rich in α-linoleic acid and monounsaturated fatty acids significantly increased leptin concentration only in men, whereas a significant decrease in leptin level was observed in women [65]. In the same study neither a diet rich only in monounsaturated fatty acids nor in *n*-6-polyunsaturated fatty acids affected leptin levels in either sex [65].

Previous reports have suggested that carbohydrate intake, rather than fat consumption, may be most strongly associated with changes in leptin level

[66]. It has been hypothesized that total body fat mass and insulin resistance in overweight and obese subjects may be more important in regulation of leptin level than dietary fat [64].

Plasma leptin level increase with weight gain and decrease with weight loss also in old age are consistent with leptin's role as a signal of the size of adipose tissue stores [60, 62, 66–70]. Short-term weight loss programs decrease leptin levels such that a subject at a reduced body weight has a lower blood leptin level than weight-stable subjects with similar body weight [67]. Long-term dietary intervention (i.e., greater than 6 months) has also been proven to significantly affect leptin level in old adults with knee osteoarthritis [68]. It is noteworthy that even modest (5–6%) weight loss resulted in significant reduction in leptin level [68, 69], whereas other circulating adipocytokines are unaffected [69]. Finally, a gender difference has been described in the response of leptin to weight loss in obese old subjects [70]. Nicklas *et al.* [70] found a greater relative decline in circulating leptin level in women despite similar changes for both genders in fat-mass percentage. The reason for this reported gender difference is, however, unclear.

Different studies have shown that lower baseline leptin level predicts subsequent weight loss [68, 71]. Lower leptin level predicts greater weight loss in a wide sample of older subjects, even after adjusting for baseline weight, age, gender, race, and type of intervention [68]. Lower leptin level may indicate the presence of a lower degree of leptin resistance with leptin receptors more sensitive to the effect of leptin itself and thus biochemically capable of producing desirable weight loss.

4.6. Leptin and Lifestyle Factors

Although several lifestyle factors have been related to leptin level [2–5, 7], relatively few papers have addressed this topic in elderly subjects. The effect of exercise on circulating leptin level is unclear due to confounding factors [72, 73]. Studies that used indirect measures of physical activity presented discrepant results, describing either no association [34, 35] or a negative association [64] between leptin and physical activity after adjustment for BMI. Data from studies examining directly the effects of physical activity suggest that single exercise bouts (short duration of <60 min or nonexhaustive exercise) did not alter serum leptin level [72, 73]. Long duration exercise bouts (>60 min), however, induced a decrease in leptin concentration [72, 73]. These short-term changes in leptin level have been attributed to circadian rhythms, hemoconcentration or changes in energy balance rather than exercise *per se* [72, 73].

Training studies, after taking into account the effects induced by weight loss, have documented that short-term exercise (<12 weeks) does not affect

leptin level [72, 73] with the exception of diabetic subjects [74]. More controversy, however, exists about the effect of long-term training (>12 weeks) [72, 73]. These studies found no effect or a reduction in leptin level even after adjusting for fat-mass changes. The mechanisms explaining the relationship between physical activity and leptin levels are unclear. Physical activity may be associated with decreased plasma leptin level via decreased adiposity and leptin production. Alternatively, exercise may increase leptin sensitivity, followed by subsequent decline in leptin production through an unknown feedback mechanism. Other hypotheses have included exercise related changes in energy balance, improvement in insulin sensitivity, and alteration in lipid metabolism.

Several studies have reported an inverse association between cigarette smoking and plasma leptin level [35, 64, 75, 76]. The study of Chu *et al.* [64] found that current smokers had significantly lower leptin level than past smokers and those who never smoked even after adjusting for BMI. These results led investigators to hypothesize that smoking may be associated with increased leptin sensitivity [35, 64]. This premise could explain in part the usual weight gain that typically follows smoking cessation [76]. Weight gain after smoking cessation, however, appears not associated with differences in leptin level. This finding was described in a small group of ex-smokers in whom leptin level did not significantly change despite increased hunger [77]. It can be hypothesized that even without significant changes in leptin level, smoking cessation could lead to decreased leptin sensitivity, thus, favoring weight gain.

It is still debated if alcohol intake is a major modulator of leptin production. Some investigators found no association between alcohol intake and leptin level [35, 64], whereas others described a positive relationship [75]. Recent results from a controlled feeding and alcohol ingestion study indicated that moderate alcohol consumption increased leptin level in postmenopausal women, even after accounting for BMI [78]. The pathophysiological mechanism that links alcohol intake with increased leptin levels is unknown. However, the study of Kiefer *et al.* [79] suggested that alcohol intake, through increased TNF-α, may enhance leptin secretion, alcohol craving, and ultimately alcohol intake, thus, creating a vicious cycle.

5. Clinical Implications of Leptin Physiology in the Elderly

Based on the above, it is certain that leptin physiology remains not particularly well understood in older patients. This confusion is, however, further exacerbated in older patients with different disease states typically found in aging.

In recent years, investigators have hypothesized that leptin may be involved in the pathogenesis of chronic disease states including diabetes, metabolic syndrome, dyslipidemia, anorexia, and malnutrition as well as hypertension, atherosclerosis, osteoporosis, and osteoarthritis [2–5]. Although these diseases are highly prevalent in old age, existing data on the role of leptin in a specific disease state has not been unequivocal. Furthermore, the available literature data were not always obtained in an elderly population. Because of this limitation, the second part of the review addresses those diseases in which the role of leptin has been supported by relevant and unequivocal findings from data collected in elderly populations.

6. Leptin and the Anorexia of the Elderly

Several cross-sectional and longitudinal studies have described a decline in food intake with aging in healthy elderly subjects [80–83]. This physiological decrease in food intake with age was defined as anorexia [80–83]. In the NHANES III an average decline in energy intake of about 1000 kcal/day (men) and 500 kcal/day (women) between the age of 20 and 75 years was described [84]. Aging was also associated with a decline in total energy expenditure that is accounted not only by a decline in physical activity but also by a decrease in resting metabolic rate [83]. When the decrease in energy intake is greater than the decrease in energy expenditure, involuntary weight loss may occur. Anorexia in the elderly and involuntary weight loss has been related to adverse outcomes, such as increased risk of sarcopenia, frailty, functional impairment, and mortality [80–83].

Anorexia has multiple causes including alterations in taste, flavor and palatability of food, increased gastrointestinal satiations signals, and decline in central feeding drive [80–83]. A role for leptin has been advanced in this multifactoral pathway due to its ability to decrease food intake and increase resting metabolic rate [80–83]. Thus, increased leptin level with age could play a role in anorexia of aging. Increased circulating leptin level in aging humans is largely due to increased fat mass. Adjustment for fat mass, however, eliminates this relationship in most [20–23, 25, 29] but not all studies [18, 24, 27]. Some investigators have speculated that increased leptin in males is due to the age-related decrease in testosterone that ultimately leads to decline in food intake.

A second hypothesis linking leptin to anorexia of aging is that aging may be associated with alteration in leptin sensitivity. Little information on this topic, however, is currently available. Ma *et al.* [85] hypothesized that the high plasma leptin level observed in different aging models may not be completely explained by the age related increase of body fat. This premise

suggested the existence of a leptin-resistant state of aging because aging rats showed impaired leptin responsiveness with respect to food intake, fat mass, and distribution compared to young animals [85]. The impact of aging was also shown in adenovirus transfected hyperleptinemic diabetic rats [86]. In old rats the decline in food intake, body weight, and body fat following increased leptin levels was significantly lower than that observed in young rats [86]. Gabriely *et al.* [87] found a marked decrease in leptin's ability to decrease food intake in aging rats that underwent calorie restriction to maintain body fat and a metabolic pattern similar to that of young animals. Cumulatively, these results [85–87] imply a resistance to the effects of leptin with aging.

Fasting normally suppresses leptin level, thus, stimulating food intake. Reduced suppression of leptin level as well as a reduced increase in hypothalamic neuropeptide Y (NPY) by fasting has been reported in old versus young rats [88]. Monkeys on long-term caloric restriction did not have significantly lower food intake despite their low-leptin level when compared to controls [89].

Given the confusing animal experimental data in the above reports, it is very difficult to derive any definitive conclusion regarding the role of leptin in physiological anorexia of aging. Clearly further research including comprehensive human studies is mandatory to elucidate the contribution of leptin in decreased food intake with old age.

7. Leptin and Glucose Metabolism

A number of epidemiological studies have examined circulating leptin level in diabetic and nondiabetic subjects with discrepant results [52, 90, 91]. It is still debated whether different degrees of glucose tolerance may affect plasma leptin level. In elderly overweight patients, plasma leptin did not differ between diabetic and nondiabetic men and women after accounting for age and fat mass [90]. Among US Pima Indians, subjects with type 2 diabetes had lower leptin concentration than nondiabetic subjects independent of body fat percentage [91].

However, there is clear evidence demonstrating a strong relationship between leptin, insulin, and indices of insulin resistance independent of body fat in both men and women with or without diabetes mellitus. A number of studies have been published in recent years on leptin and its relationship to body fat and insulin resistance [2–4]. However, only a few of these were performed in older subjects [34, 92–94]. In a group of 107 nondiabetic women aged 67–78 years with a wide range of BMI, we previously showed that leptin was significantly related to insulin level and insulin

resistance, evaluated by homeostasis model assessment of insulin resistance (HOMA), even after adjusting for age and fat mass [92]. In this study leptin, waist size, and age as a group accounted for up to 31–33% of insulin level and HOMA total variance, respectively [92]. In contrast, about 22% of insulin level and HOMA were accounted for by leptin alone [92]. An independent association between leptin and insulin concentration after adjustment for BMI has also been observed by Zimmet *et al.* [34]. This study examined Polynesian men and women with a wide age-range with and without diabetes. Donahue *et al.* [93] reported an association between leptin and insulin resistance as evaluated by euglycemic-hyperinsulinemic clamp in 49 young to middle age men and women even after adjusting for body fat percentage. The role of leptin in determining insulin resistance in elderly men and women has recently received further support in a study comparing metabolic characteristics of normo- and hyperlipemic elderly subjects [94].

Several mechanisms link leptin to glucose metabolism. Some of these studies have been clearly defined, whereas areas of uncertainty exist for others [95, 96]. In human and animal models, prolonged hyperinsulinaemia leads to increased leptin gene expression and increased circulating leptin level [51, 53, 97]. Soderberg *et al.* [98] demonstrated a significant association between proinsulin and leptin in healthy men and women. This association, however, was lost with increased central adiposity [98]. The association between insulin and leptin thus appears dysfunctional in insulin resistance syndrome.

On the other hand, leptin itself has been shown to reciprocally regulate insulin production. Functional leptin receptors have been shown to be expressed in pancreatic islets [99]. Leptin treatment of pancreatic islets resulted in a marked suppression of insulin secretion as well as decreased proinsulin mRNA expression [100]. These results support a negative feedback mechanism for leptin on insulin production. Inhibition of insulin secretion increases as leptin levels increase and adipose mass enlarges. However, in susceptible obese patients chronic hyperleptinemia could desensitize leptin receptors in pancreatic islets and lead to increased insulin production and eventually hyperinsulinemia. This mechanism has been hypothesized to be involved in the pathogenesis of obesity and adipogenic diabetes mellitus [95].

Leptin has an important site of action in the liver. Rapid elevations in leptin level have been associated with increased hepatic gluconeogenesis as well as decreased glycogenolysis [101]. Leptin induces a redistribution of intrahepatic glucose fluxes switching hepatic substrate oxidation from carbohydrates to lipids favoring β-oxidation of fatty acids [95, 96].

The role of leptin on glucose metabolism in skeletal muscle is still not clear [95, 96]. Leptin mimics the effects of insulin on glucose transport and glycogen synthesis in skeletal muscle through a mechanism mediated in part by the

CNS [95, 96]. In contrast, leptin, and insulin have opposite effects on lipid metabolism in skeletal muscle. Leptin has been shown to favor lipid oxidation in skeletal muscle, whereas insulin promotes lipid storage as triglyceride [102]. It is interesting to note that both obesity and type 2 diabetes are associated with decreased fatty acid oxidation and increased concentration of muscle triglycerides.

8. Leptin, Leptin Resistance, and the Metabolic Syndrome

Metabolic syndrome is defined by the concomitant presence of at least three of the following characteristics: waist greater than 102 cm (men) and 88 cm (women), serum high-density lipoprotein cholesterol concentration lower than 1.0 (men) and 1.3 mmol/liter (women), serum triglyceride concentration greater than 1.7 mmol/liter, blood pressure greater than 130/85 mmHg, and serum glucose concentration greater than 6.1 mmol/liter [103].

The prevalence of the metabolic syndrome increases with aging, rising from about 4% at the age of 20 years to almost 50% at the age of 60 years [104]. Concomitant with increased prevalence of metabolic syndrome is increased body fat [105, 106] and particularly visceral fat. Visceral fat has been recognized as a main factor in the pathogenesis of the metabolic syndrome [107]. Body fat distribution is associated with several components of the metabolic syndrome in the elderly [108]. In addition to the role of total visceral fat, it was hypothesized that peptides produced by adipocytes might also explain the higher prevalence of metabolic syndrome in older age groups [109]. Leptin can modulate many of the metabolic changes that are characteristic of aging [2–4, 95, 96, 110].

Aging has been considered the most common cause of impaired leptin sensitivity at both hypothalamic and peripheral levels [111]. Unger *et al.* [111] suggested that the physiological role of hyperleptinemia might be to permit the storage of surplus calories in adipocytes (without injury to other tissues from ectopic lipid deposition) in order to extend survival during famine [111]. However, at some point, a substantial subset of patients with hyperleptinemia may undergo decompensation. As such, ectopic lipid deposition may occur with subsequent development of metabolic syndrome [111]. The mechanism hypothesized for this proposed decompensation is decreased leptin sensitivity. In rats there are consistent findings supporting a causative role for leptin resistance in metabolic decline of aging [85–87, 112]. Hyperleptinemia induced by leptin infusion [85] or adenovirus transfection [86] failed to produce a significant change in food intake, fat mass and distribution, and in hepatic and peripheral insulin action in old rats compared to young

animals. Similar results suggesting a failure in leptin action were described in aging rats that underwent calorie restriction throughout their life to be metabolically similar to young rats [87]. Leptin resistance was also reflected in the inability of older rats to suppress leptin gene expression in response to leptin infusion [85].

The mechanisms for leptin resistance are under intensive investigation. Decreased availability of leptin in the hypothalamus, impaired peripheral leptin action, or both have been proposed as mechanisms of leptin resistance during aging [111]. Scarpace *et al.* [112] described a metabolic response typical of leptin resistance following both central and peripheral leptin infusion in older rats. Following central leptin infusion, NPY mRNA levels did not significantly change in older rats compared to young rats [112]. STAT3 phosphorylation was found to be significantly diminished with age [112]. These data indicate that there may be two components to leptin resistance with aging, peripheral, and hypothalamic. Further research is clearly necessary to extend these interesting animal model findings to humans.

Leptin resistance may play a central role in the development of metabolic syndrome with aging. Leptin resistance may be due to obesity and elevated serum leptin with age, age itself, or both. Impressive evidence has accumulated over the past decade that generalized and abdominal obesity constitute low-grade inflammatory states with adipose tissue playing an active role with the production of different proinflammatory cytokines [113]. Diabetes mellitus with concomitant metabolic syndrome is characterized by increased levels of several acute-phase markers [114]. As such, pathways leading to metabolic syndrome may involve not only leptin but also a number of different adipokines and mediators of the inflammatory response [115]. For example, we have recently shown a positive association between insulin, insulin resistance, and leptin as well as a negative relationship with levels of adiponectin, another cytokine, exclusively produced by adipose tissue in a small group of elderly men and women [36]. In this study leptin and adiponectin were significant independent predictors of HOMA explaining about 40% of its total variance [36].

Our understanding of the molecular basis for metabolic syndrome has consistently increased in recent years. A central role for the nuclear transcription factor-κB (NF-κB) has been hypothesized as a possible point of signal convergence from different adipocytokines that ultimately lead to the clinical features of metabolic syndrome (Fig. 2) [115]. Aging can directly modulate adipocytokines expression from adipose tissue or indirectly through increased body weight and fat mass. TNF-α has been shown to stimulate leptin production from adipose tissue [59] as well as positively correlate to leptin level in old age [116].

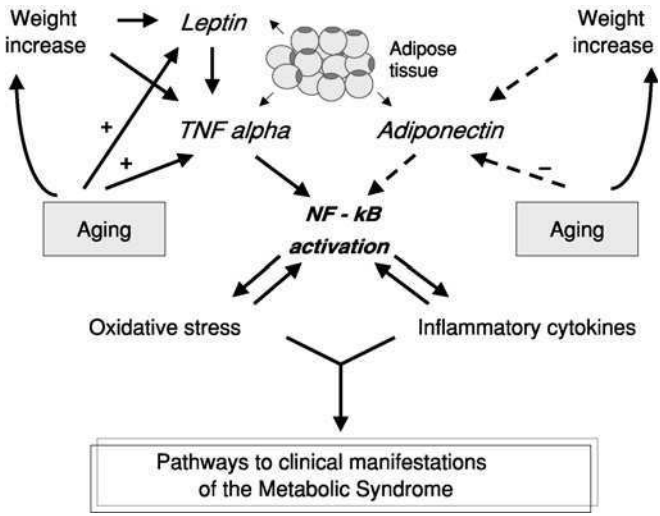

FIG. 2. The role of different adipocytokines and aging in the pathways leading to clinical manifestations of the metabolic syndrome. TNF-α = tumor necrosis factor alpha; NF-κB = nuclear transcription factor-κB; dashed lines = inhibitory effect. Aging can modulate adipocytokines expression from adipose tissue directly or indirectly through an increase in body weight and fat mass. TNF-α and leptin may stimulate NF-κB activation, antagonizing the effect of adiponectin. NF-κB activation contributes to the development of insulin resistance, dyslipidemia, endothelial dysfunction, and atherogenesis through increased oxidative stress and increased production of inflammatory cytokines and adhesive molecules.

It has been suggested that TNF-α and adiponectin are antagonistic to stimulating NF-κB activation [115]. NF-κB activation contributes to the development of insulin resistance, dyslipidemia, endothelial dysfunction, and atherogenesis through increased oxidative stress and increased production of inflammatory cytokines and adhesion molecules [115].

9. Leptin and Bone Metabolism

Leptin has been proposed as mediating the protective effects of obesity on the skeleton similarly to traditional factors, such as mechanical loading and increased levels of estrogens and insulin [117]. Leptin shows both a peripheral and positive action on bone as well as a central and negative control of bone metabolism [118–122]. Considering the conflicting and sometimes contradictory data published in the literature, it remains difficult to draw a clear picture harmonizing these dual effects of leptin on bone metabolism.

9.1. Peripheral Effect of Leptin on Bone Metabolism

The expression of both short and long forms of leptin receptor was first recognized in human marrow stromal cells [123] and more recently in primary cultures of normal human osteoblasts [124]. In different cell models, leptin stimulates human osteoblastic cell differentiation [123, 125], bone matrix mineralization [123–125], and collagen synthesis [125] with protective effects against osteoblastic apoptosis [125]. Leptin could be considered one of the factors that could shift the differentiation of stromal cells from an adipocytic to an osteoblastic pathway. This would occur through activation of the mitogen-activated protein kinase (MAPK) cascade. MAPK is involved both in osteoblastic differentiation as well in the phosphorylation of peroxisome proliferator-activated receptor γ (PPARγ), a mechanism shown to inhibit adipogenesis [121].

In addition to its positive effect on the osteoblastic lineage, leptin may modulate osteoclast differentation and function. In experimental studies [126, 127], leptin inhibited osteoclast generation via reduction in expression of the soluble receptor activator of nuclear factor-κB (sRANK) as well as through increased secretion of osteoprotegerin (OPG), its decoy receptor, by stromal cells.

Several *in vivo* studies, conducted in different animal models confirm and support the findings obtained from *in vitro* experiments. Steppan *et al.* [128] showed that leptin administration to leptin-deficient (ob/ob) mice led to a significant increase in bone mineral content and density. Similarly, leptin administration to ovariectomized rats prevented bone loss induced by estrogen deficiency or disuse [127]. Both *in vitro* and *in vivo* studies appear to support the hypothesis that leptin directly and positively affects bone metabolism by modulating both osteoblast and osteoclast generation and function.

9.2. Central Effect of Leptin on Bone Metabolism

In contrast to the evidence for a positive peripheral role of leptin on bone metabolism, ob/ob, and leptin receptor-deficient (db/db) mice presented a phenotype characterized by increased bone mass with an increased rate of bone formation despite hypogonadism and hypercortisolism [129]. In this study, there was no expression of leptin receptors on osteoblasts, whereas the intracerebroventricular infusion of leptin decreased bone formation rate in ob/ob mice as well as in wild-type animals [129]. Mice knock-in of LacZ in the leptin locus with increased circulating leptin level had a dramatically reduced bone mass [130]. In this experimental model, leptin synthesis was not detected in the CNS. As such, circulating leptin must therefore account for the observed antiosteogenic effect with similar amounts of leptin needed to affect both body weight and bone mass [130].

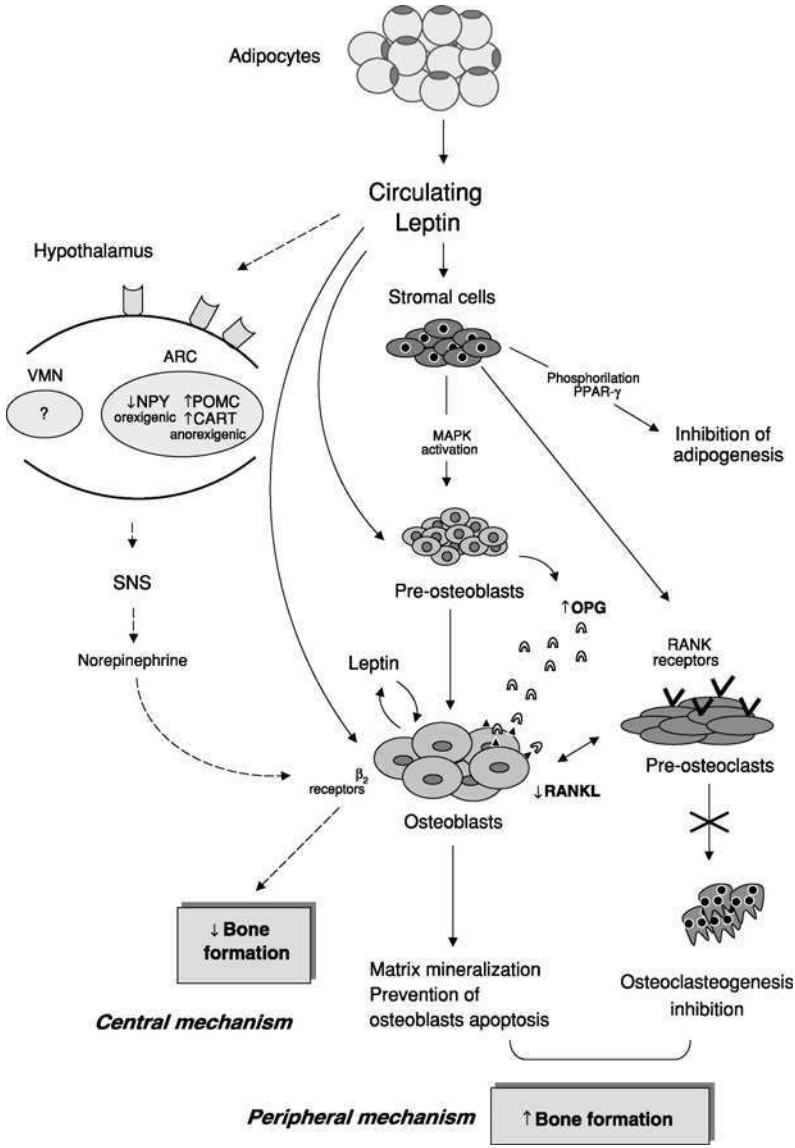

FIG. 3. Central and peripheral leptin control of bone metabolism. OPG = osteoprotegerin; RANK = receptor activator of NF-κB; RANKL = receptor activator of NF-κB ligand; VMN = ventromedial hypothalamic nucleus; ARC = arcuate nucleus; NPY = neuropeptide Y; POMC = pro-opiomelanocortin; CART = cocaine amphetamine-regulated transcript; SNS = sympathetic nervous system. Peripherically circulating leptin secreted by fat tissue or locally produced by osteoblasts and/or bone marrow adipocytes, inhibits osteoclast precursors

It has also been shown that leptin action on bone may be dependent on activation of different hypothalamic nervous circuits than those mediating anorexigenic action of leptin [131]. In fact, experiments of selective destruction of hypothalamic nuclei suggest that neurons of the ventromedial nucleus may be implicated in leptin antiosteogenic function [131]. However, there is also evidence that peptides produced by the arcuate nucleus, under leptin control, could play a role in regulating bone metabolism [118, 119]. NPY decreases bone mass when administered centrally [129], whereas mice knockout for NPY receptors have increased bone formation [132]. Similarly α-Melanocyte stimulating hormone has been shown to have stimulatory effects on osteoblast function and osteoclasteogenesis [133]. When systemically administered to mice, this hormone decreased bone volume via increased bone turnover [133].

Leptin activates the sympathetic nervous system with a stimulation of β_2-adrenergic receptors on the surface of the osteoblasts and a final reduction in bone formation [131]. In fact, mice lacking a key enzyme in the production of norepinephrine and epinephrine have a higher bone mass than wild-type animals [131]. Intracerebral infusion of leptin resulted in decreased fat mass but not bone mass. This finding showed that osteogenic action of leptin, not its anorexigenic effect, is dependent on the sympathetic nervous system [131].

In conclusion, the findings from *in vitro* and *in vivo* studies appear to support the existence of two conflicting pathways in leptin regulation of bone mass (Fig. 3): a central negative mechanism opposed to a peripheral and positive control of bone metabolism.

An explanation for the existence of these conflicting effects of leptin on bone has been recently proposed by Khosla [120]. Leptin level falls in response to starvation. This phenomenon triggers a hormonal compensative response necessary for survival but devastating to the skeleton. To overcome this effect, a central mechanism may have naturally developed to increase bone formation despite low leptin level [120]. Conversely, under conditions of rising fat stores, leptin levels increase. A situation of central inhibition of bone formation may thus occur if the positive peripheral effects of leptin did

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differentiation and proliferation through an increased production of osteoprotegerin from pre-osteoblasts as well as through a reduction of the expression of RANKL on osteoblasts surface; osteoprotegerin prevent the interaction between RANK receptors on osteoclast progenitors and RANKL on the surface of osteoblastic cells. Leptin through an increased production of IGF-1 and TGF-β from osteoblasts, stimulates the proliferation of osteoprogenitor cells, bone matrix mineralization and prevent osteoblasts apoptosis. Centrally (dashed lines) leptin activates neurons in the hypothalamus, in particular ARC and VMN through still not well-characterized neuropeptides; leptin determines an activation of the sympathetic nervous system with an increase in norepinephrine that stimulates β_2 receptors on osteoblasts surface with a reduction in bone formation.

not come into play giving the skeleton more weight bearing strength [120]. It may be concluded that there is a balance between these two pathways, with a predominance of peripheral and positive effects of leptin only in conditions of central leptin resistance, as in obesity, or when serum leptin levels rise above a threshold. This hypothesis appears supported by recent observations in hemodialysis patients in which bone mineral density (BMD) was positively associated with leptin only when circulating levels were higher than the threshold for blood–brain transport saturation [134].

However, it must be kept in mind if data obtained from mice models is relevant to humans. This question particularly needs to be addressed because leptin-deficient mice and humans are phenotypically different [118]. As such, further clinical studies are clearly required to better define the effect of leptin on bone metabolism in humans.

9.3. Clinical Studies

9.3.1. *Cross-Sectional Studies*

Several cross-sectional studies have been published reporting conflicting results about the relationship between circulating leptin level and bone mass in humans [135–150]. It is difficult, however, to compare these findings because they differ considerably in study sample characteristics (age, gender, BMI, pre- or postmenopausal status, physical activity level, health status), bone mass evaluation (X-ray or DXA), parameter of bone density considered, bone site examined, availability of bone turnover markers and finally in the type of adjustments used in the statistical analysis.

Table 2 presents the main findings from studies including healthy elderly subjects (>60 years) with bone density measured by DXA and/or bone turnover markers [135–147]. Some studies [138, 139, 141, 142] support the existence of a significant positive association between leptin and BMD and/or metabolism, whereas others do not [135–137, 140, 143–147].

When interpreting these discordant results, it is important to examine the type of adjustments used in the analyses. Most [139, 141–143, 147], but not all studies [135–137, 138, 140, 144–146] adjusted for fat mass. Because leptin is almost exclusively produced by fat [2–5], and a strong relationship between fat and bone mass exists [121], adjusting only for weight or BMI could not remove the confounding effect of this factor from the analyses. Moreover, BMI is only an indirect measure of fat mass and is not a reliable estimate of adiposity in the elderly as a consequence of age-dependent increase of fat and of age-dependent decrease in height [17, 151].

The majority of studies [135–143, 147] were adjusted for age because they included populations with wide age-range. Only two studies [138, 139] focused exclusively on elderly subjects, whereas in the others [135–137,

TABLE 2

CROSS-SECTIONAL STUDIES ON THE RELATION BETWEEN BONE MASS (EVALUATED BY DXA AND/OR MARKERS OF BONE TURNOVER) AND LEPTIN CIRCULATING LEVELS IN SAMPLES OF SUBJECTS INCLUDING MEN AND WOMEN OLDER THAN 60 YEARS

Author	Sample m/w	Age	BMI kg/m^2	Measures	Adjustments	Results
Zhong *et al.* [135] Clin Chim Acta 2005	340 pre-m w 336 post-m w (China)	20–80 years M = 45.4	M = 21.7 kg/m^2 M = 23.7 kg/m^2	DXA (BMD whole body, spine, femur, forearm)	Age, body weight, BMI	No significant correlation between BMD and leptin at all sites after adjustments in pre- and post-m w
Kontogianni *et al.* [136] J Bone Miner Res 2004	80 w (Greece)	42–68 years	M = 29.2 kg/m^2	DXA (BMC, BMD whole body)	Menopausal status	Negative correlation between BMC, BMD, and leptin but not after including insulin in the regression
Sun *et al.* [137] Acta Diabetol 2003	50 m (USA cauc)	18–66 years	—	DXA (BMD whole body)	Age, BMI	Negative correlation between BMD and leptin after adjustments
Scariano *et al.* [138] Mech Ageing Dev 2003	100 w 31 m (USA cauc)	62–97 years 72–92 years	—	DXA (BMD whole body) Markers of bone turnover	Age, BMI, BMD	Positive association between a marker of bone formation and leptin after adjustment
Zoico *et al.* [139] Clin Endocrinol 2003	171 w 92 m (Italy)	68–75 years	18–37 kg/m^2	DXA (BMC, BMD whole body, femur)	Analysis by gender; Age, fat mass %	8.9% (m), 18.2% (w) of BMC variance, 10.6% (w) of BMD variance explained by leptin
Ruhl *et al.* [140] J Bone Miner Res 2002	1906 pre-m w 1148 post-mw 2761 m (USA cauc, non hisp blacks, mex-am)	M = 36 years M = 66 years M = 43 years	M = 25.8 kg/m^2 M = 27.4 kg/m^2 M = 26.6 kg/m^2	DXA (BMD femur)	Analysis by gender; Age; BMI; other confounding factors	No relation between BMD and leptin in men older than 60 years after adjustments. No association also in post-m w after adjustment

(*continues*)

TABLE 2 (*Continued*)

Author	Sample m/w	Age	BMI kg/m^2	Measures	Adjustments	Results
Blain *et al.* [141] J Clin Endocrinol Metab 2002	107 w (post-m) (France)	50–90 years M = 68.9 years	<30 kg/m^2	DXA (BMD whole body, spine, femur) Markers of bone turnover	Years since menop., lean and fat mass, creatinine Cl., IGF-1	3–7% of BMD variance at different sites explained by leptin. After adjustments negative correlation between leptin and CTx
Pasco *et al.* [142] J Clin Endocrinol Metab 2001	214 w (pre-, post-m) (Australia)	20–91 years M = 44.5 years	<30 kg/m^2	DXA (BMC, BMD whole body, spine, femur, ultradistal, and midforearm)	Age, weight, fat mass, bone area	1–4% of BMC variance (only bearing sites) explained by leptin
Thomas *et al.* [143] Bone 2001	137 pre-m w 165 post-m w 343 m (USA cauc 96%)	M = 35.1 years M = 69.7 years M = 55.1 years	—	DXA (BMD spine, femur, mid-distal radius), markers of bone turnover	Analysis by gender, menopausal status; Age, fat mass	No significant correlation between leptin and BMD in women, negative in men, after adjusting for fat mass. Significant unadjusted correlations between leptin and markers
Martini *et al.* [144] Bone 2001	123 w (post-m) (Italy)	39–82 years M = 62.1	17–44 kg/m^2	DXA (BMD whole body) Markers of bone turnover	BMI	No association between leptin and BMD and markers of bone turnover after adjustment

Sato *et al.* [145] J Clin Endocrinol Metab 2001	221 m (Japan)	21–73 years M = 52.1 years	17–31 kg/m^2	Single photon absorptimetry (calcaneal BMD); fat mass (BIA); bone markers	Weight	Negative association between BMD and leptin after adjustment. Negative correlation between a marker of bone formation and leptin
Iwamoto *et al.* [146] Acta Obstet Gynecol Scand 2000	76 w (47 pre-m) (29 post-m) (Japan)	29–67 years M = 40.8 years M = 62.5 years	M = 23.5 kg/m^2 M = 24.2 kg/m^2	DXA (BMD whole body, arm, rib, spine, pelvis, leg, head); bone markers	Analysis by menopausal state BMI	No significant relation between leptin, BMD, or markers of bone turnover in post-m w before and after adjustment
Goulding *et al.* [147] Calcif Tissue Int 1998	54 w (post-m) (New Zeland)	54–79 years	15–42 kg/m^2	DXA (BMC, BMD whole body); bone markers	Age, fat mass	No significant correlation between leptin, BMC, BMD, or markers after adjustment

m = Men; w = women; cauc = Caucasian; non hisp blacks = non-Hispanic blacks; mex-am = mexican americans; M = mean; pre-m = premenopausal; post-m = postmenopausal; BMI = body mass index; DXA = dual energy X-ray absorptiometry; BMC = bone mineral content; BMD = bone mineral density.

140–147] mean age was not always above 60 years. Pooling subjects of different ages confuses the role of leptin on bone. It is conceivable that as leptin and bone physiology changes with age, their relationship may also differ with aging.

Blain *et al.* [141] examined the relationship between BMD and bone turnover markers with serum leptin in 107 postmenopausal women. In a stepwise multiple linear regression analysis, leptin explained 7.2% and 3.7% of whole body and femoral neck BMD independently of years since menopause, fat and lean mass, Dehydroepi-androsterone (DHEA), IGF-1, creatinine clearance, and calcium intake [141]. In another study conducted in 214 nonobese women, a significant association was described between leptin and whole body bone mineral content (BMC) and BMD in premenopausal women and between leptin and BMD measured at the whole body and trochanter level in postmenopausal women [142]. Leptin explained a small, but significant proportion (1–3%) of BMC and BMD variance at different bone sites, independently of age, body weight, and body fat mass [142]. In elderly postmenopausal women, leptin was significantly related to whole body, total hip, and femoral neck BMC and BMD, accounting for a percentage of the total variance up to 18% [139]. However, these results are in contrast with a large cross-sectional study in which there was no association of leptin and BMD in pre- or postmenopausal women in multivariate analysis [140]. Similarly in a wide population of Chinese women with a wide age-range, the relationship between leptin and BMD at all skeletal sites disappeared after adjustment for age, body weight, and BMI [135]. The lack of association between leptin and bone mass was previously described in other smaller groups of pre- and postmenopausal women [143, 144, 146]. This finding could be partially explained by failure to account for the confounding effect of sex steroid hormones on bone metabolism, seldom available in these clinical studies. Moreover, as Ruhl *et al.* [140] pointed out, another hypothesis could be due to the presence of leptin resistance. Despite high circulating leptin levels, decreased leptin function occurs. Thus, no direct correlation of leptin and bone density was observable.

The first published studies including men, lead to the hypothesis of a sexual dimorphism even in the relationship between leptin and bone mass [143, 145]. Studies conducted in male subjects [137–140] appear to confirm previous observations. The results of Thomas *et al.* [143] were first to show an inverse association between serum leptin and BMD at different sites. These findings were recently confirmed in a smaller population of healthy men [137]. Ruhl *et al.* [140] analyzed a wider population of men with a similar mean age. This study, however, failed to find any evidence of an association between leptin and BMD. Similarly, in a study conducted in dialysis patients [152], age-adjusted z-score for BMD was directly correlated to serum leptin level in women but not men. Conversely, when older population was considered [138, 139], the

data support the existence of a positive association between leptin, BMD, and markers of bone formation. This relationship was, however, weaker in men versus women.

A sexual dimorphism in the relationship between leptin and BMD could be partially explained by gender differences in leptin secretion itself, with leptin concentration substantially higher in women versus men [2–5], by different body fat content and distribution, reproductive hormonal status as well as genetic influences between genders. Moreover, as leptin has been shown to correlate negatively with testosterone, and testosterone increases bone density in men [23], it is also possible that the inverse association of bone density with leptin in adult men is related to serum testosterone.

As such, further studies are clearly warranted to confirm a gender specific relationship between leptin and bone mass and specifically to clarify if this association changes with age.

The lack of literature data prevents any conclusions to be drawn regarding the role of leptin in bone metabolism in obese subjects. In some, but not all, reports BMI ranges were wide, and obese subjects were not always numerically well represented in each BMI category [139, 144, 147]. In some studies [141, 142], subjects with BMI higher than 30 kg/m^2 were intentionally excluded because the authors hypothesized that leptin resistance, frequently displayed in obese subjects [2–5], may be a reason for lack of significant association between leptin and BMD in obese subjects in previous studies [144, 147]. In a single study using NHANES II data [140], subjects were stratified by BMI values and the class of overweight and obesity was well represented. This study found that there was an inverse association between bone density and leptin level in unadjusted analyses in overweight men but not in normal weight or obese subjects [140].

Another unresolved question is whether the bone site selected and/or type of bone measure may influence results of studies that evaluated leptin–skeleton interaction. In evaluating the relationship between BMC or BMD and leptin level, many studies often considered only weight bearing bone sites [136–141, 144, 145, 147]. Only a few studies also measured bone density at non-weight-bearing sites [135, 142, 143, 146, 149]. Among these, Thomas *et al.* [143] and Yamauchi *et al.* [149] reported a significant relationship at non-weight-bearing sites, at least in unadjusted analysis. In contrast, three other reports [135, 142, 146] found no association between leptin and BMD at the forearm and midforearm level. Despite these findings, it should be noted that accuracy of measure at some bone sites could be suboptimal due to high prevalence of osteoarthritis in the elderly [153].

Although diagnostic imaging studies are better suited to describe long-term skeletal consequences of leptin, biochemical markers of skeletal turnover may be useful for short-term endpoints. Both measurements, however,

together provide relevant information in defining the role of leptin in acute changes in skeletal metabolism. Thus, many studies [138, 141, 143–147, 149, 152] were designed to obtain information on bone turnover markers in addition to measuring BMD. In postmenopausal women Blain *et al.* [141] observed an inhibiting role of leptin on bone resorption, with a negative correlation between leptin and urine C-telopeptide of type I collagen (CTx) after adjustment for fat mass and creatinine clearance. These results expanded findings of previous studies [143, 146, 152]. The association of leptin was positive with markers of bone formation such as bone-specific alkaline phosphatase (BAP) [138, 141, 146]. These findings suggested a positive role of leptin on osteoblast function. However, other cross-sectional studies [144, 147, 149] did not observe any association between leptin and either bone formation or resorption markers. Others described a negative association between leptin and markers of bone formation [143, 145].

To evaluate leptin–skeleton interactions, some studies focused on parameters different from BMD measures obtained with DXA [148–150]. Yamauchi *et al.* [149] evaluated the presence of vertebral fractures with standard radiographs in 139 postmenopausal women. Leptin level was significantly lower in women with vertebral fractures. In a multiple logistic regression analysis, high-leptin level was significantly associated with a reduced risk of vertebral fractures independent of fat mass [149]. In a group of osteoporotic postmenopausal women, a positive correlation between leptin and spine BMD was found [148]. In contrast, no association was described in an age- and BMI-matched group of control subjects [148]. However when leptin was acutely measured after hip fracture, leptin did not change the significant relationship between total body adiposity and femur BMD [150].

In conclusion, cross-sectional studies leave many unanswered questions. It seems important to design further studies providing measurements of hormones involved in bone metabolism in order to examine leptin–skeletal interactions in concert with the existing hormonal milieu, rather than in isolation. Moreover, in future studies of leptin–skeletal interaction, leptin resistance should also be taken into account. Finally, in all cross-sectional studies, leptin is measured at single point in time, whereas bone mass is accumulated and lost over a lifetime. Longitudinal and interventional studies seem more suitable to obtain a clearer picture of the complex relationship between leptin and bone mass in humans.

9.3.2. *Longitudinal and Interventional Studies*

To our knowledge, only one longitudinal study has been published on the relationship between circulating leptin level and changes in bone density [154]. This study is a 4-year follow-up longitudinal study conducted on about 300 elderly men and women of the Hertfordshire Cohort Study

[154]. After adjustment for confounding variables, no association was found between baseline leptin level and changes in BMC or BMD at all bone sites examined [154]. However, in 25 type 2 diabetic patients treated for 1 year with troglitazone, percent changes in whole body BMD were inversely correlated to serum leptin level [155]. In these patients, leptin level significantly fell during the treatment without change in BMI and fat percentage [155].

A few interventional studies have been conducted in humans [156, 157]. After 16–18 months of leptin therapy in two women with generalized lipodystrophy and hypoleptinemia, no significant change in either BMD at different sites or markers of bone metabolism was observed [156]. However, subcutaneous administration of leptin in three morbidly obese children with congenital leptin deficiency for 4 years was reported to increase bone mineral mass despite a dramatic decrease in body weight [157]. These data should be considered preliminary, and confirmation of leptin effects on bone metabolism will require large-scale clinical trial with adequate statistical power.

In conclusion, at ages when the prevalence of osteopenia and osteoporosis is progressively increasing and the protective role of other hormonal factors declines, a potential protective role of leptin on bone mineral tissue could be important. Leptin–skeletal interaction should be examined in concert with the existing hormonal milieu to exclude indirect effects and those mediated through different endocrine systems. The effect of leptin on bone metabolism appears to result from a complex balance between central negative and peripheral positive effects. Interventional studies should better clarify in which type of patients this complicated system could be better balanced to favor of a positive and protective role of leptin on bone metabolism.

10. Conclusions

Although leptin was discovered more than 10 years ago, it has only been in the last several years that an increased interest in leptin physiology and pathophysiology in geriatric medicine has been raised.

Data is scarce on elderly populations. Discrepant findings on leptin physiology and pathophysiology in the elderly generate equivocal interpretation. Review of the literature does, however, support the following conclusions.

1. A strong association exists between leptin and body fat, at least in older-aged healthy elderly subjects. However, the relationship between leptin and fat distribution is still not clear.
2. Sexual dimorphism in leptin level is well described in elderly populations. However, the mechanisms explaining higher leptin level in women are not completely understood.

3. Only a few studies have accurately addressed the relationship between leptin level and aging albeit with discrepant findings. Additional cross-sectional and specifically longitudinal studies are necessary to completely understand this relationship.
4. Leptin level increases with weight gain and decreases with weight loss in older aged subjects, consistent with leptin's role as a signal of the size of adipose tissue stores.
5. A role for increased leptin level with age as well as for leptin resistance has been hypothesized in the pathogenesis of the anorexia of the elderly. However, little data is available to support a definitive conclusion.
6. There is, however, clear evidence demonstrating a strong relationship between leptin, insulin, and insulin resistance in elderly men and women with different degrees of glucose tolerance.
7. Leptin resistance may play a central role in the development of the metabolic syndrome with aging. However, further research is necessary to extend these interesting findings from animal studies to humans.
8. Leptin has been shown to exert important effects on bone metabolism in the elderly. These appear to be the result of a complex balance between central negative and peripheral positive effects. However, interventional studies should better clarify which patients may benefit more of a positive and protective role of leptin on bone metabolism.

In conclusion, many interesting questions with important implications in clinical practice remained unanswered and suggest relevant areas for future leptin research.

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BIOCHEMICAL PATHWAYS OF WOUND HEALING: IMPLICATIONS FOR DEVELOPMENT OF DISEASE-SPECIFIC DIAGNOSTICS

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1. Abstract

Acute soft tissue wounds undergo a highly orchestrated healing process involving the following overlapping stages: hemostasis, inflammation, proliferation, and remodeling. Platelets, neutrophils, macrophages, and fibroblasts are the key cells involved in the various stages of healing and they use cytokines and growth factors as chemical messengers. When an acute wound fails to progress to the healing stage, a chronic wound results characterized by excessive inflammation resulting in collagen destruction that outweighs collagen synthesis. The other end of the abnormal healing spectrum is marked by fibrosis where the synthesis of collagen is much more prevalent than the remodeling and as a result, a large and inordinate amount of extracellular matrix is deposited. The underlying mechanism responsible for the development of chronic, nonhealing wounds or alternatively fibrosis is an alteration in the controlling feedback loops that normally lead to a well-defined process of wound healing.

2. Introduction

The normal response to tissue injury is a timely and orderly reparative process that results in sustained restoration of anatomic and functional integrity [1]. In contrast, in chronic dermal ulcers the healing process is prolonged, incomplete, and proceeds in an uncoordinated manner resulting in poor anatomical and functional outcome. On the opposite end of the spectrum are the numerous clinical problems associated with excessive healing and fibrosis. Clinically, wounds can be categorized as acute or chronic based on the timeliness of healing. This chapter reviews the dynamics and normal sequence of cellular, humoral, and biochemical events that occur during acute wound healing and then provides some models based upon this information. The complexities of chronic nonhealing wounds are then addressed and models proposed to reflect predicted outcomes. Fibrosis and contractures represent a third wound healing scenario in which the anatomical structures are so distorted that normal function is lost. Models are proposed to describe the pathological events responsible for these types of abnormal healing responses.

3. Acute Wound Healing

Wound repair is a complex process involving a highly regulated cascade of events requiring interactions among many cell types, soluble factors, and matrix components. There are four distinct phases of acute wound healing

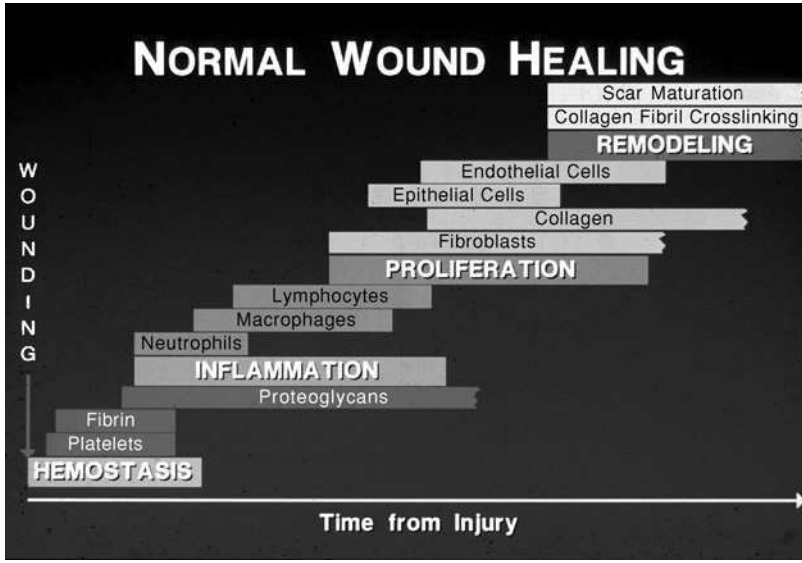

FIG. 1. The sequence of events during normal wound healing. Reproduced from Wound Healing: Biochemical and Clinical Aspects, Figure 22–2, IK Cohen, RF Diegelmann and WJ Lindblad, eds.; WB Saunders, Philadelphia, PA., 1992, with permission from Elsevier.

consisting of hemostasis, inflammation, proliferation, and remodeling (Fig. 1). Following injury, exposure of platelets to the extracellular matrix (ECM) induces them to adhere and aggregate, thus, releasing the contents of their secretory granules. This hemostasis response occurs immediately following tissue disruption and is needed to control bleeding and seal off the site of injury. The wide variety of platelet factors liberated by degranulation include those which facilitate hemostasis and also act as chemoattractants [platelet derived growth factor (PDGF) and transforming growth factor beta (TGF-β)] for cells involved in the next stage of repair, the inflammatory phase.

3.1. HEMOSTATIC PHASE

The process of hemostasis begins immediately upon injury. The immediate goal is to stabilize the wound and thereby prevent exsanguination. The exposed blood vessels vasoconstrict and platelets that are exposed to collagen are activated and begin the process of aggregation at the wound site. Activated platelets release growth factors, cyclic adenosine monophosphate (cAMP), and adhesive glycoproteins, which activate receptors that cause platelets to become sticky and aggregate. The glycoproteins released from activated platelet alpha granules, include fibrinogen, fibronectin, thrombospondin, and

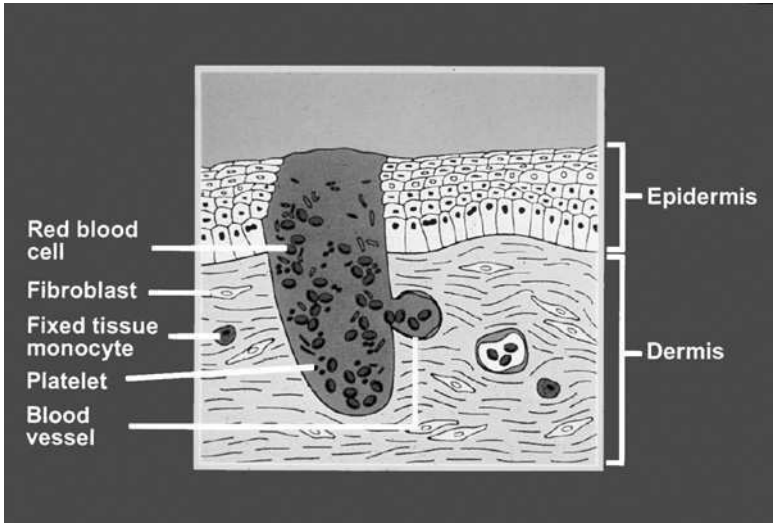

FIG. 2. At the time of injury, the tissue is disrupted and the platelets adhere to the exposed collagen and to each other. The platelets release clotting factors, PDGF, and TGF-β to initiate the repair process. Reproduced with permission from Wound Healing in Surgery: Scientific Principles and Practice, 1st edition, edited by LJ Greenfield, Lippincott Williams & Wilkins, 1993.

von Willebrand factor. The next step in hemostasis involves the coagulation cascade as platelets release coagulation factors. The result is a fibrin clot that not only prevents further blood loss but also serves as a provisional wound healing matrix [2]. Platelets become entrapped in the fibrin clot providing further bulk as well as a membrane on which inactive clotting enzymes are bound and activated further promoting the clotting cascade (Fig. 2).

The process of hemostasis also provides the impetus for the next stage in wound healing through the growth factors released by the platelet alpha granules. A list of some of the most critical growth factors and cytokines involved in wound healing is presented in Table 1. Neutrophils and monocytes are recruited and activated by PDGF and TGF-β. Endothelial cells are activated by vascular endothelial growth factor (VEGF), transforming growth factor alpha (TGF-α), and basic fibroblast growth factor (bFGF). Fibroblasts are activated by PDGF to begin migration to the wound site and produce collagen and glycosaminoglycans, which facilitates cellular migration and interactions with the cell matrix-supporting framework. In addition to factors released from platelets, breakdown fragments from complement, such as C5a and f-Met-Leu-Phe, a bacterial waste product, also signal and recruit neutrophils to the wound site. To summarize, the wound healing response begins with the process of hemostasis, platelet activation at

TABLE 1
CYTOKINES AND CHEMOKINES INVOLVED IN WOUND HEALING

Cytokine	Symbol	Source	Functions
Platelet-derived growth factor (including isoforms AA, AB, and BB)	PDGF	Platelets, macrophages, endothelial cells, keratinocytes, smooth muscle cells	Chemotactic for PMNs, macrophages, fibroblasts and smooth muscle cells. Activates PMNs, macrophages, and fibroblasts. Mitogenic for fibroblasts endothelial cells and smooth muscle cells. Stimulates production of MMPs, fibronectin, and HA. Stimulates angiogenesis and wound contraction. Remodeling. Inhibits platelet aggregation. Regulates integrin expression
Transforming growth factor beta (including β1, β2 and β3	TGFβ	Platelets, T lymphocytes, macrophages, endothelial cells, keratinocytes, smooth muscle cells, fibroblasts	Chemotactic for PMNs, macrophages, lymphocytes, fibroblasts, and smooth muscle cells. Stimulates TIMP synthesis, keratinocyte migration, angiogenesis, and fibroplasia. Inhibits MMPs production and keratinocyte proliferation. Regulates integrin expression and other cytokines. Induces TGFβ production
Epidermal growth factor	EGF	Platelets, macrophages, saliva, urine, milk, plasma	Mitogenic for keratinocytes and fibroblasts. Stimulates keratinocyte migration and granulation tissue formation
Transforming growth factor alpha	TGFα	Macrophages, T lymphocytes, keratinocytes, and many tissues	Similar to EGF
Fibroblast growth factor family	FGF	Macrophages, mast cells, T lymphocytes, endothelial cells, fibroblasts, and many tissues	Chemotactic for fibroblasts. Mitogenic for fibroblasts and keratinocytes. Stimulates keratinocyte migration, angiogenesis, wound contraction and matrix deposition

(*continues*)

TABLE 1 (*Continued*)

Cytokine	Symbol	Source	Functions
Keratinocyte growth factor (also called FGF-7)	KGF	Fibroblasts	Stimulates keratinocyte migration proliferation and differentiation
Insulin-like growth factor-1	IGF-1	Liver, macrophages, fibroblasts and other tissues	Stimulates synthesis of sulfated proteoglycans, collagen, keratinocyte migration, and fibroblast proliferation. Endocrine effects similar to growth hormone
Connective tissue growth factor	CTGF	Endothelial cells, fibroblasts	Chemotactic and mitogenic for various connective tissue cells
Vascular endothelial cell growth factor	VEGF	Keratinocytes	Increases vaso-permeability. Mitogenic for endothelial cells
Tumor necrosis factor	TNF	Macrophages, mast cells, T lymphocytes	Activates macrophages. Mitogenic for fibroblasts. Stimulates angiogenesis. Regulates other cytokines
Interleukins	IL-1, etc.	Macrophages, mast cells, keratinocytes, lymphocytes and many tissues	Chemotactic for PMNs (IL-1,8) and fibroblasts (IL-4). Stimulates MMP-1 synthesis (IL-1), angiogenesis (IL-8), TIMP synthesis (IL-6). Regulates other cytokines
Interferons	IFN gamma, etc.	Lymphocytes and fibroblasts	Activates macrophages. Inhibits fibroblast proliferation and MMP synthesis. Regulates other cytokines

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the site of the injury, and the interactions of soluble mediators and growth factors with the ECM to provide the provisional wound-healing matrix that sets the stage for subsequent events.

3.2. Inflammatory Phase

The inflammatory phase begins as early as 2 h after injury (Fig. 2). Activated neutrophils leave the circulation to begin the debridement of devitalized tissue and phagocytosis of any infectious agents or foreign bodies. Neutrophils also secrete cytokines and other specific chemical "signals" needed to attract specialized cells that are important in repairing the injured tissue. In addition, neutrophils release a battery of proteolytic enzymes, such as elastase and matrix metalloproteinase-8 (MMP-8), to assist in their movement through the tissue and needed for the removal of damaged ECM [3–5]. Mast cell granules are filled with enzymes, such as chymase and tryptase, histamine, and other active amines, which when released cause the classic signs of inflammation: *rubor* (redness), *calor* (heat), *tumor* (swelling), and *dolor* (pain). The crucial inflammatory cells during the inflammatory phase are neutrophils and activated monocytes (macrophages). Neutrophils and macrophages are responsible for bound wound bed preparation and the initial milieu of the healing wound. In order to expedite healing, cellular debris and bacteria are cleared. The inflammatory cells also release cytokines [including interleukins 1 (IL-1), IL-6, IL-8, and tumor necrosis factor alpha (TNF-α)] and growth factors [PDGF, TGF-β, TGF-α, insulin-like growth factor-1(IGF-1), and FGF] that not only serve to continue the inflammatory phase but also serve to initiate the proliferative phase by recruiting fibroblasts and epithelial cells (Table 1).

3.2.1. *Neutrophils*

In the first 24 h, neutrophils are the predominant inflammatory cell type. The neutrophils are recruited and activated by the soluble mediators released by platelets and the coagulation cascade during the hemostatic phase. The initial goal of the neutrophils is to prepare the wound bed by killing bacteria and removing devitalized tissue. Not only does this begin the process of wound healing but also helps fight infection. Neutrophils are chemoattracted to the wound site by the initial inflammatory cytokines released at the time of injury and subsequent hemostasis process. Normally, leukocytes weakly adhere to endothelial cells, which slow their momentum resulting in a rolling type motion along the surface of the endothelial cells. As a result, there is more time for binding between the adhesion molecules [selectins, cell adhesion molecules (CAMs), and cadherins] and receptors (integrins) of circulating leukocytes and vascular endothelial cells. Once activated, leukocytes firmly

adhere to epithelial cells as a result of the binding between integrin receptors and ligands, such as vascular CAM (VCAM) and intercellular adhesion molecule (ICAM), which are expressed on activated endothelial cells. The activated leukocytes are induced to migrate between the endothelial cells and move into the injured tissue using their integrin receptors to bind to ECM components. At that point, inflammatory cells secrete elastase and collagenase in order to migrate through the basement membrane of the endothelial cell and into the ECM. Once the neutrophils have entered the wound, they begin to phagocytose bacteria and subsequently produce oxygen free radical to kill the entrapped bacteria. In addition, they degrade damaged components of the ECM by releasing large amounts of proteases (neutrophil elastase and neutrophil collagenase). Neutrophils also produce and release inflammatory mediators, such as TNF-α and IL-1, which further recruit and activate more neutrophils as well as fibroblasts and epithelial cells. After 2–3 days, neutrophils are no longer the dominant cell type as they are depleted by apoptosis allowing tissue monocytes to predominate.

3.2.2. *Macrophages*

After the first 24 h, activated monocytes (macrophages) are the most numerous inflammatory cell types present in the wound bed (Fig. 3).

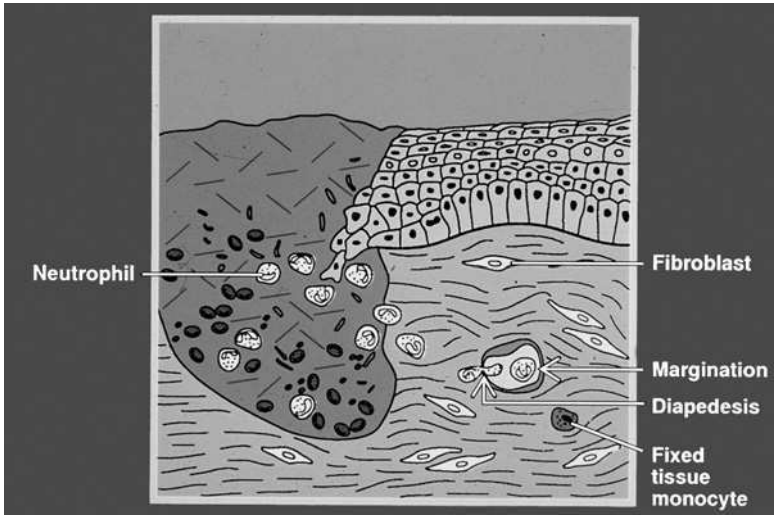

FIG. 3. By the first day following injury, neutrophils attach to endothelial cells in the vessel walls surrounding the wound (margination), then change shape to move through the cell junctions (diapedesis) and migrate to the wound site (chemotaxis). This is the beginning of the inflammatory phase. Reproduced with permission from Wound Healing in Surgery: Scientific Principles and Practice, 1st edition, edited by LJ Greenfield, Lippincott Williams & Wilkins, 1993.

Macrophages are not only active in healing the wound but also in the regulation and progression of the healing process. Macrophages are mandatory components of the normal healing process [6]. Circulating monocytes are attracted to the wound site and extravasate in the same manner as described for neutrophils. Once the monocyte has entered the wound site, they are activated into tissue macrophages by chemokines, cytokines, growth factors, and soluble fragments of ECM components produced by proteolytic degradation of collagen and fibronectin. Macrophages play a similar role as neutrophils in the healing process. They are responsible for killing bacteria and wound debridement through the actions of secreted MMPs and elastase. In addition to the functions shared with neutrophils, the macrophages also play a crucial role in modulating the inflammatory response by regulating the proteolytic destruction of wound tissue and initiating the transition from the inflammatory phase to the proliferative phase. Macrophages downregulate tissue destruction by secrete inhibitors of the proteases. They also release a wide variety of growth factors and cytokines including PDGF, TGF-β, TGF-α, FGF, IGF-1, TNF-α, IL-1, and IL-6 that serve to recruit and activate fibroblasts and promote angiogenesis. The addition of fibroblasts that synthesize, deposit, and organize the provisional ECM, combined with the ability to decrease proteolytic destructs lead to the initiation of the proliferative phase. As the number of macrophages decrease and the number of fibroblasts increase the wound begins to enter the proliferative phase and exit the inflammatory phase.

3.3. Proliferative Phase

During the ''proliferation'' phase, keratinocytes proliferate, migrate, and differentiate, thus, restoring surface integrity. Endothelial cells from damaged blood vessels begin to send out new capillary buds, while fibroblasts from nearby connective tissue enter the wound site and deposit ECM [7]. In the normal dermis, fibroblasts are quiescent and have a low concentration, but in granulation tissue and the provisional matrix of the wound they are active and exist in high concentrations. Fibroblasts are recruited to the wound site by the soluble products of activated platelets and macrophages (Fig. 4). The migration of fibroblasts is far more complicated than that of neutrophils and macrophages as they have to change their morphology and clear a path for their movement from the ECM to the wound site.

Fibroblasts begin their migration by first binding to matrix components (fibronectin, vitronectin, and fibrin) via their integrin receptors. The receptors bind to specific amino-acid sequences (R-G-D or arginine–glycine–aspartic acid) or binding sites on the matrix components. The fibroblasts move in a step-wise pattern by extending a cytoplasmic projection from the

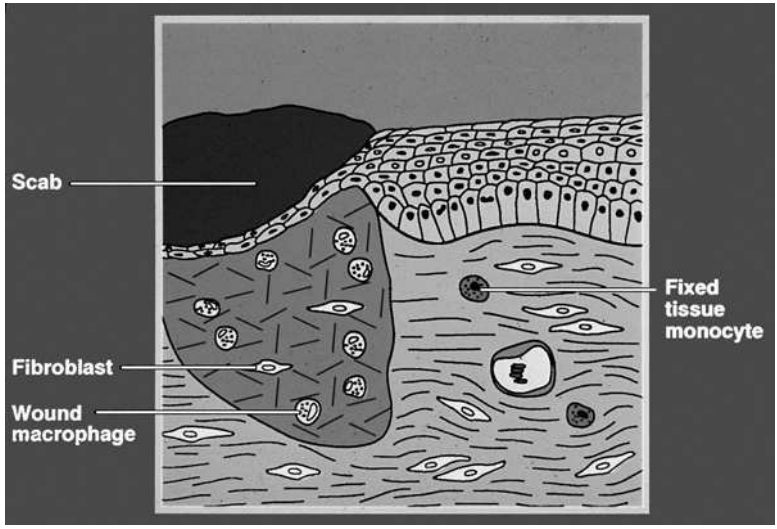

FIG. 4. The inflammatory phase continues as fixed tissue macrophages become active and move into the site of injury and transform into very active wound macrophages. These highly phagocytic cells also release PDGF and TGF-β to recruit fibroblasts to the site and thus begin the proliferative phase. Reproduced with permission from Wound Healing in Surgery: Scientific Principles and Practice, 1st edition, edited by LJ Greenfield, Lippincott Williams & Wilkins, 1993.

unbound side of its membrane to find the next binding site. Once a new site is successfully bound, the original site is then released (by local protease activity), and the cell pulls itself forward using its cytoskeleton network of actin fibers.

The concentration gradient provided by chemotactic growth factors, cytokines, and chemokines in conjunction with the alignment of the fibrils in the ECM and provisional matrix determine the direction of fibroblast migration. The fibroblast moves along the fibrils as opposed to across them. The proteolytic enzymes used to facilitate their forward motion include collagenase (MMP-1), gelatinases (MMP-2 and MMP-9) that degrade gelatin substrates, and stromelysin (MMP-3) that has multiple protein substrates in the ECM.

Collagen is the major ECM component providing strength, integrity, and structure to normal tissues. It is also required to repair defects created by injuries, thereby restoring tissue structure and function [7, 8]. During repair, collagen is initially laid down in irregular bundles possessing modest mechanical strength, but as the wound matures, the collagen is cross-linked and then the scar undergoes remodeling indicating the "maturation" phase of healing (Fig. 5). The collagen is then organized into a dense structure with increasing tensile strength and the matrix begins the process of contraction. However, the

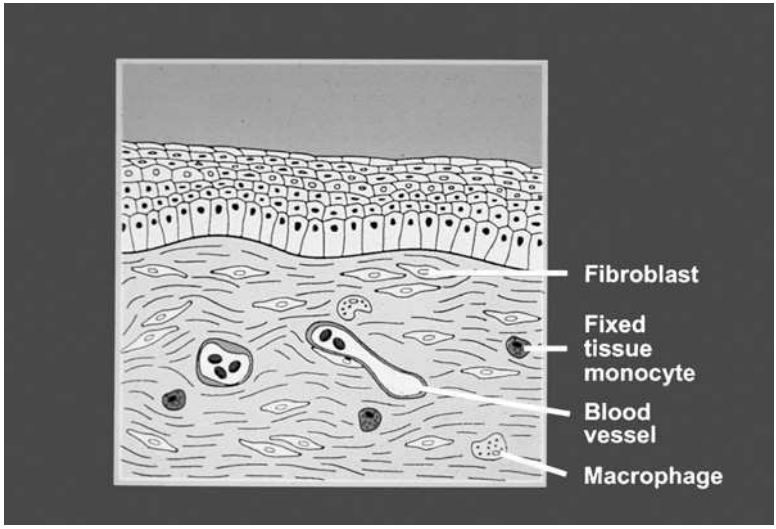

FIG. 5. The remodeling phase is characterized by continued synthesis and degradation of the extracellular matrix components trying to establish a new equilibrium. Reproduced with permission from Wound Healing in Surgery: Scientific Principles and Practice, 1st edition, edited by LJ Greenfield, Lippincott Williams & Wilkins, 1993.

regained tensile strength will never reach normal, and the maximum a wound can ever achieve is approximately 80% of normal tensile strength [7, 8]. Other components of the ECM that exhibit significant structural and functional roles include fibronectin, laminin, proteoglycans, and glycosaminoglycans [9]. Fibronectin and laminin contain important binding sites for cells, collagen, as well as glycosaminoglycans [10]. Figure 1 summarizes the sequence of events with the appearance of specialized cells into the wound site.

Wounds with significant tissue loss heal by the mechanism of contraction, a process whereby the edges of a wound are drawn toward the center, due to forces generated within the wound. Contraction facilitates spontaneous wound closure by reducing the volume required to be filled by granulation tissue as well as the area required to be reepithelized [8, 11].

In order to protect the underlying wound it is necessary to create an epithelial barrier. Epithelization is a complex process that involves epithelial cell detachment phenotypic alterations, migration, proliferation, and then differentiation [12]. An undamaged mature epidermis consists of five layers of differentiated epithelial cells ranging from the cuboidal basal keratinocytes nearest to the dermis up to the flattened, hexagonal, and tough keratinocytes in the uppermost layer. Only one layer is capable of replication (the basal epithelial cells). These basal cells are normally attached to their neighboring

cells by intercellular connectors called desmosomes and to the basement membrane by hemidesmosomes. In order to begin replicating basal cells must become activated by growth factors such as EGF, keratinocyte growth factor (KGF), and TGF-α. The growth factors bind to receptors on the epithelial cells and stimulate migration and proliferation. Not only do the growth factors activate the basal cells but also they trigger the desmosomes and hemidesmosomes to dissolve so that the cells can detach in preparation for migration. Integrin receptors are then expressed and the normally cuboidal basal epithelial cells flatten in shape and begin to migrate as a monolayer over the newly deposited granulation tissue, following along collagen fibers. The monolayer is provided with new cells from the proliferating basal cells near the wound margin (cells that are actively migrating are incapable of proliferation). In order for the epithelial cells to penetrate the newly formed scab, necrotic tissue, or scar, the leading edge of the monolayer produces and secretes proteolytic enzymes (MMPs). The migration ceases after the epithelial cells contact each other forming a confluent sheet. Once this contact has been made, the entire epithelial monolayer enters a proliferative mode and the stratified layers of the epidermis are reestablished and begin to mature to restore barrier function. The intercellular desmosomes and the hemidesmosome attachments to the newly formed basement membrane are also reestablished. TGF-β is one growth factor that can speed up the maturation (differentiation and keratinization) of the epidermal layers. Clinically, epithelization is the hallmark of healing, but it is not the final event, as the granulation tissue must be remodeled to form the permanent scar.

3.4. Remodeling Phase

During late phases of wound healing, scar remodeling involves a variety of matrix degrading enzymes including matrix metalloproteinases, serine proteinases, cathepsins, and glycosidases [11, 13]. The goal of the remodeling phase is the conversion of the preliminary wound matrix to the production of a final scar that maximizes wound tensile strength. Fibroblasts are the cells responsible for remodeling. There is an equilibrium between collagen formation and degradation that results in a constant amount of collagen. There are several different classes of proteolytic enzymes in the wound bed that are responsible for the degradation of collagen: matrix metalloproteinases, serine proteinases, cathepsins, and glycosidases [11, 13]. Specific MMP proteases required for remodeling include collagenases (which degrade intact fibrillar collagen molecules), the gelatinases (which degrade damaged fibrillar collagen molecules), and the stromelysins (which very effectively degrade proteoglycans). The most important serine protease is neutrophil elastase, which can degrade almost all types of protein molecules. In order to balance the

destructive capabilities of the matrix degrading enzymes, inhibitors are also needed. The specific inhibitors of the MMPs are the tissue inhibitors of metalloproteinases (TIMPs) and specific inhibitors of serine protease are α1-protease inhibitor (α1-PI) and α2-macroglobulin. The process of remodeling is dependant on the relative activities of the proteases and their inhibitors.

Tightly controlled regulatory systems maintain a delicate balance between these complex synthetic and degradative processes; normal tissue repair ultimately results from a fine-tuning of this equilibrium. There is now a growing recognition that abnormal wound healing often results from a disruption of degradative/synthesis equilibrium. When the balance tips in favor of matrix degradation, the end result may be a chronic ulcer, whereas reduced degradation and/or increased matrix synthesis results in fibrosis. An ultimate goal in the clinical intervention in abnormal wounds is to restore the *balance* between synthesis and degradation so that healing can proceed in an optimal fashion.

4. Chronic Wounds: Overview

Most chronic ulcers are associated with a small number of well-defined clinical entities particularly chronic nonhealing pressure ulcers, diabetic ulcers, and venous stasis ulcers. These conditions are collectively responsible for approximately 70% of all chronic ulcers [14]. The incidence and prevalence of chronic pressure ulcers vary considerably but are especially high in spinal cord injury patients as well as elderly patients who are nonambulatory. Because these ulcers cause a major disability and are characterized by chronicity and frequent relapse, they have a significant impact on the socioeconomic well-being of the population and attract enormous health care expenditures that exceed $3 billion a year [14].

Improved treatment regimens for these chronic ulcers require a better understanding of the pathophysiologic mechanisms underlying the failure of the wound healing process. Therapeutic strategies for chronic ulcers must therefore be designed with due attention to the underlying abnormalities.

5. Pressure Ulcers

Pressure ulcers are characterized by deep tissue necrosis and loss of deep tissues (muscle and fat) that is disproportionately greater than the overlying skin defect [15, 16]. Pressure ulcers are a serious and frequent occurrence among the immobile and debilitated. Patients with spinal cord injuries are

particularly vulnerable to pressure ulcer formation. There are approximately 225,000 spinal cord injury patients in the United States, with approximately 9000 new patients each year. Approximately 60% of these patients develop pressure ulcers, and the annual cost estimates range from $18,000–29,000 per patient for medical, surgical, and nursing care [13]. If the elderly nursing home population with pressure ulcers is added to the spinal cord injury population, then the figure for the care of all pressure ulcers is enormous [14].

There are several primary etiological factors believed to be important in the formation of pressure ulcers. Pressure over bony prominences is a key factor but shear forces, friction, and moisture are probably also important in the development of pressure ulcers [15]. Muscle and subcutaneous tissues are more susceptible to pressure-induced injury than the skin. As a result, increased pressure over bony prominences creates a cone-shaped pressure gradient with the base over the bone. When tissue necrosis occurs, a three-dimensional area of volume loss is created under a deceptively minor skin defect.

5.1. Neutrophils in Pressure Ulcers

It has been noted that neutrophils appear to be present in very high numbers in pressure ulcers [17, 18]. In addition there is indirect evidence correlating neutrophil products with chronic pressure ulcers. This includes neutrophil elastase, the gelatinase, MMP-9, and the collagenase, MMP-8 [19–22].

The microcirculation constitutes the functional interface between the circulating blood and the interstitial space. To gain access to sites of inflammation, polymorphonuclear leukocytes (PMNs) must pass through the endothelial barrier. The recruitment paradigm encompasses a number of steps including a response to chemotactic factors, margination, capture, rolling, activation, firm adhesion, and transmigration or diapedesis. Once the neutrophil arrives at the site of inflammation, it then undergoes degranulation. Any of these steps represent potential targets for modulating the activity of neutrophils with regard to repair in chronic pressure ulcers.

5.2. Inactivation of PMN Products

Neutrophil products may be good targets for strategies seeking to modify the progression of repair. Although more than 50 different products have been identified in the various neutrophil granules only a few have been implicated as having important roles in neutrophil-mediated tissue injury [3]. Neutrophil-derived elastase is an abundant primary granule serine proteinase with the ability to degrade a wide range of connective tissue macromolecules. There is growing evidence suggesting that this enzyme may also

play a role in the pathophysiology of chronic ulcers. Compared to fluids from healing surgical wounds, chronic ulcer fluids contain increased amounts of degraded fibronectin and vitronectin [23, 24]. Based on the ability to block this activity with specific proteinase inhibitors it has been shown that neutrophil elastase is the responsible enzyme [19, 23, 24]. In addition, we have demonstrated that neutrophil elastase in chronic ulcers degrades peptide growth factors such as TGF-β and PDGF [25]. This finding could have significant implications for the use of topical peptide growth factors to treat chronic ulcers and probably explains the disappointing results to date for the use of topical growth factors.

The collagenase, MMP-8, is a major constituent of neutrophil secondary granules. The collagenases are critical enzymes in the breakdown of the ECM as they are unique in their ability to initiate degradation of collagen. We have demonstrated that MMP-8 is found in abundance in human excisional wounds but the majority is in the inactive proenzyme form [5]. In contrast, we have found that both levels and activity of this enzyme are significantly higher in pressure ulcers [5, 19].

All of the MMPs can be inhibited by the nonspecific proteinase inhibitor, α_2-macroglobulin, and specifically by a small group of proteins called the TIMP. A number of other naturally occurring and synthetic inhibitors also exist. Included in this group are the tetracyclines. The inhibitory concentration-50 (IC_{50}) for doxycycline is approximately 25 mM for MMP-8 and 300 mM for MMP-1 [26]. There are growing numbers of studies suggesting that tetracycline derivatives can reduce the activity of MMPs as well as elastase, although the latter may be an indirect action [27–29]. Once the proteolytic environment of pressure ulcers is brought under control, then the positive production of matrix components can proceed with the development of granulation tissue, contraction, epithelization, and healing.

6. Diabetic Ulcers

Diabetic ulcers represent the most complex and perhaps the most challenging chronic dermal ulcers for the clinician to treat. The underlying causes are neuropathy, vascular disease, and ischemia. Diabetic patients are susceptible to a process termed ''nonenzymatic glycosylation'' in which many proteins in their bodies have excess glucose attached and that ultimately interferes with biologic function [30]. For example, the vascular basement membrane collagen alters filtration when it becomes excessively glycosylated and thus the delivery of oxygen and nutrients to tissues is impaired. Although this is a problem throughout the body, it is most evident in the peripheral circulation in the extremities. A secondary effect of this nonenzymatic glycosylation

process is localized tissue ischemia. Diabetics are also subject to neuropathy and this causes problems especially in the feet where they can develop diabetic ulcers because of the lack of pain sensation [31].

7. Venous Stasis Ulcers

The underlying mechanism responsible for venous stasis ulcers is also thought to be ischemia in which a fibrin cuff develops around vessels and thus impairs the free flow of oxygen and nutrients to sustain viability. The inability of chronic venous stasis ulcers to heal is believed to be related to a failure to adequately reepithelize. In normal wounds, the epithelization process is regulated by the expression of integrin receptors by keratinocytes. The epidermal cells are activated by exposure to plasma components that cause them to begin disassembling its attachments from basement membrane and neighboring cells. The cells then proceed to migrate a provisional matrix containing fibrinogen, fibronectin, vitronectin, and tenascin and stop when they encounter laminin. In parallel, keratinocytes produce fibronectin until the epithelial cells contact, at which time they again begin manufacturing laminin to regenerate the basement membrane.

There is evidence that the interaction between the integrin receptors on keratinocytes with the ECM will transform resting cells to a migratory phenotype. Integral in this transformation is the alteration in the pattern of integrin receptors expressed. After epithelization is completed, integrin expression reverts back to the resting pattern. To further complicate this process, growth factors are involved in mediating keratinocyte activation, integrin expression, and in alterations in the matrix. Growth factors are able to differentially affect these processes, for example, TGF-β is able to promote epithelial migration while inhibiting proliferation.

In venous stasis ulcer, despite optimal condition for keratinocyte recruitment, proliferation, and differentiation, the wound edges show hyperproliferative epidermis. Although TGF-β induces the necessary integrin expression for migration, the cells behind those at the leading edge have little proliferative ability and so epithelial coverage of the wound is inhibited. Therefore, it is hypothesized that some chronic wounds may be deficient in TGF-β and its receptor [32].

8. Fibrosis

Keloids are characterized by an altered wound healing response resulting in excessive matrix deposition and reduced remodeling and represent a good human model to study fibrosis in general. Keloids and most other fibrotic

conditions are associated with an increased density of mast cells [33]. We have found that mast cell-derived chymase can facilitate collagen processing [34] and may actually cause a blockade in the normal feedback mechanism controlling downregulation of collagen synthesis. Studies also suggest that there may be some abnormal signaling between fibroblasts and epithelial cells in keloids resulting in an upregulation of collagen expression [35]. Over the years a wide spectrum of therapies have been used to treat keloids including intralesional injections of triamcinolone, radiation, pressure therapy, Imiquimod (an inducer of interferons), fluorouracil, and topical silicone dressings. These therapies are all designed to reestablish the equilibrium between collagen synthesis and collagen degradation. Once a better understanding of the underlying mechanisms responsible for both excessive matrix deposition seen in fibrosis as well as inadequate healing seen in pressure ulcers are understood, then there should be an advancement in new therapeutic strategies to treat these many debilitating and costly clinical problems [36].

9. Modeling

An important concept while discussing normal wound healing is the concept of feedback loops (Fig. 6). In order for wound healing to progress

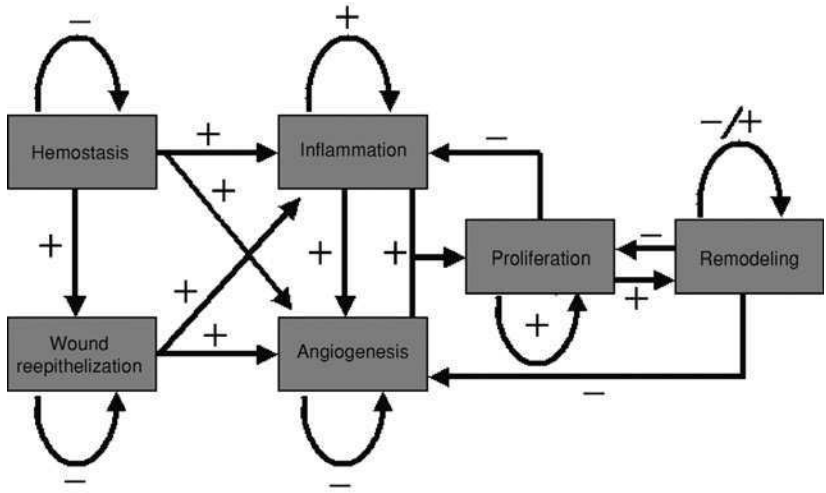

FIG. 6. Model of the normal feedback signaling that controls the normal wound healing response.

in a coordinated manner, each stage in the healing process must not only self-perpetuate but also initiate the next stage. Furthermore, each progressive stage must have the ability to turn off the previous stage once it has reached the critical point at which it is no longer dependant on the previous stage for positive feedback to continue. The interlocking feedback loops allow an orderly progression of wound healing and prevents any one stage from preventing progress into the next stage.

The mechanism by which the different phases inhibit and promote each other is through the expression of soluble mediators like growth factors and cytokines. Each cell type involved is capable of responding to and releasing a multitude of factors that allows the formation of feedback loops to control cell concentrations as well as activity.

In chronic wounds, there is a failure of the normal wound healing process that does not allow for normal wound closure. There are multiple etiologies for the formation of chronic wounds that include diabetic ulcers, venous stasis ulcers, and pressure ulcers as discussed earlier. Each of these wounds results from different pathological mechanisms, but they have remarkably similar wound healing trajectories as well as similar inflammatory profiles. In the chronic pressure ulcer, neutrophils are the predominant cell type with large amounts of proteases and inflammatory cytokines [18]. Because of the excessive amount of inflammation, it is likely that these wounds have failed to progress from the predominantly inflammatory phase to a predominantly proliferative phase (Fig. 7). The wound is not able to mature, and the disorganized ECM degrades liberating breakdown products, thus, causing further inflammation. As a result, the chronic pressure ulcer becomes enmeshed in an inflammatory phase that self-perpetuates without providing

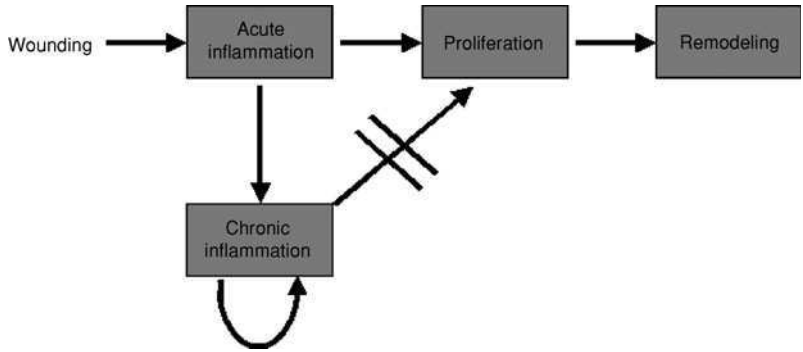

FIG. 7. Model of altered feedback signaling responsible for nonhealing chronic dermal pressure ulcers.

enough forward momentum to propel the process to the next stage of wound healing (Fig. 7). The effect of chronic inflammation is further tissue destruction and worsening of the chronic ulcer thereby causing more inflammation and preventing any progress.

If the feedback model is correct, not only is it imperative to slow down the self-perpetuating inflammatory cycle but also the wound must be matured into the proliferative phase. It is not enough to inhibit neutrophils, but the wound must enter a self-sustaining mode of tissue repair. It is likely that it is necessary to create a milieu in which the wound is forced to proliferate and move forward to the remodeling stage.

10. Summary

Normal wound healing is a carefully choreographed process that results in a mature scar, which restores the anatomic and functional integrity of the dermis. The process is divided into an orderly progression of individual phases. The hemostatic phase is controlled by platelets, which ensure wound stabilization and recruits inflammatory cells into the wounded tissue. The inflammatory phase, which is initially dominated by neutrophils and subsequently by macrophages, prepares the wound bed by killing bacteria and removing devitalized tissue as well as recruiting fibroblasts. The proliferative phase is characterized by fibroblasts laying down a provisional ECM matrix. The remodeling phase is a balance of collagen synthesis and degradation that results in a mature scar that maximizes wound tensile strength.

In contrast to normal wound healing, chronic wounds are characterized by pathological inflammation that inhibits wound closure. Pressure ulcers are the result of deep tissue necrosis and loss of deep tissues among immobile and debilitated patients. Venous stasis ulcers are due to failure of wounds to adequately epithelize. In chronic wounds, neutrophils are present in high numbers resulting in excessive amounts of neutrophil elastase, MMP-9, and MMP-8 resulting in excessive wound degradation and failure to heal. Research has targeted neutrophils and their products as ways to help heal chronic nonhealing ulcers.

Fibrosis is the process of excessive matrix deposition resulting in excessive scar formation. Therapies to prevent keloid formation are designed to restore the proper equilibrium between collagen formation and degradation.

A new model for looking at wound healing involves feedback loops. The positive and negative loops allow the wound to progress in an orderly fashion. The failure to progress results in chronic wound formation and abnormal feedback results in excessive scar formation.

Acknowledgment

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CLINICAL LABORATORY TOOLS TO DIAGNOSE INFLAMMATION

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1. Abstract

Inflammation is essential for survival. In this chapter an outline of various components and mediators of inflammation and their role has been given. Recent studies suggest that low-grade systemic inflammation occurs in obesity, metabolic syndrome X, type 2 diabetes mellitus, hypertension, and coronary heart disease. In view of this, particular emphasis has been given

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to the role of C-reactive protein (CRP), free radicals, nitric oxide, and pro-inflammatory cytokines in the pathobiology of these diseases. High-sensitive CRP has been studied in detail as a marker of low-grade systemic inflammation. In view of this, its role and importance in cardiovascular disease is outlined in this review.

2. Introduction

Inflammation is a complex reaction to injurious agents, either external or internal, that consists of both vascular and cellular responses. Inflammation may be local or systemic, and it can be acute or chronic. During the inflammatory process, the reaction of blood vessels is unique that leads to the accumulation of fluid and leukocytes in extravascular tissues. The reaction of blood vessels can be in the form of vasodilatation seen in the form of hyperemia at the site(s) of injury. This phenomenon performs the essential function of increasing the blood supply to the injured tissue/organ so that adequate elimination of the inflammation-inducing agent is achieved and/or repair process can occur after the inflammation subsides. Thus, both injury and repair are two components of the inflammatory process that are very closely intertwined so that it is difficult to separate these two processes. In fact, in majority of the instances, both inflammation to injury and repair occur almost simultaneously.

Inflammation is fundamentally a protective response whose ultimate goal is to eliminate the injury-inducing agent (that could be a microorganism, physical stimuli, chemical agent, etc.), prevent tissue damage, and/or initiate the repair process. Without inflammation there would be no life. In the absence of adequate inflammation, cell/tissue injury would go unchecked. Damage done to the cells/tissues/organs would never heal and ultimately lead to the death of the organism itself. Thus, inflammation is both beneficial and potentially harmful. In order to comprehend the significance of the existing clinical laboratory tools of inflammation and develop new diagnostic tools, it is important to understand pathophysiological mechanisms of inflammation.

2.1. Components of Inflammatory Response

The inflammatory response mainly consists of two components: a vascular response and a cellular response. These responses are integral and essential parts of the inflammatory reaction. The vascular and cellular reactions of both acute and chronic inflammation are mediated by chemical factors that are proteins, lipids, or lipoproteins in nature. These factors are secreted by various cells that take part in the inflammatory process either directly and/or responding to the inflammatory stimulus. These chemical mediators can act

singly, in combination with other factors/mediators, or sequentially to amplify the tissue/organ response to the stimulus and influence the course of inflammation. In addition, necrosis or apoptosis of cells or tissues during inflammation/repair result in the elaboration or liberation of certain chemicals that also take part in inflammation. Once the inflammatory process is initiated, tissues/organs attempt to elaborate antiinflammatory chemicals and signals to minimize tissue damage and eliminate the harmful effects of inflammation. Thus, the ultimate recovery (i.e., regain of function) of a tissue/organ from the inflammatory process depends, to a large extent, on the balance between pro- and antiinflammatory chemicals and events that occur as a result of these mutually antagonistic processes. Once inflammation is terminated either by endogenous mediators/repair processes and/or by modern medical intervention (i.e., antibiotics, antiinflammatory drugs, chemical, and surgical measures) and the offending agent is successfully removed, all secreted mediators and cellular responses are either broken down or dissipated and the tissues/organs in question revert to their natural physiological state. As can be expected, return to normal function depends on degree of damage and success of repair.

Circulating cells and chemical mediators participate in both acute and chronic inflammation. Thus, the expression of certain molecules on the surface of these circulating cells or release of chemicals by these circulating cells, or both can be used as markers of inflammation. When the inflammatory process is on the surface of the body and is in an acute form, as evidenced by *rubor*, *tumor*, *calor*, *dolor*, and *functiolaesa* (redness, swelling, heat, pain, and loss of function respectively), no specific tests are probably necessary to measure the presence or absence of inflammation. Specific tests do become necessary when certain chronic inflammatory processes need to be detected. These conditions may occur deep inside the body or within internal organs and, as such, would not be typically obvious on physical examination. This is especially important since many chronic diseases presently thought to be due to degenerative processes or aging result from low-grade systemic inflammation. Thus, obesity, hyperlipidemia, essential hypertension, type 2 diabetes mellitus, coronary heart disease (CHD), and metabolic syndrome X (characterized by abdominal obesity, hypertension, hyperlipidemia, and insulin resistance) appear to be diseases of low-grade systemic inflammation [1]. Because of this finding, several studies are examining markers of inflammation to predict disease development and/or course.

2.2. Components of Acute Inflammation

Acute inflammation, a rapid response to an injurious agent, has three main components: (1) alterations in blood vessel diameter (vasodilatation) to

increase blood flow to the site of inflammation, (2) changes in microvascular structure to permit plasma proteins and leukocytes to leave the circulation to aid in the pathobiology of inflammation both in injury and repair, (3) accumulation of leukocytes at the site of inflammation and their activation to release chemical mediators of inflammation to eliminate the offending organism or agent. Various agents that generally trigger acute inflammation include: infection by bacterial, viral, fungi, and parasitic organisms and their toxins; trauma; physical and chemical agents such as burns, radiation, and environmental or man-made chemicals; foreign bodies such as splinters, thorns, sutures; and abnormal immune reactions especially hypersensitivity reactions. Although inflammation induced by these various agents could have some distinct features, in general, all inflammatory reactions share the same basic features (see Table 1).

2.2.1. *Vascular Changes*

2.2.1.1. *Vasodilatation.* Vasodilatation is one of the most essential components of inflammation. It is an early manifestation of acute inflammation.

TABLE 1
COMPONENTS OF INFLAMMATORY RESPONSE: CIRCULATING CELLS AND PROTEINS, CELLS OF BLOOD VESSELS, AND CELLS AND PROTEINS OF THE EXTRACELLULAR MATRIX

Cellular components	Corresponding proteins/molecules
1. Connective tissue cells	
Mast cells	Histamine, serotonin, lysosomal enzymes, nitric oxide, ROS,
Fibroblasts	eicosanoids, cytokines, growth factors, kinins, adhesion
Macrophages	molecules, etc.
2. Vascular tissue cells	
Smooth muscle cells	
Endothelial cells	Nitric oxide, eicosanoids, ROS, growth factors, cytokines, CRP, etc.
3. Circulating cells	
Polymorphonuclear leukocytes	Platelet activating factor, growth factors, ROS, NO, eicosanoids, cytokines, histamine, serotonin, kinins, adhesion molecules, CO,
Lymphocytes	complement system, coagulation and fibrinolysis system, etc.
Platelets	
Monocytes	
Eosinophils	
Basophils	
4. Connective tissue matrix	
Elastin fibers	Several matrix metalloproteinases, etc.
Collagen fibers	
Proteoglycans	

It is clear that some proteins/molecules are common to several cells. This list is by no means exhaustive.

Interestingly, early vasodilatation is sometimes followed by transient vasoconstriction. The main purpose of vasodilatation is to increase blood flow to the site of inflammation to carry circulating proteins and other mediators to aid inflammation. Initially, existing blood vessels undergo dilatation. At later stages of inflammation, depending on demand and necessity and mediator release, new capillary beds are opened. Vasodilatation is followed by increased permeability of the microvasculature that ultimately allows for outpouring of protein-rich fluid and extravasation of leukocytes to site(s) of inflammation. Stasis of blood flow may also occur due to increased red blood cell concentration in smaller vessels and contribute to increased blood viscosity. As a result of this stasis, leukocytes, especially polymorphonuclear neutrophils (PMNs), accumulate along the vascular endothelium and escape into the interstitial tissue over time.

The exact mechanism(s) and the mediators involved in vasodilatation process during inflammation are still not known. Studies showed that nitric oxide (NO) produced by endothelial cells and possibly other cells seem to have a pivotal role in the vasodilatation process. NO is a potent vasodilator and platelet antiaggregator. Its local production could indeed be one of the important mediators of vasodilatation seen during inflammation. Several other mediators of vasodilatation may include carbon monoxide (CO); prostaglandins (PGs) including other eicosanoids, bradykinin, and other kinins; and histamine. The final degree of vasodilatation at a given site of inflammation could depend on the amount of each of these possible mediators released from various cells, the balance between vasodilator and vasoconstrictor mediators released, and their respective inactivators. These various mediators are released by macrophages, monocytes, infiltrating leukocytes, lymphocytes, endothelial cells, and other cells present at the site of inflammation. Furthermore, there appears to be a close interaction between these various vasoactive molecules. For instance, it was observed that myeloperoxidase (MPO), released by activated PMNs, not only generates cytotoxic oxidants but also impacts deleteriously on NO-dependent signaling cascades thereby influencing vasodilatation during inflammation. MPO increased tyrosine phosphorylation and p38 mitogen-activated protein kinase (PK) activation. Myeloperoxidase-treated PMNs released increased amounts of free radicals and enhanced PMN degranulation [2]. MPO, a highly abundant, PMN-derived heme protein, facilitates oxidative NO consumption and impairs vascular function in animal models of acute inflammation [3]. Superoxide anion ($O_2^{\bullet -}$), produced by PMNs during acute inflammation, has the ability to inactivate NO thereby reducing NO half-life and activity. Thus, there appears to be a close interaction between various mediators of acute inflammation. This property may have relevance to the pathogenesis of inflammation including vasodilatation seen during this process.

2.2.1.2. *Vascular Leakage.* A hallmark of inflammation is leakage of circulating protein into the extravascular tissue resulting in edema. This leakage of proteinaceous fluid is due to the formation of endothelial gaps in venules, direct endothelial damage, necrosis or detachment, leukocyte-mediated endothelial injury [4].

Exact details as to the chemical mediators and the sequence of their production is not clear. However, for the present discussion it is sufficient to know that cytokines, such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), vascular endothelial growth factor (VEGF), histamine, substance P, free radicals, NO, and other yet unidentified chemicals, seem to play a significant role in vasodilatation, vascular leakage, and diapedeses of leukocytes [5]. On the other hand, PMN-induced damage to vascular endothelial cells is believed to be due to increased production of reactive oxygen species (ROS), inducible NO (iNO) and its metabolites (such as hypochlorite (OCl), ozone, and release of cytokines. The main purpose of ROS, iNO, and ozone is to kill and eliminate invading microorganisms. These factors contribute to collateral damage to the surrounding cells/tissues due to their ability to diffuse across cell membranes and tissues and their potent mechanisms of action. In addition to their proinflammatory actions, ROS, iNO, IL-1, TNF, IFN, and, to some extent, VEGF, also have modulatory influence on vascular reactivity, endothelial cell function, smooth muscle cell proliferation, expression of adhesion molecules, leukocyte function, and extracellular matrix (ECM) production. These actions ultimately influence the inflammatory process, repair of the inflamed tissues/organs, and functional integrity of the target tissues/organs. The therapeutic application of the knowledge gained from the fundamental understanding of inflammation and its various molecular events has led to development of various monoclonal antibodies to specifically neutralize the actions of IL-1, TNF-α, IFN, and VEGF. For example, it is now known that age-related macular degeneration (AMD) is due to increased production of VEGF in retinal tissue. Studies showed that anti-VEGF antibody therapies are of significant benefit in AMD [6]. On the other hand, monoclonal antibodies directed against IL-1 and TNF-α failed to show any significant benefit in acute systemic inflammatory conditions, such as sepsis and septic shock [7]. This finding indicates that our understanding of inflammation is still inadequate to successfully develop therapeutically meaningful strategies. In this context, the role of free radicals in vascular reactivity during inflammation may prove to be interesting. Free radicals such as hydrogen peroxide (H_2O_2), $O_2^{\bullet -}$, NO, nitrated lipids, etc., have vasoactive actions. NO is a vasodilator, whereas $O_2^{\bullet -}$ and other free radicals have vasoconstrictor actions. In fact, it is believed that $O_2^{\bullet -}$ could be the vasoconstrictor that produces coronary vasospasm leading to acute angina. In view of the contrasting actions of NO

and $O_2^{\bullet -}$ on vascular reactivity, final blood vessel diameter may depend on the balance between NO and $O_2^{\bullet -}$ produced at the site of inflammation. Tissue antioxidant defenses [superoxide dismutase (SOD), catalase, and glutathione] act to neutralize, suppress, or antagonize the actions of free radicals. Thus, tissue destructive properties and vasoconstrictor actions of free radicals are largely determined by the tissue antioxidant concentration. Furthermore, NO itself can neutralize the actions of $O_2^{\bullet -}$. Hence, the balance between these two molecules could be yet another modulator of inflammation (Fig. 1).

2.2.2. *Cellular Events*

2.2.2.1. *Leukocyte Extravasation and Chemotaxis.* In order to eliminate the inciting agent responsible for inflammation and initiate the repair process, it is critical that leukocytes are delivered to the site of injury. One of the major functions of leukocytes is to ingest the offending agent; kill bacteria and other microbial organisms; and remove the necrotic tissue, debris, and foreign material. In the process of performing these important functions, leukocytes also induce tissue damage and in some instances may prolong inflammation. Leukocytes need to extravasate from inside the blood vessels

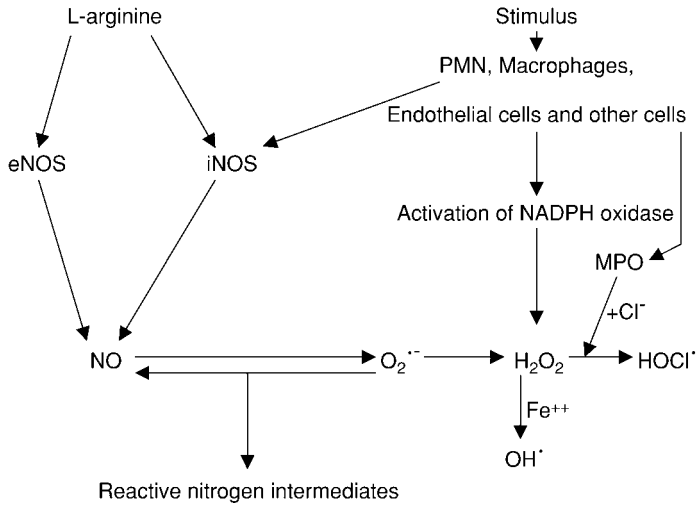

FIG. 1. Scheme showing generation of ROS and NO and formation of RNI (reactive nitrogen intermediates). Stimulus could be injury, foreign particles, or release of various proinflammatory cytokines. There is a close interaction between NADPH oxidase and MPO (see the text). Superoxide anion ($O_2^{\bullet -}$) can inactivate NO and in turn NO can inactivate $O_2^{\bullet -}$. NO and $O_2^{\bullet -}$ interact to form reactive nitrogen intermediates that are potent inflammatory substances.

in order to bring about these actions. To accomplish this, leukocytes adhere to the endothelial lining of blood vessels, transmigrate across the endothelium (a process termed diapedesis), and migrate through interstitial tissues toward the chemotactic stimulus to reach the site of inflammation or injury [8]. For extravasation to occur and for the leukocytes to adhere and transmigrate from the blood into tissues, both leukocytes and endothelial cells express complementary adhesion molecules, the expression of which is largely regulated by cytokines. The adhesion receptors involved in this process belong to four major molecular families: selectins, immunoglobulin superfamily, integrins, and mucin-like glycoproteins. Endothelial cell adhesion molecules, their complementary leukocyte receptor, and major function(s) are shown in Table 2. The multistep process of leukocyte migration through blood vessels involves: leukocyte rolling, activation and adhesion of leukocytes to endothelium, transmigration of leukocytes across the endothelium, piercing the basement membrane, and finally migration toward chemoattractants emanating from the site of injury or inflammation. Although almost all molecules may have a role in several of these processes, certain molecules play a dominant role. For example, selectins play a major role in neutrophil rolling. Chemokines are involved in activating the neutrophils to increase avidity of integrins. Integrins are important in firm adhesion. CD31 [platelet endothelial cell adhesion molecule (PECAM-1)] is involved in cell transmigration [9].

TABLE 2
MAJOR ADHESION MOLECULES THAT ARE EXPRESSED ON THE SURFACE OF ENDOTHELIAL CELLS AND THEIR COMPLEMENTARY ADHESION MOLECULES ON LEUKOCYTES

Endothelial molecule	Leukocyte receptor	Major function
P-selectin	Sialyl-Lewis X, PSGL-1	Rolling neutrophils, monocytes, and lymphocytes
E-selectin	Sialyl-Lewis X	Rolling, adhesion of neutrophils, monocytes, and T cells to activated endothelium
ICAM-1	CD11/CD18 (integrins) LFA-1, Mac-1	Adhesion and transmigration of leukocytes
VCAM-1	$\alpha 4\beta 1$ (VLA4) (integrins) $\alpha 4\beta 7$ (LPAM-1)	Adhesion of eosinophils, monocytes, and lymphocytes
GlyCam-1	L-selectin	Lymphocyte homing
CD31 (PECAM)	CD31	Leukocyte migration through endothelium

ICAM-1, VCAM-1, and CD31 belong to the immunoglobulin family of proteins; PSGL-1 = P-selectin glycoprotein ligand 1.

Induction of adhesion molecules on endothelial cells may occur by a number of mechanisms. For example, histamine, thrombin, and platelet activating factor (PAF) stimulate the redistribution of P-selectin from intracellular stores to the cell surface.

Macrophages, mast cells, and endothelial cells secrete proinflammatory cytokines, such as IL-1, TNF-α, and chemokines that act on endothelial cells to induce the expression of several adhesion molecules. This phenomenon results in the expression of E-selectin on the surface of endothelial cells. Simultaneously, leukocytes express carbohydrate ligands that allow them to bind to endothelial selectins [10]. This binding of leukocytes to the endothelium is a low-affinity interaction that is easily disrupted by blood flow. The process of binding, disruption, and rebinding results in rolling of leukocytes on the surface of endothelium (see Table 2).

Interleukins-1, TNF-α, and possibly other proinflammatory cytokines induce the expression of ligands for integrins, such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). Chemokines produced at the sites of inflammation or injury act on endothelial cells such that proteoglycans (e.g., heparan sulfate glycosaminoglycan) are expressed at high concentrations on their surface. Chemokines can also activate leukocytes to convert low-affinity integrins, such as integrin very late antigen 4 (VLA4) and lymphocyte function-associated antigen 1 (LFA-1), to high-affinity state. These events ultimately lead to firm binding of activated leukocytes to activated endothelial cells. As such, leukocytes stop rolling. Subsequent cytoskeletal reorganization results in leukocyte spreading out on the endothelial surface. Binding to the endothelial surface induces endothelial dysfunction and damage due to leukocyte production of ROS and iNO. Adherent leukocytes migrate through interendothelial spaces toward the site of injury or infection by binding to certain molecules of the immunoglobulin superfamily called PECAM-1 or CD31. Leukocytes pierce the basement membrane by secreting collagenases, enzymes that can digest collagen.

One of the mechanisms by which leukocytes migrate toward the sites of injury or inflammation is chemotaxis, a process induced by chemotaxins. These chemoattractants can be either endogenous or exogenous molecules. The most common exogenous chemoattractants are bacterial products, some of which are peptides that contain *N*-formyl-methionine terminal amino acid. Some of the endogenous chemoattractants include (but are not limited to): components of the complement system such as C5a, lipoxygenase (LO) pathway products such as leukotriene B_4 (LTB_4), and some cytokines such as IL-8. The exact mechanism by which leukocytes sense and are attracted toward a chemosensory agent is not clear. However, studies have suggested that the majority of these chemoattractants bind to seven specific

transmembrane G-protein-coupled receptors (GPCRs) on the surface of leukocytes [11]. GPCRs, in turn, activate phospholipase C (PLC), phosphoinositol-3-kinase (PI3K), and PKs. Both PLC and PI3K act on cell membrane phospholipids to generate lipid second messengers, such as inositol triphosphate (IP3) that increase cytosolic calcium (Ca^{2+}) and activate small GTPases of the Rac/Rho/cdc2 family as well as numerous kinases. Interestingly, GTPases induce polymerization of actin thus helping leukocyte motility. In fact, Bucci *et al.* [12] demonstrated that endothelial NO (eNO) synthase activation is critical for vascular leakage during acute inflammation. Early-phase inflammation (0–6 h), induced by intraplantar injection of carrageenan, was eliminated in congenic eNO synthase-deficient (eNOS−/−) mice. Secondary-phase inflammation (24–96 h) was found to be markedly reduced compared to wild type (WT) mice. Although zymosan-induced inflammatory cell extravasation was similar in WT and eNOS−/− mice, extravasation of plasma protein was lower in eNOS−/− mice. Inhibition of phosphatidylinositol 3-kinase and heat shock protein 90 (hsp90) also blocked protein leakage, but not leukocyte influx [12]. These results clearly established the critical role of eNOS in vascular leakage during acute inflammation. However, the exact relationship between selectins, VCAM-1 and ICAM-1, GPCRs, small GTPases of the Rac/Rho/cdc2 family, as well as numerous kinases and eNOS, and how the interaction between these molecules influences the inflammatory process remains unclear.

2.2.2.2. *Leukocyte Activation.* In order to kill microbes that produce inflammation, leukocytes generate ROS by a process termed activation. Products of necrotic cells, antigen–antibody complexes, cytokines, and chemokines also induce leukocyte activation. Different classes of leukocyte cell surface receptors recognize different stimuli. For instance, chemokines, lipid mediators, and *N*-formyl-methionyl peptides increase integrin avidity and produce cytoskeletal changes that aids leukocyte chemotaxis; microbial lipopolysaccharide (LPS) binds to toll-like receptors (TLRs) on leukocyte membrane leading to their activation and production of cytokines and ROS that are essential for the killing of microbes; and binding of microbial products to mannose receptor augments leukocyte phagocytic process that aids in the elimination of the invading organisms. Activation of leukocytes by various stimuli triggers several signaling pathways that result in increased cytosolic Ca^{2+} and activation of PKC and PLA_2 that are ultimately seen in the form of various functional leukocyte responses. In this context, it is interesting to note that PLA_2 activation leads to the release of lipids such as arachidonic acid (AA, 20:4 ω-6), eicosapentaenoic acid (EPA, 20:5 ω-3), and docosahexaenoic acid (DHA, 22:6 ω-3) from the cell membrane lipid pools. Studies showed that AA, and possibly EPA and DHA themselves could increase cytosolic Ca^{2+} and PKC concentrations in various cells [13,

14]. Furthermore, AA itself has the ability to activate leukocytes [15]. These findings suggest that simple dietary lipids have the ability to modulate leukocyte response and the inflammatory process. Products of AA, EPA, and DHA such as PGs, LTs, lipoxins (LXs), and resolvins are also known to have both positive and negative influences on leukocyte activation, chemotaxis, inflammation, and its resolution [16, 17]. Some products released by activated leukocytes include AA and its metabolites, lysosomal enzymes, ROS, NO, various cytokines and leukocyte adhesion molecules, and other cell surface receptors such as TLRs, GPCRs, receptors for opsonins, etc.

2.2.2.3. *Phagocytosis and Killing of Microbes by ROS.* To eliminate the invading microorganism, leukocytes first have to phagocytose them and release appropriate amounts of ROS and NO to kill them. Although leukocytes use mannose receptors and scavenger receptors to specifically bind and ingest bacteria, it can engulf bacteria and other particles without attachment to specific receptors. Opsonins greatly enhance the efficiency of phagocytosis. Once the bacteria or other foreign particle is recognized by leukocytes, they are engulfed for destruction.

Killing and degradation of ingested bacteria or foreign particles by leukocytes (as well as macrophages) is accomplished by ROS, NO, and ozone. In general, phagocytosis stimulates reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase accompanied by a burst of oxygen consumption, glycogenolysis, and increased glucose oxidation via the hexose-monophosphate shunt pathway. ROS, NO, and ozone have the ability to kill bacteria. The azurophilic granules of neutrophils contain MPO, which, in the presence of a halide such as Cl^-, converts H_2O_2 to hypochlorite (HOCl). HOCl is a potent antimicrobial agent that acts through binding covalently to cellular constituents or via oxidation of proteins and lipids [18]. Once leukocytes have performed their function of killing the bacteria, they rapidly undergo apoptosis and are ingested by macrophages.

It should be noted, however, that bacterial killing could also occur by oxygen-independent mechanisms. A study by Reeves *et al.* [19] showed that mice made deficient in neutrophil-granule proteases but normal with respect to ROS production and iodinating capacity were unable to resist staphylococcal and candidal infections. They showed that activation of neutrophils provoked influx of high amounts of ROS into the endocytic vacuole. This resulted in an accumulation of anionic charge that was compensated for by a surge of K^+ ions. These K^+ ions crossed the membrane in a pH-dependent manner and induced a steep rise in ionic strength resulting in release of cationic granule proteins, including elastase and cathepsin G. This protease release was primarily responsible for destruction of bacteria. Thus, there appears to be a close relationship between ROS and protease release, and bactericidal action of neutrophils. Given this finding it appears that proteases

are primarily responsible for bactericidal action not ROS itself. These observations have important clinical implications because the relative importance of MPO and NADPH oxidase generated ROS in fighting various infections remains a contentious issue. For example, Aratani *et al.* [20] demonstrated that mice with no MPO activity in their neutrophils and monocytes developed normally, were fertile, and showed normal clearance of *Staphylococcus aureus*. These animals, however, showed increased susceptibility to infection with *Candida albicans*. Lack of MPO was found to significantly enhance the dissemination of *Candida albicans* into various organs. These results suggest that MPO is important for early host defense against fungal infections. In contrast, the same authors reported that both MPO (MPO−/−) and NADPH oxidase deficient [X-linked chronic granulomatous disease (X-CGD)] mice were susceptible to pulmonary infections with *Candida albicans* and *Aspergillus fumigatus* compared with normal mice, and the X-CGD mice exhibited shorter survival than MPO−/− mice [21]. Increased mortality in X-CGD mice was associated with a 10–100-fold increased outgrowth of fungi in their organs. These results suggest that $O_2^{\bullet -}$ produced by NADPH oxidase was more important than HOCl produced by MPO against pulmonary infection with those fungi. It is interesting to note that at the highest dose of *Candida albicans*, the mortality of MPO−/− mice was comparable to X-CGD mice but was the same as normal mice at the lowest dose [22]. At the middle dose, the number of fungi disseminated into various organs of the MPO−/− mice was comparable to the X-CGD mice at 1 week postinfection, but it was significantly lower at 2 weeks postinfection. These results suggest that MPO and NADPH oxidase are equally important for early host defense against large inocula of *Candida albicans*. Hereditary MPO deficiency is a common defect with an estimated incidence of 1 in 2000 in the United States. The results of the studies performed by Aratani *et al.* [20–22] suggest that MPO-deficient individuals could exhibit similar problems as chronic granulomatous disease (CGD) patients if exposed to a large amount of fungi/microorganisms. It is likely that MPO deficient diabetics are more susceptible to fungal infections if the dose of inocula is small compared to normal patients.

3. Mediators of Inflammation

There are many chemical mediators of inflammation. Although the exact function and the source of some chemical mediators are unclear, certain generalizations are, however, possible. It should also be noted that there could be some unidentified chemical mediators of inflammation. Some of

the important mediators of inflammation include histamine, serotonin, lysosomal enzymes, PGs, LTs, PAFs, ROS, NO, HOCl, various cytokines, the kinin system, the coagulation/fibrinolysis system, and the complement system. Some of the general properties of mediators of inflammation are given later.

Plasma-derived mediators such as complement proteins and kinins are present in plasma in precursor form. These circulating mediators must be activated by a series of proteolytic cleavages to acquire their biologic properties. On the other hand, cell-derived mediators need to be secreted (e.g., histamine in mast cell granules) or synthesized *de novo* (e.g., PGs, cytokines) in response to a given stimulus. Major cellular sources of these mediators are platelets, neutrophils, monocytes/macrophages, and mast cells. Mesenchymal cells such as endothelium, smooth muscle, fibroblasts, and most epithelia can also be induced to elaborate some of these mediators. Invading microorganisms trigger the production of most of these mediators or host-derived products, such as complement, kinins, etc., that are activated by microbes or tissues under attack. These mediators generally bind to specific receptors on target cells to produce their action. In some instances, however, they can have direct enzymatic activity or induce the production of ROS or NO that, in turn, either mediate their actions or induce tissue damage. It is also interesting to note that in majority of the instances, one mediator triggers the release of another mediator that acts on the target tissue. These secondary mediators either potentiate the action of the initial mediator or paradoxically abrogate its action. Thus, the ultimate degree of inflammation depends on the balance between pro- and antiinflammatory mediators. In some instances, the antiinflammatory chemicals or signals initiated may not only act on the target tissue but also on other tissues to suppress inflammation. Thus, pro- and antiinflammatory mediators may act on specific or diverse tissues. Once released or activated, most of these mediators are either inactivated or decay quickly. For instance, AA and its metabolites have a short half-life, whereas specific or nonspecific enzymes inactivate kinins. In contrast, ROS and NO are scavenged by specific or nonspecific antioxidants [9]. This suggests that under normal physiological conditions positive and negative checks and balances are in place. An imbalance likely contributes to the generation of a pathological state.

The involvement of histamine, serotonin, bradykinin, the complement system, and coagulation cascade in the inflammatory process are well known and not addressed in this review. The role of AA and other polyunsaturated fatty acids and their products in inflammation warrants a brief discussion in view of their diverse role in inflammation and other various physiological and pathological processes.

3.1. Polyunsaturated Fatty Acids and Their Products

Cis-linoleic acid (LA, 18:2 ω-6) and α-linolenic acid (ALA, 18:3 ω-3) cannot be synthesized by mammals, but are essential nutrients and hence are referred to as "essential fatty acids" (EFAs). LA is converted to gamma-linolenic acid (GLA, 18:3, ω-6) by the action of the enzyme Δ^6 desaturase. GLA is elongated to form dihomo-GLA (DGLA, 20:3, ω-6), the precursor of

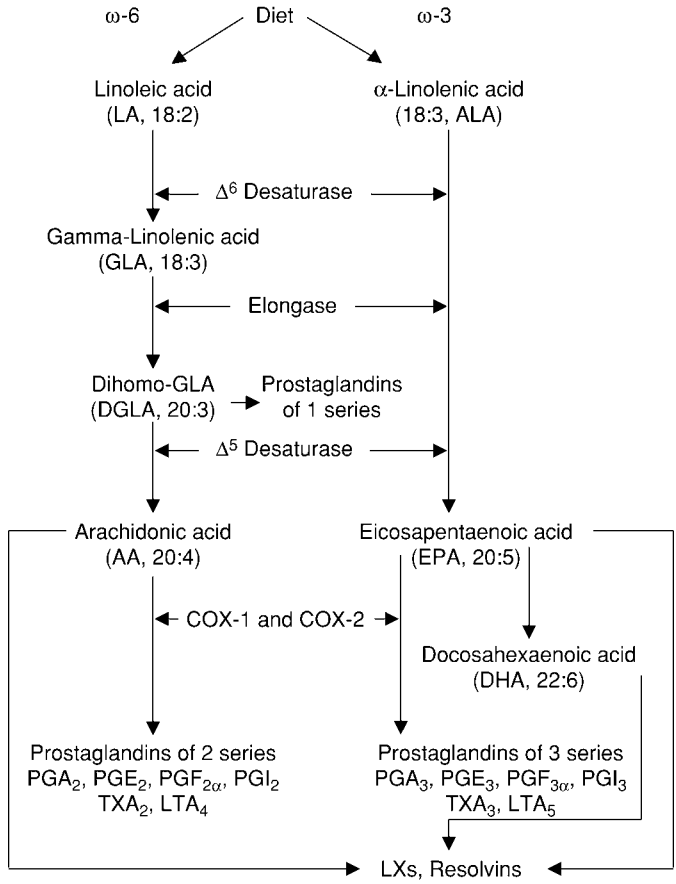

FIG. 2. Scheme showing metabolism of essential fatty acids and their metabolites. Resolvins are formed from AA, EPA, and DHA that have antiinflammatory action and inhibit leukocyte migration. LXs and resolvins reduce inflammation. PGE_2, PGE_3, $PGF_{2\alpha}$, $PGF_{3\alpha}$, LTA_4, and LTA_5 are proinflammatory in nature. PGE_1 appears to have antiinflammatory action. TXA_2 and TXA_3 are platelet aggregators and vasoconstrictors, whereas PGI_2 and PGI_3 are potent platelet antiaggregators and vasodilators.

the 1 series of PGs. The reaction catalyzed by Δ^6 desaturase is the rate-limiting step in the metabolism of EFAs. DGLA can also be converted to AA by the action of Δ^5 desaturase. AA is the precursor of the two series of PGs, thromboxanes (TXs), and the four series of LTs. ALA is converted to EPA by Δ^6 and Δ^5 desaturases. EPA forms the precursor of the three series of PGs and the five series of LTs. EPA can be elongated to form DHA. AA, EPA, and DHA form precursors to a group of novel compounds called LXs and resolvins [16, 17, 23]. These molecules have been shown to have antiinflammatory action [16]. The metabolism of EFAs is shown (Fig. 2). Eicosanoids bind to GPCRs on many cell types and mediate virtually every step of inflammation. They are found in inflammatory exudates and their synthesis is increased at sites of inflammation. The ability of nonsteroidal antiinflammatory drugs (NSAIDs), such as aspirin, to inhibit cyclooxygenase (COX) activity is believed to be responsible for their antiinflammatory action. Based on the role of eicosanoids in inflammation, COX-2 inhibitors have been developed to reduce inflammation *in vivo* without gastric side effects [24].

3.1.1. *Cyclooxygenase, Lipoxygenase, Lipoxins, and Resolvins Pathways*

There are two COX enzymes, the constitutively expressed COX-1 and the inducible enzyme COX-2 that lead to generation of PGs. The type of PG formed depends on the substrate fatty acid from which they are derived. It is important to know that different PGs have different actions as well as actions that are sometimes diametrically opposed. For example, PGE_2, PGF_{2a}, thromboxane A_2 (TXA_2), and LTs have proinflammatory action whereas PGE_1 and prostacyclin (PGI_2) may show antiinflammatory action. This finding is further complicated by the fact that the distributions of COX-1 and COX-2 enzymes have restricted tissue distribution. For example, the presence of TX synthetase in platelets results in production of TXA_2, a potent platelet-aggregator and vasoconstrictor. In contrast, vascular endothelial cells lack TX synthetase but do possess PGI_2 synthetase. This leads to formation of PGI_2, a potent platelet antiaggregator and vasodilator. PGI_2 potentiates the permeability-increasing and chemotactic effects of other mediators and thus, may participate in inflammation. The balance between TXA_2 and PGI_2 plays a significant role in thrombus formation in coronary and cerebral blood vessels. PGs also have a role in the pathogenesis of pain and fever of inflammation. PGE_2, a hyperalgesic, causes a marked increase in pain produced by intradermal injection of suboptimal concentrations of histamine and bradykinin, and is involved in cytokine-induced fever during infection. Major metabolites of the COX pathway in mast cells (PGD_2, PGE_2, and PGF_{2a}) cause vasodilatation and increase the permeability of postcapillary venules, thus, potentiating edema formation.

The COX-2 enzyme is absent in most tissues under normal "resting" conditions and is expressed in response to various proinflammatory stimuli. In contrast, COX-1 is constitutively expressed in most tissues. This suggests that PGs produced by COX-1 primarily serve a homeostatic function (fluid and electrolyte balance in the kidneys, cytoprotection in the gastrointestinal tract), but are also involved in inflammation. In contrast, COX-2 stimulates the production of PGs that are principally involved in inflammatory reactions.

Three types of LOs are present in only a few types of cells. 5-Lipoxygenase is the predominant enzyme in neutrophils. The main product of this enzyme is 5-hydroxyeicosatetraenoic acid (5-HETE), a chemotactic molecule, which is converted into LTs. LTB_4 itself is a potent chemotactic agent and activator of neutrophils. LTB_4 induces aggregation and adhesion of leukocytes to vascular endothelium, generation of ROS, and release of lysosomal enzymes. The cysteinyl-containing LTs C_4, D_4, and E_4 (LTC_4, LTD_4, and LTE_4) induce vasoconstriction, bronchospasm, and vascular permeability. Vascular leakage, as with histamine, is restricted to venules. LTs are more potent than histamine in increasing vascular permeability and causing bronchospasm. They mediate their actions by binding to cysteiny leukotreine 1 (CysLT1) and CysLT2 receptors.

Lipoxins are generated from AA, EPA, and DHA by transcellular biosynthetic mechanisms (involving two cell populations). Leukocytes, particularly neutrophils, produce intermediates in LX synthesis. These intermediates are converted to LXs by platelet–leukocyte interaction. LXA_4 and LXB_4 are generated by the action of platelet 12-LO on neutrophil-derived LTA_4. Cell–cell contact enhances transcellular metabolism. Blocking adhesion inhibits LX production. LXs inhibit leukocyte recruitment and cellular components of inflammation. They also inhibit neutrophil chemotaxis and adhesion to endothelium [17]. LXs serve as endogenous negative regulators of LT synthesis and action and may, thus, play a role in the resolution of inflammation. The inverse relationship between LXs and LTs suggest that the balance of these two molecules is critical in degree of inflammation as well as its final resolution.

3.1.2. *Aspirin-Triggered 15 Epimer LXs and Resolvins*

It is evident from the preceding discussion that AA not only forms precursors to proinflammatory compounds, such as TXs and LTs, but also gives rise to LXs. In this context, it is interesting to note that aspirin-triggered 15 epimer LXs (ATLs) are potent counter-regulators of polymorphonuclear neutrophil (PMN)-mediated injury and acute inflammation [25, 26]. Acetylation of COX-2 by aspirin prevents the formation of prostanoids, but the acetylated enzyme remains active *in situ* to generate 15R-HETE from AA. This is released and converted by activated inflammatory cells such as PMNs

to the 15-epimeric LXs. These LXs possess potent antiinflammatory properties [27–29]. Cross talk between endothelial cells and PMNs leads to formation of 15R-HETE and subsequent conversion to 15-epimeric LXs (aspirin-acetylated COX-2). This phenomenon is a protective mechanism to prevent local inflammation on the vessel wall by regulating the motility of PMNs, eosinophils, and monocytes [30]. Furthermore, endothelial cells oxidize AA via the cytochrome P450 enzyme system to 11,12-epoxy-eicosatetraenoic acid(s) that blocks endothelial cell activation [29]. Nonenzymatic oxidation products of EPA inhibit phagocyte–endothelium interaction and suppress the expression of adhesion molecules [31]. This suggests that when potent COX-2 inhibitors are used, these novel and highly beneficial LXs are not formed. Thus, PMN and endothelial cell interaction leads to endothelial damage, thrombus formation, and coronary artery disease.

It is known that akin to the formation of 15R-HETE and 15-epimeric LXs from AA, similar compounds are also formed from EPA and DHA. Human endothelial cells, in which IL-1β induced COX-2, pulsed with EPA and treated with aspirin, converted EPA to 18R-HEPE, 18-HEPE, and 15R-HEPE. Similar to the ability of PMNs to convert aspirin triggered, COX-2 derived 15R-HETE to 15-epi-LXA_4 and EPA to 5-series LXs, activated human PMNs converted 18R-HEPE to 5,12,18R-triHEPE and 15R-HEPE to 15-epi-LXA_5 by their 5-LO. Both 18R-HEPE and 5,12,18R-triHEPE inhibited LTB_4-stimulated PMN transendothelial migration similar to 15-epiLXA_4. 5,12,18R-triHEPE effectively competed with LTB_4 for its receptors and inhibited PMN infiltration suggesting that it can suppress LT-mediated responses if present in adequate amounts at the sites of inflammation. Similar to aspirin, other NSAIDs, such as acetaminophen and indomethacin, also induced the formation of 18R-HEPE and 15R-HEPE when tested with recombinant COX-2 and EPA. This finding suggested that NSAIDs permit oxygenation of AA and EPA by activated endothelial cells at sites of inflammation to form the novel antiinflammatory compounds [32].

In a similar fashion, murine brain cells expressing COX-2 and treated with aspirin transformed enzymatically DHA to 17R series of hydroxy DHAs (HDHAs) that, in turn, were converted enzymatically by PMNs to di- and tri-hydroxy containing docosanoids [33]. It is interesting to note that similar small molecular weight compounds are generated from AA, EPA, and DHA. These include 15R-hydroxy containing compounds (AA), 18R series (EPA), and 17R-hydroxy series (DHA). All these compounds have potent antiinflammatory action and are involved in resolution of the inflammatory process and hence have been termed as ''resolvins.'' These compounds inhibited cytokine generation, leukocyte recruitment, leukocyte diapedesis, and exudate formation. These AA, EPA, and DHA-derived products (resolvins) from acetylated COX-2 are formed via transcellular biosynthesis (due to

cell–cell communication between endothelial cells and PMNs) and are probably meant to suppress inflammation. This suggestion is supported by the observation that resolvins inhibit brain ischemia-reperfusion injury [16, 33]. Hence, it is likely that some of the cardioprotective actions of EPA and DHA can be related to their conversion to resolvins and LXs. Thus, both LXs and resolvins derived from AA, EPA, and DHA have antiinflammatory actions and are involved in the resolution of inflammation. In view of this, any defect in their synthesis or their inappropriate degradation may lead to continuation of the inflammatory process and/or progression of acute inflammation to the chronic phase.

3.2. Platelet Activating Factor

Platelet activating factor is another bioactive phospholipid-derived mediator known to have multiple proinflammatory effects. Chemically, PAF is acetyl-glyceryl-ether-phosphorylcholine (AGEPC), a phospholipid with a glycerol backbone, a long-chain fatty acid in the **A** position, an unusually short chain substituent in the **B** location, and a phosphatidylcholine moiety. PAF mediates its effects via a single GPCR. A family of inactivating PAF acetylhydrolases regulates its effects. Platelets, basophils, mast cells, neutrophils, monocytes/macrophages, and endothelial cells can elaborate PAF. PAF not only causes platelet activation, but also causes vasoconstriction and bronchoconstriction. At extremely low concentration, PAF induces vasodilatation and increased venular permeability with potency many times greater than that of histamine. PAF also causes leukocyte adhesion to endothelium by enhancing integrin-mediated leukocyte binding, chemotaxis, degranulation, and the oxidative burst. It boosts the synthesis of eicosanoids by leukocytes and other cells. Thus, PAF can elicit all the cardinal features of inflammation [34]. PAF receptor antagonists inhibit inflammation in some experimental models.

3.3. Cytokines and Chemokines in Inflammation

Cytokines are proteins produced by many cell types including activated lymphocytes and macrophages, endothelial cells, epithelial cells, and connective tissue cells. They have the ability to modulate the functions of various other cells. Cytokines not only have a regulatory role in cellular immune responses but also participate in both acute and chronic inflammation. The major cytokines that are involved in inflammation and have proinflammatory actions are TNF-α, IL-1, and IL-6. On the other hand, IL-4 and IL-10 have antiinflammatory actions, restrict inflammation and generally antagonize the actions of IL-1, IL-6, and TNF-α. Activated macrophages and T cells are sites of production for these cytokines. Studies, however, have

shown that a variety of other cells and tissues are also capable of producing these cytokines. For instance, endothelial cells, adipose tissue, Kupffer cells, and glial cells are capable of producing them. Endotoxin and other microbial products, immune complexes, physical injury, and a variety of inflammatory stimuli can stimulate TNF-α and IL-1 secretion. They activate endothelial cells, stimulate leukocytes, and fibroblasts, and induce systemic acute-phase reactions. Activation of endothelial cells by TNF-α, IL-6, and IL-1 induces a spectrum of changes, mostly regulated at the level of gene transcription. These induce the synthesis of endothelial adhesion molecules and chemical mediators of inflammation such as other cytokines, chemokines, growth factors, eicosanoids, and NO [35]. These events increase the thrombotic tendency on the surface of the endothelium. TNF-α primes neutrophils, leading to augmented responses of these cells to other mediators, and stimulates neutrophils to produce ROS [36]. IL-1, IL-6, and TNF-α induce the systemic acute-phase responses associated with infection or injury such as fever, loss of appetite, slow-wave sleep, the release of neutrophils into the circulation, and the release of corticotropin and corticosteroids. When large amounts of these cytokines are released they may produce hemodynamic effects of septic shock (hypotension, decreased vascular resistance, increased heart rate, and decreased blood pH) that may ultimately cause death. Sustained and increased production of TNF-α occurs during chronic intracellular infections such as tuberculosis and neoplastic diseases. Lipid and protein mobilization also occur and lead to development of cachexia in these patients. IL-1, IL-6, and TNF-α suppress appetite that contributes to cachexia [37]. Increased production of IL-1, IL-6, and TNF-α is also seen in rheumatoid arthritis, systemic lupus erythematosus (SLE), and other collagen vascular diseases. This discovery led to the development of anti-TNF-α antibodies and TNF-α receptor blockers for use in the therapeutic treatment of these conditions.

3.4. Low-Grade Systemic Inflammation in Metabolic Syndrome X

Studies suggested that low-grade systemic inflammation plays a significant role in the pathogenesis of type 2 diabetes [38, 39]. This proposal is based on the observation that plasma concentration of C-reactive protein (CRP), TNF-α, IL-6, and resistin, all markers of inflammation, are elevated whereas the concentration of adiponectin that shows antiinflammatory actions are reduced in type 2 diabetes mellitus [40–42].

3.5. Cytokines and C-Reactive Protein

Elevated plasma concentration of CRP, TNF-α, and IL-6 may produce their harmful effects in type 2 diabetes mellitus, hypertension, and obesity by

inducing endothelial dysfunction. TNF-α and IL-6 damage endothelial cells, cause apoptosis of endothelial cells, and trigger procoagulant activity and fibrin deposition [38–43]. It was shown that forearm blood flow responses to acetylcholine (ACh) were inversely correlated with CRP serum concentration indicative of endothelial dysfunction [44]. High CRP concentration was associated with decreased endothelial nitric oxide (eNO) generation [45]. In this context, it is interesting to note that in earlier studies by our group and others showed that NO levels were low in patients with diabetes mellitus [46]. These results suggested that elevated CRP, IL-6, and TNF-α concentration in patients with type 2 diabetes may lead to decrease in eNO production and consequent endothelial dysfunction. Because NO is a potent vasodilator and platelet antiaggregator, low eNO level may, in turn, lead to increased peripheral vascular resistance and higher incidence of thrombosis and atheroslcerosis.

However, it is still debated whether inflammation is the primary event or is secondary to development of type 2 diabetes. For instance, CRP levels do not correlate with the extent of atherosclerosis. This finding suggests that CRP levels reflect the body's response to inflammation elsewhere. On the other hand, CRP functions as a chemoattractant, increases the expression of adhesion molecules, and activates complement proteins. All are important mediators of inflammation. Furthermore, CRP binds to LDL cholesterol and increases the uptake of LDL by macrophages. Studies in animals revealed that CRP enhances the size of myocardial infarction [47]. These results suggest that inflammation plays a role in the pathobiology of type 2 diabetes and diseases associated with it.

Interleukin-6 and TNF-α increase neutrophil $O_2^{\bullet -}$ generation [36, 48]. Superoxide anion inactivates NO and PGI_2 causing endothelial dysfunction, enhanced thrombosis, and atherosclerosis [49, 50]. All are common in type 2 diabetes. On the other hand, optimal production of NO inactivates $O_2^{\bullet -}$ thereby preventing/arresting thrombosis and atheroslcerosis [50, 51]. This observation indicates that increased oxidative stress could be another factor that contributes to development of type 2 diabetes, hypertension, and other components of metabolic syndrome X [38, 39, 50–52].

It is now known that adipose tissue produces several biologically active molecules that have important actions on the immune response and inflammation. Three of these molecules are adiponectin, resistin, and corticosterone. Adiponectin has antiinflammatory actions and its plasma concentration is inversely related to insulin resistance and the severity of type 2 diabetes whereas resistin induces insulin resistance and has proinflammatory actions [53, 54]. A study suggested that transgenic mice overexpressing 11 β-hydroxysteroid dehydrogenase type 1 (11βHSD-1) selectively in adipose tissue developed abdominal obesity and exhibited insulin-resistant diabetes

(type 2 diabetes), hyperlipidemia, and hyperphagia [55]. This finding suggests that type 2 diabetes could behave similar to localized Cushing's syndrome.

Several other studies also revealed that elevated plasma concentration of CRP and possibly, IL-6 and TNF-α predict the future development of type 2 diabetes mellitus, hypertension, and CHD [56–58]. Furthermore, reduction in the level of CRP, IL-6, and TNF-α achieved by diet control, exercises, and statin therapy predicted better outcome in these patients. This finding suggests that measurement of these inflammatory markers could be used to predict the development of metabolic syndrome and response to various therapies.

3.6. Chemokines

Chemokines are a family of small (8–10 kD) proteins that act primarily as chemoattractants for specific types of leukocytes [59–61]. About 40 chemokines and 20 chemokine receptors have been identified. They are classified into four major groups, according to the arrangement of the conserved cysteine (C) residues in the mature proteins. Chemokines mediate their action by binding to seven transmembrane GPCRs that usually exhibit overlapping ligand specificities. Leukocytes generally express more than one receptor type. Certain chemokine receptors (CXCR-4, CCR-5) act as coreceptors for a viral envelope glycoprotein of human immunodeficiency virus (HIV-1) and are involved in viral binding and entry into cells. Chemokines have the ability to stimulate leukocyte recruitment in inflammation and control normal migration of cells through various tissues [62]. Some chemokines are produced transiently in response to inflammatory stimuli and promote the recruitment of leukocytes to the sites of inflammation, whereas others are produced constitutively in tissues and participate in organogenesis. In both situations, chemokines are displayed at high concentration attached to proteoglycans on the surface of endothelial cells and in the ECM.

3.7. Nitric Oxide

NO was originally discovered as a vasodilatation factor released from endothelial cells and hence was called as endothelium-derived relaxing factor [63]. It is a soluble gas produced not only by endothelial cells but also by a variety of cells including macrophages and brain neurons. It is now evident that many (if not all) cells produce NO and that NO also participates in inflammation. It acts in a paracrine manner on target cells through induction of cyclic guanosine monophosphate (cGMP) that, in turn, initiates a series of intracellular events leading to the desired response such as relaxation of vascular smooth muscle cells, neurotransmission, tumoricidal, cytotoxic,

and bactericidal actions. The half-life of NO is only few seconds and hence, it has to be produced in close proximity to where it is needed.

NO is synthesized from L-arginine by the action of NO synthase (NOS) [64]. There are three types of NOS: eNOS, neuronal (nNOS), and iNOS. NOS exhibits two patterns of expression. eNOS and nNOS are constitutively expressed at low levels but can be activated rapidly by an increase in cytoplasmic calcium ions. Influx of calcium into cells leads to a rapid production of NO. In contrast, iNOS is induced in macrophages and other cells when activated by cytokines such as TNF-α and IFN-γ. It is paradoxical that eNO and nNO have many beneficial properties whereas iNO shows proinflammatory action.

NO plays an important role in the vascular and cellular components of inflammatory responses. It is a potent vasodilator and prevents platelet aggregation. It inhibits vascular smooth muscle cell proliferation, reduces platelet adhesion, and also inhibits several features of mast cell-induced inflammation. It serves as an endogenous regulator of leukocyte recruitment. Inhibition of endogenous NO production promotes leukocyte rolling and adhesion in postcapillary venules. On the other hand, delivery of exogenous NO reduces leukocyte recruitment. Thus, under normal physiological conditions, NO is an inhibitor of inflammatory response and possibly, increased production of NO in inflammatory conditions could be a compensatory mechanism to block inflammatory responses [65]. But, it should be understood that increased production of NO in response to various inflammatory stimuli might itself perpetuate inflammation. In these situations NO may be converted to peroxynitrite radical that has potent proinflammatory actions (Fig. 1). Decreased production of eNO occurs in insulin resistance, obesity, atherosclerosis, diabetes, and hypertension [66–69].

NO and its derivatives have microbicidal actions and, thus, NO functions as an endogenous mediator of host defense against infection [70]. This premise is supported by the observation that: (1) reactive nitrogen intermediates (RNI) derived from NO possess antimicrobial activity, (2) NO interacts with ROS to form multiple antimicrobial metabolites, (3) NO production is increased by macrophages and other immune cells in response to infection, and (4) inactivation of iNOS enhances the incidence of infection and augments multiplication of microbial organisms in experimental animals. Enhanced production of NO by macrophages and other immune cells has been shown to inhibit the growth of several bacteria, viruses, fungi, and other organisms. It is relevant to note that NO also had tumoricidal action.

Although NO is unstable, its concentration in plasma and various cells *in vitro* could be measured using various colorimetric techniques and specific NO probes. NO is measured as its stable metabolites nitrite and nitrate in plasma, which provides an indication as to the concentration of NO

released by endothelial cells. Highly sensitive NO probes are commercially available to measure intracellular NO concentration and NO released by *in vitro* cell cultures. These sensitive techniques provide a means by which to study the influence of chemicals, drugs, and factors on the generation of NO. Thus, it is now possible to assess NO generation both *in vivo* and *in vitro* by various cells and tissues.

3.8. Leukocyte Lysosomal Enzymes

Two types of lysosomal granules are present in neutrophils and monocytes. These are small specific (secondary) granules and large azurophil (primary) granules. The small specific secondary granules contain lysozyme, collagenase, gelatinase, lactoferrin, plasminogen activator, histaminase, and alkaline phosphatase. The large azurophil primary granules contain MPO, lysozyme, defensins, acid hydrolases, and a variety of neutral proteases such as elastase, cathepsin G, proteinase 3, and nonspecific collagenases [71]. To bring about their action, both granule types release their contents into phagocytic vacuoles that form around engulfed material. Granule contents can also be released into the extracellular space. Release of lysosomal granules contributes to inflammation. Granule enzymes have specific functions. For instance, acid proteases degrade bacteria and debris within the phagolysosomes under acidic pH conditions, whereas neutral proteases degrade various extracellular components. Neutral proteases attack and degrade collagen, basement membrane, fibrin, elastin, and cartilage. This action ultimately results in tissue destruction typically seen in acute and chronic inflammatory processes. Neutral proteases also have the ability to cleave C3 and C5 directly resulting in the release of anaphylatoxins and kinin-like peptide from kininogen. Neutrophil elastase degrades virulence factors of bacteria and thus helps to control bacterial infection [72]. Both monocytes and macrophages participate in chronic inflammatory reactions due to their granule composition including acid hydrolases, collagenase, elastase, PL, and plasminogen activator. In view of the destructive nature of lysosomal enzymes released by neutrophils, it is important that therapies control leukocyte infiltration at sites of injury and infection. Unchecked leukocyte infiltration can lead to increased vascular permeability and subsequent tissue destruction. Antiproteases are present in serum and tissue fluid to control the harmful effects of these proteases. One of the best examples is α_1-antitrypsin, an antiprotease that inhibits neutrophil elastase. Deficiency of α_1-antitrypsin leads to uncontrolled action of leukocyte elastase, a protease is associated with pulmonary damage in emphysema. α_2-Macroglobulin is another important antiprotease found in serum and various secretions.

3.9. Reactive Oxygen Species

ROS or oxygen-derived free radicals are released by leukocytes, macrophages, and other similar cells present in various organs into the extracellular compartment following exposure to noxious agents such as microbes, foreign objects, and in response to chemokines, ingestion of immune complexes, or phagocytic challenge [73]. The production of ROS is due to activation of the NADPH oxidative system. Known ROS species are $O_2^{\bullet -}$, H_2O_2, and hydroxyl radical (OH). ROS are produced mainly within the cell and are capable of reacting with NO to form RNI toxic to various intracellular organelles [74]. Because ROS and RNI are highly toxic, their release into the extracellular space even at low concentrations may prove harmful. Furthermore, even at very low concentrations they are capable of increasing the expression of chemokines (e.g., IL-8), cytokines, and endothelial leukocyte adhesion molecules, that is, events that amplify the inflammatory cascade [75]. The physiological function of ROS and RNI are to destroy bacteria, viruses, fungi, and cancer cells. Conversely, increased production of ROS and RNI is potentially harmful and could cause acute and chronic inflammation, sepsis, and other pathological conditions. Thus, ROS and RNI can cause endothelial cell damage resulting in increased vascular permeability, insulin resistance, and thrombosis. In this context, it is important to note that activated adherent neutrophils not only produce ROS and RNI but also stimulate xanthine oxidase in endothelial cells that, in turn, elaborates further generation of $O_2^{\bullet -}$. ROS and RNI inactivate antiproteases such as α_1-antitrypsin. This can lead to unopposed protease activity resulting in increased destruction of ECM. ROS by themselves damage many cells and tissues including, but not limited to, parenchymal cells. It is now believed that several clinical conditions are due to excess production of ROS. For instance, there is reasonable evidence to suggest that ROS and RNI are responsible for diseases such as rheumatoid arthritis, systemic lupus erythematosus, and other collagen vascular diseases; ulcerative colitis, ischemia-reperfusion injury to myocardium (following coronary bypass surgery), and cerebral cortical damage (after ischemic stroke); and several pathophysiological processes such as insulin resistance, metabolic syndrome X, atherosclerosis, schizophrenia, and Alzheimer's disease. In view of this, research efforts are currently underway to develop antioxidants and free radical quenchers that might mitigate these diseases and pathophysiologic processes. Evidence also indicates that various features of metabolic syndrome X are due to low-grade systemic inflammation due to excess production of ROS and RNI in the specific tissues in question. For example, excess ROS production in endothelial cells (or close to endothelial cells) produces damage resulting in endothelial dysfunction. Interestingly, obesity, hypertension, type 2 diabetes mellitus, hyperlipidemia, and CHD (all

components of metabolic syndrome X) are characterized by endothelial dysfunction. This is further supported by increased ROS generation in obesity, hypertension, type 2 diabetes mellitus, hyperlipidemia, and insulin resistance. Despite these findings, it is not yet clear why and how increased generation of ROS occurs. Once identified, it will be possible to develop reasonable therapeutic approaches to prevent or treat these conditions. It is important to know when increased ROS generation occurs so that preventive or therapeutic measures can be appropriately timed.

In order to abrogate the harmful actions of ROS, several antioxidants are present in serum, various tissue fluids, and cells. These antioxidants include: (1) the copper-containing serum protein ceruloplasmin; (2) the iron-free fraction of serum, transferrin; (3) the enzyme SOD, which is found or can be activated in a variety of cell types; (4) the enzyme catalase, which detoxifies H_2O_2; and (5) glutathione peroxidase, another powerful H_2O_2 detoxifier. The influence of ROS in inflammatory conditions is dependent on the balance between the production and inactivation of these metabolites by cells and tissues.

Following its identification, NO has been shown to have an important role in the pathogenesis of both acute and chronic inflammation. Excess production of NO, especially by macrophages, is harmful to several tissues. Activation of iNOS, as occurs in response to various stimuli or by itself sometimes is sufficient to initiate and perpetuate the inflammatory process. More often than not, excess production of both ROS and NO occur in the majority of inflammatory conditions. As such, it is extremely difficult to separate the individual roles of ROS and NO in a given pathology or inflammatory condition.

It is important to note that NO has many useful actions as well. It is a potent platelet antiaggregator and vasodilator and has been thought to prevent atherosclerosis. Production of appropriate amounts of eNO is possible only when endothelial cells are healthy. Hence, measurement of NO plasma concentration and/or NO endothelial production can be used to assess endothelial cell integrity and health. In obesity, hypertension, type 2 diabetes mellitus, insulin resistance, hyperlipidemia, and CHD the plasma concentration of NO is low. This observation suggests that endothelial dysfunction is common to all these conditions. NO levels revert to normal following weight loss achieved by diet restriction and exercise, control of hypertension, normalization of plasma glucose levels in type 2 diabetes mellitus, and reduction of plasma lipid levels. Thus, measurement of plasma NO concentration could be used as a biomarker of endothelial function and may also be useful in judging adequacy of treatment given to patients with these conditions. Since many factors could influence the synthesis and half-life of NO, it is important to keep note of them. For example, decreased NO production could be due to deficiency of its precursor L-arginine and/or lack

of an essential cofactor such as tetrahydrobiopterin (BH_4). One or both could contribute to low plasma NO levels [76]. These factors should be considered before a cause of decreased NO levels is made.

3.10. Neuropeptides in Inflammation

Neuropeptides are known to play a significant role in the initiation and propagation of inflammation. Substance P and neurokinin A, produced both in the central and peripheral nervous systems, influence transmission of pain signals, regulation of blood pressure, stimulation of secretion by endocrine cells, and increase vascular permeability [77–79]. The involvement of these neuropeptides in the inflammatory process explains the neurogenic component of inflammation. Sensory neurons produce certain proinflammatory molecules that link sensing of dangerous stimuli to development of protective host responses form the basis of neurogenic inflammation [79].

4. Clinical Laboratory Tools to Diagnose Inflammation

It is evident from the preceding discussion that many biological molecules are involved in the pathobiology of inflammation. At bedside, it is relatively simple to diagnose acute inflammation generally characterized by *rubor*, *tumor*, *calor*, *dolor*, *and functiolaesa* (redness, swelling, heat, pain, and loss of function, respectively). Because these acute inflammatory events are visible and easily assessed, no specific laboratory tests may be required to measure the presence or absence of inflammation. However, when the inflammatory process is low-grade and localized to the internal organs it is difficult, if not impossible, to detect and confirm the presence of inflammation. This is especially true in low-grade systemic inflammation. Assessment of chronic inflammation as a result of infection or infestation requires specific tests. For example, chronic malaria (especially *Plasmodium malariae* and *ovale*) infection itself and in partially immune individuals is extremely difficult to diagnose. This is because the classical signs of malaria such as fever with chills and rigors do not manifest themselves clearly. Under these circumstances, one has to carefully examine the peripheral blood smear for the malarial organism. This test, however, is ordered only when the clinician suspects the presence of malaria or has a high degree of clinical suspicion. The physician may suspect malarial infection in an individual with significant hepatospleenomegaly, loss of weight and appetite, and whether the patient is from an endemic area or has recently traveled to a tropical country where malaria is common. In this modern era the incidence of infections is less common whereas degenerative conditions and geriatric disease are more frequent, it has become increasingly difficult to diagnose diseases in

which low-grade systemic inflammation is common. Examples of diseases in which low-grade systemic inflammation is common include obesity, insulin resistance, type 2 diabetes mellitus, hypertension, CHD, dyslipidemia, and atherosclerosis. The belief that inflammation plays a significant role in these conditions has come from the observation that subjects with these diseases have enhanced plasma levels of CRP, IL-6, and TNF-α. These patients also show low circulating NO levels and increased generation of ROS. Increased ROS decreases antioxidant content of the cells/tissues due to their utilization. Thus, these patients may show decreased vitamin E, SOD, and glutathione levels. As such, the delicate balance between the pro- and antioxidant status is tilted in favor of pro-oxidants ultimately leading to tissue damage and disease. However, it is not yet certain as to what actually triggers the initiation of the disease process. Once this phenomenon is understood, it will be possible to take adequate steps or device methods/drugs to arrest early disease process development.

In view of the above low-grade systemic inflammation is integral in the pathobiology of metabolic syndrome X and its associated conditions. Clinical laboratory tools to analytically assess and diagnose inflammation will be as discussed in the following section.

4.1. High-Sensitive CRP

Present knowledge indicates that inflammation plays a significant role in the initiation and development of atherosclerosis. Molecules that participate in inflammation of the arterial wall are also detected in the circulation. Hence, these molecules have been evaluated as potential indicators, that is, "biomarkers," for predicting risk of CHD. These biomarkers include acute-phase reactants such as fibrinogen, CRP, and serum amyloid A (SAA). The presence of these markers at low concentration in the circulation suggests low-grade systemic (albeit chronic) inflammation. More specific primary inflammatory signals, such as IL-6, and TNF-α; and VCAM, such as soluble ICAM-1, may also be enhanced in CHD. There is now sufficient evidence to suggest that these inflammatory markers could be used to clinically predict and prognose CHD. Large interest in this clinical application has led to the development of a variety of commercial assays for these markers.

CRP is a 135,000-dalton nonimmunoglobulin protein. It is the most reliable marker of inflammation and increases several hundred-fold in response to acute injury, infection, or other inflammatory stimuli. CRP is probably more reliable than erythrocyte sedimentation rate (ESR). One of the most attractive features of CRP is its preanalytical long-term stability in serum or plasma, at room temperature or frozen. A commonly assigned cutoff value for CRP is $\sim$10 mg/liter in serum or plasma. CRP serum concentration

between 10 and 40 mg/liter is associated with mild inflammation whereas a concentration between 40 and 200 mg/liter is associated with acute inflammation and bacterial infection. Commonly used procedures usually detect CRP values at ~3 mg/liter. The concentration of CRP in low-grade systemic inflammatory conditions is, however, substantially lower than in acute inflammation. Hence, a much more sensitive assay is necessary to measure CRP in low-grade systemic inflammation. High-sensitivity CRP (hs-CRP) assays accurately and reproducibly measure CRP concentration as low as 0.3 mg/liter.

4.1.1. *Physiological Variables and Interpretation of hs-CRP Results*

Several studies revealed that no sex- or ethnicity-specific alterations are seen with CRP. No significant differences were found in the distribution of CRP concentration among Caucasian, African-American, and Mexican-American men [80]. A comparable CRP distribution was found in Japanese men, with slightly lower concentrations in Japanese women [81]. Indian Asians, who are racially at high-risk of developing metabolic syndrome X, have been shown to have a 17% higher geometric mean for CRP concentration than whites [82]. This difference was not, however, statistically significant when results were adjusted for central obesity and insulin resistance [82]. Most studies have reported that there is no relation between age and CRP concentration for individuals between healthy 20 and 70 years old [80, 83]. CRP concentration was found to vary slightly between age groups in women in a large study ($n = 15{,}770$). Median CRP concentrations for women aged 45–54, 55–64, 65–74, and >75 years old were 1.31, 1.89, 1.99, and 1.52 mg/liter, respectively [84]. There is no seasonal variation in CRP concentration. Despite this, it is recommended that the average of two independent CRP measurements (fasting or nonfasting), taken at least two weeks apart, be used to establish a person's risk for future CHD. There is no diurnal variation for CRP. The presence of heparin or ethylene diamine tetra acetic acid (EDTA) does not influence CRP measurement. Thus, plasma or serum for CRP determination could be collected any time of the day. CRP is stable at 4°C for up to 60 days and remains unchanged for months or years when stored at −70°C [85].

CRP cutoff points recommended for CHD risk assessment are >1.0 mg/liter (low risk), 1.0–3.0 mg/liter (average risk), and >3.0 mg/liter (high risk). These cutoffs can be applied irrespective of sex and race. If hs-CRP levels are >10 mg/liter, the test should be repeated and careful clinical and laboratory studies performed to rule out the presence of infection or inflammation. Recommended hs-CRP units of measure are milligrams per liter with results expressed to one decimal point.

4.1.2. *High-Sensitive CRP and In-Hospital Events*

CRP has a prothrombotic role [86]. Some clinical data has suggested that CRP could be a valuable short-term marker of risk in patients [87–89]. In fact, CRP and SAA protein were shown to significantly predict prognosis in patients with severe unstable angina. In this study, hs-CRP values >3 mg/liter showed a fivefold increased risk of recurrent attacks [90]. However, the large c7E3 Anti-Platelet Therapy in Unstable Refractory angina (CAPTURE) trial [91] failed to show a significantly increased risk of in-hospital events among patients with unstable angina, non-ST-elevation myocardial infarction (NSTEMI), and elevated CRP levels or both NSTEMI and elevated CRP. On the other hand, Morrow *et al.* [92] showed a 18-fold increased risk of death among patients with unstable angina and NSTEMI who had elevated hs-CRP levels. Several other studies confirmed that in-hospital mortality was significantly higher in patients having hs-CRP levels >6 mg/liter versus patients with hs-CRP <3 mg/liter [93–98]. Elevated hs-CRP level appears to predict adverse events. As such, hs-CRP could be used as a marker of low-grade systemic inflammation to predict future development of cardiovascular disease/events and prognosis.

4.1.3. *High-Sensitive CRP and Prediction and Prognosis of Long-Term Cardiovascular Events*

There is evidence to suggest that hs-CRP is useful to predict and prognosticate the recurrence of cardiovascular events in mid- to long-term states. CRP levels predicted not only the composite end point of death, acute myocardial infarction (AMI), recurrent angina, and the need for coronary revascularization procedures but also the incidence of death in follow-up ranging from 90 days to 4 years [88, 91, 95, 97–106]. For example, the large European Concerted Action on Thrombosis and disabilities (ECAT) study showed a twofold increase in coronary events in patients with CRP >3.6 mg/liter. Similar results were also reported by other studies [97–106]. These studies suggested that patients with CRP levels >3 mg/liter at discharge are at increased risk of developing new ischemic events, including death, AMI, and unstable angina. This was true in patients who underwent an interventional procedure, such as angioplasty/bypass surgery, or were on medical treatment. All these studies were consistent with the observation that high CRP level (>3 mg/liter) is an independent predictor of prognosis and progression of coronary disease. With this consideration in mind, it is interesting to note that hs-CRP can also be used to predict the future development of hypertension, insulin resistance, stroke, and peripheral vascular disease. All appear to be low-grade systemic inflammatory conditions. Furthermore, subjects with high hs-CRP levels (>3 mg/liter) are more likely to respond

poorly to treatment and develop complications much earlier than those with low hs-CRP levels. Thus, subjects with high hs-CRP levels should be treated more aggressively and require close and careful follow-up. Subjects who are otherwise normal, but have high hs-CRP levels are most likely to develop features of metabolic syndrome X within 5 years. As such, hs-CRP could be used both to predict future development of cardiovascular diseases and to prognose disease development. Thus, patients can be advised accordingly. In fact, it has been suggested that hs-CRP should be measured in all subjects (normal or those with disease) and used to assess the underlying disease process, potential response to treatment, and predict development of cardiovascular disease, stroke, peripheral vascular disease, and features of metabolic syndrome X.

4.2. Cytokines and Chemokines

A number of studies showed that other inflammatory markers could be used to predict the development of various cardiovascular diseases and atherosclerosis and predict their prognosis. IL-1, IL-6, IL-8, IL-10, TNF-α, and monocyte chemoattractant protein (MCP-1) are some factors studied. Adhesion molecules such as ICAM-1, and soluble VCAM-1 and proinflammatory cytokines (IL-1, IL-6, IL-8, IL-10, and TNF-α) have been associated with a risk of new coronary events in ischemic heart disease and clinical recurrence of symptoms [107–112]. Despite these findings, these markers are less reliable than hs-CRP. In contrast to CRP, these markers are relatively unstable in serum. Serum and plasma needs to be rapidly separated from the cellular blood components. Samples need to be assayed rapidly or stored frozen to prevent degradation of labile cytokines and adhesion molecules. These assays are typically performed using an enzyme-linked immunosorbent assay (ELISA) technique, a labor-intensive methodology. The advent of automated assay methods (such as a microplate chemiluminescent assay) may perhaps promote use and popularity. Multiplex assays for several cytokines have been developed and show great promise. Despite these advances, these techniques have not become part of routine clinical laboratory medicine practice.

Another limitation of most cytokine assays is their general lack of precision. Also, cytokine assays typically lack a sufficiently lower limit of quantification for use in apparently healthy subjects. It is hoped that as the field advances, more accurate and precise methods will be available to accurately quantify cytokine levels within the normal reference interval (i.e., concentration range in an apparently healthy population).

Studies showed that fibrinogen was consistently associated with long-term CHD risk [113], although its association differs among studies. This discrepancy could, in part, be due to the differences in analytical methodology used.

SAA was observed to be a reliable CHD marker [114, 115]. Some results, however, have been inconsistent. In one study, SAA, but not hs-CRP, was found to be associated with the extension of CHD. This finding suggesting that both markers have a similar association with CHD events, but may possess different roles in the pathogenesis of atherosclerosis, but is not in the prediction of future events.

IL-18, originally described as IFN-inducing factor, is present in atherosclerotic plaques [116]. IL-18 has been shown to be associated with future cardiovascular death in a 3.9-year long follow-up of patients with stable angina and unstable angina pectoris. The predictive value of IL-18 was similar to that of hs-CRP suggesting that it does not add significant value in predicting future CHD compared to hs-CRP [117].

Myeloperoxidase is a proinflammatory leukocyte-derived enzyme present in abundant amounts in ruptured plaques. Studies showed that MPO could be associated with the recurrence of CHD and other cardiovascular events even in those patients negative for the cardiac marker troponin [118]. It is also interesting to note that the predictive value of MPO was found to be independent of both troponin and hs-CRP levels [119]. It remains to be determined if MPO can be used routinely to predict prognosis of patients with CHD.

4.3. Other but More Conventional Markers of Inflammation

Leukocytosis is known to be an excellent marker of inflammation. Studies revealed that a higher leukocyte count could be associated with a greater cardiovascular risk. Because many extraneous factors can influence leukocyte count, one needs to be careful in using this analytical measure as a marker for predicting or prognosing cardiovascular risk. Sample preparation is important since leukocyte count has to be performed on fresh whole blood specimens. It should be noted that current cigarette smoking increases leukocyte count. Any unnoticed or subclinical infection may also increase leukocyte count, thus, limiting the clinical utility of this measurement.

Elevated fibrinogen levels have been shown to be a major independent risk factor for cardiovascular diseases and stroke outcome [120, 121]. Higher fibrinogen level was shown to enhance CHD risk in patients with hypertension or diabetes, as well as in cigarette smokers.

5. High Sensitive-CRP and Other Proinflammatory Indices as Markers of Cardiovascular Diseases: But, Why and How?

It is evident from the preceding discussion that hs-CRP and other proinflammatory indices could be used as independent risk factors for cardiovascular diseases and atherothrombosis. High levels of hs-CRP, IL-6, IL-18,

TNF-α, amyloid A, MPO, fibrinogen, and leukocytosis appear to predict future cardiovascular risk in apparently healthy men and women. Despite these findings, the exact mechanism(s) involved in cardiovascular disease process remains unclear. It is likely that markers such as CRP and MPO are closely linked to the underlying pathophysiology, that is, low-grade systemic inflammation. High hs-CRP concentration may thus simply reflect the underlying inflammatory process ultimately responsible for the initiation and progression of atherosclerosis that finally results in CHD. Since atherosclerosis occurs as a result of failure of the antithrombotic properties of endothelium, it is possible that increased hs-CRP is an indication that endothelial cells are no longer able to perform their antithrombotic actions adequately. In other words, increased hs-CRP concentration and other proinflammatory markers provide an indication that endothelial cells have failed to produce antithrombotic molecules (NO and PGI2) and that inflammation is likely responsible.

This premise is supported by the observations that CRP induced matrix metalloproteinase-1 (MMP-1) expression through the Fc region of the IgG with cell surface receptor II (Fc gamma RII) and extracellular signal-related kinase pathway, upregulated IL-8 in human aortic endothelial cells via NF-κ B, promoted MCP-1-mediated chemotaxis by upregulating CC-chemokine receptor 2 expression in monocytes, and attenuated endothelial progenitor cell survival, differentiation, and function via inhibiting NO generation [122]. These events initiate and perpetuate inflammation and atherosclerosis and induce atherosclerotic plaque instability. Studies using human CRP transgenic animal models showed that CRP promoted atherothrombosis and increased plasminogen activator inihibitor-1. There is substantial evidence to suggest that CRP is not only produced by liver but also by endothelial cells indicating that localized production of CRP could be responsible for atherosclerotic lesions. CRP binds to Fc gamma receptors on leukocytes. It significantly upregulated surface expression of Fc gamma receptors, CD32, as well as CD64 on human aortic endothelial cells and colocalized with CD32 and CD64. The increase in IL-8, ICAM-1, and VCAM-1, and decrease in eNO and PGI_2 induced by CRP was abrogated by specific antibodies to CD32 and CD64. These results suggest that the biological effects of CRP are mediated via binding and internalization through Fc gamma receptors, CD32, and CD64 [123]. CRP selectively enhanced intracellular generation of ROS in monocytes and neutrophils [124], decreased PGI_2 release from human aortic endothelial cells by inactivating PGIS (PGI_2 synthase) via nitration [125], and also directly inhibited NO generation by cytokine-stimulated vascular smooth muscle cells [126], and most importantly, induced apoptosis in human coronary vascular smooth muscle cells [127]. All these actions of

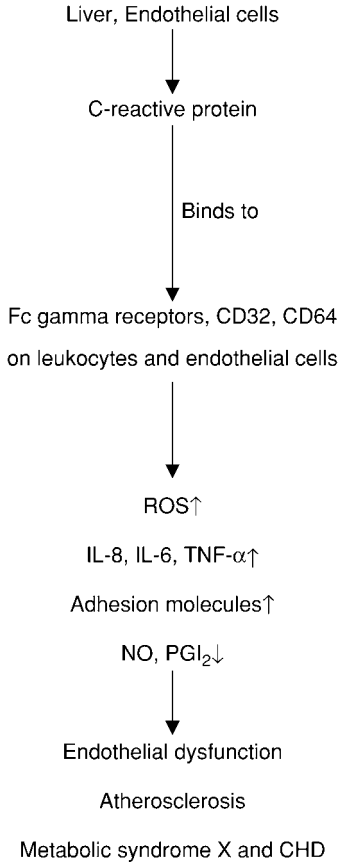

FIG. 3. Actions of CRP that is relevant to its role in atherosclerosis and as a predictor of future development of CHD and metabolic syndrome X and their prognosis.

CRP ultimately lead to the development and progression of atherosclerosis and CHD (Fig. 3).

Despite these findings, the role of CRP in atherosclerosis is not without controversy. Taylor *et al.* [128] reported that CRP-induced *in vitro* endothelial cell activation was an artifact caused by azide and LPS present in many CRP preparations. It was reported that cCRP (*Escherichia coli*-derived CRP) but not in-house-generated azide-free recombinant and ascites-purified CRP were responsible for changes in cell proliferation, morphology, apoptosis, and expression of eNO synthase and ICAM-1, MCP-1, IL-8, von Willebrand factor secretion by endothelial cells [128]. It was observed that this ability of

cCRP to induce activation of endothelial cells was lost on extensive dialysis, suggesting that low-molecular weight contaminants were responsible. Indeed, the effects of cCRP were mirrored by the presence of azide or LPS thus indicating that contaminated cCRP commercial preparations were likely responsible for the endothelial activation events reported in this study. These results led to the conclusion that CRP, *per se*, does not activate endothelial cells. This finding, however, does not mean that there is no relationship between the elevated plasma CRP levels and their correlation with prediction and prognosis of cardiovascular events. This finding suggests that CRP may not be solely responsible for all biological events associated with atherosclerosis, it may only be a marker of events that are occurring.

6. Conclusions

It is evident from the preceding discussion that inflammation is a complex multifactoral process in which it is extremely difficult to pinpoint the initiating event. Although advances in molecular biology and laboratory techniques have significantly improved our fundamental understanding of inflammation, it is apparent that the exact biochemical mechanisms involved in this process remain unclear. Lack of understanding has contributed to a paucity of treatment strategies to control inflammation, especially low-grade systemic inflammation seen in CHD, metabolic syndrome X, hypertension, etc. Studies have shown that low-grade inflammation plays a significant role in many of these hitherto believed to be degenerative conditions. It is not yet clear whether low-grade systemic inflammation occurs with the aging process. If so, it will be interesting to study whether suppressing low-grade systemic inflammation can slow the aging process itself. Because inflammation is a fundamental process of all living organisms, it remains to be seen how it can influence several other cellular processes such as longevity and development of cancer. In fact, there is significant evidence to suggest that inflammation has a role in pathogenesis of cancer. However, it is not yet certain how and where inflammation occurs in the cancerous process. In this context, it is interesting to note that platelets also play a significant role in inflammation, especially, markers of platelet activation such as regulate on activation, normal T expressed and secreted (RANTES) and in particular CD40L. The fact that platelets, in some unknown way, participate in metastasis of tumor cells once again underscores the relationship between inflammation and various diseases processes. Platelet antiaggregators have been shown to either prevent or substantially reduce tumor cell metastasis in experimental animals. This finding did not, however, find application in clinical practice, thus, reinforcing the large gap between animal and human

studies. Despite this limitation, it is believed that basic laboratory advances will eventually find clinical application. It is likely that a better understanding of inflammation will eventually lead to the use of existing or new antiinflammatory compounds in conditions such as cardiovascular diseases, cancer, and stroke.

The observation that low-grade systemic inflammation plays a role in various cardiovascular diseases and metabolic syndrome X has led to the development of new laboratory tests. These tests, however, have yet to have a significant impact on routine clinical laboratory practice. Based on the above findings, it is evident that some of these new tests would eventually become mainstream diagnostic tools. High-sensitive CRP, various proinflammatory cytokines, and adhesion molecules are revolutionizing prediction and prognosis of cardiovascular diseases and metabolic syndrome X. Further developments in the assay standardization and assay methodology are essential before these biomarkers can be routinely measured. It is also possible that many more such markers of inflammation will be developed in the future as our understanding of the inflammatory process and its role in the pathobiology of various diseases unfolds. In this context, the laboratory pathologist will have a significant role to play.

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ADVANCES IN PROSTATE-SPECIFIC ANTIGEN TESTING

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1. Abstract

Prostate cancer (PCa) is the most common cancer in men and it is a major health problem in industrialized countries. Prostate-specific antigen (PSA) is a sensitive diagnostic marker for PCa, and measurement of serum PSA is widely used for early detection, screening and monitoring of patients with PCa. However, the usefulness of PSA testing is limited because of the high rate of false-positive results. Another problem with the use of PSA for early detection and screening is overdiagnosis, i.e. detection of slowly growing prostate cancers that do not need to be treated clinically. These can be reduced by assaying the proportion of free PSA in relation to total PSA. However, further development of more cancer-specific diagnostic methods facilitating identification of aggressive cancers is desirable. This chapter summarizes existing and new approaches for detection of various forms of PSA. PSA-based multivariate algorithms are also discussed.

2. Introduction

Prostate cancer is the most common cancer and the second leading cause of cancer mortality in men in industrialized countries after lung cancer [1]. The incidence of prostate cancer has increased substantially during the past decades and it represents a major health problem worldwide. Prostate cancer can be detected by screening or case finding based on the determination of prostate-specific antigen (PSA) in serum and biopsy of men with elevated concentrations of PSA [2]. By this approach prostate cancer may be diagnosed at an early stage, in practice 5–10 years before giving rise to symptoms [3, 4]. However, so far it is not known whether population-based screening with PSA actually reduces prostate cancer mortality and results from randomized trials will not be ready for several years [5–7]. Less controversial is the use of PSA in the monitoring of prostate cancer. Furthermore, a rapid rise in PSA level before diagnosis has been shown to predict high risk of death from prostate cancer after radical prostatectomy [8].

When introduced into clinical practice PSA revolutionized prostate cancer diagnosis, staging, and follow-up of the disease [2, 9, 10]. Serum PSA is now the most important marker for diagnosis and monitoring of prostate cancer. However, the use of PSA in early detection of prostate cancer is problematic due to the high false-positive rate caused by benign prostatic hyperplasia (BPH) [9, 11]. In serum, the majority of the immunoreactive PSA consists of the PSA-α1-antichymotrypsin complex (PSA-ACT) and free PSA. The proportion of PSA-ACT is higher and that of free PSA is lower in serum from patients with prostate cancer than in those with BPH, and measurement of

these major PSA forms can improve the cancer specificity of the PSA test [12, 13]. In addition to the PSA-ACT, PSA also forms complexes with α2-macroglobulin (A2M) and α1-protease inhibitor (API, also called α1-antitrypsin). The complexes between PSA and API (PSA-API) and A2M (PSA-A2M) have been detected immunologically in serum from patients with prostate cancer [14–16]. The measurement of serum PSA-API and PSA-A2M can be used to improve the accuracy of diagnosis of early prostate cancer [17–19]. About 5–35% of serum PSA is free [12, 13], and several variants of free PSA occur in serum, that is, proPSA, enzymatically active PSA, and internally cleaved, enzymatically inactive PSA [20–22]. Some studies have suggested that enzymatically active PSA and proPSA in serum are preferentially found in men with prostate cancer, while the cleaved forms are more specific for BPH [21–23]. Thus, determination of these PSA variants may reduce the number of false-positive results in early diagnosis of prostate cancer [24, 25].

Another problem with the use of PSA for early detection and screening is overdiagnosis, that is, detection of small and slowly growing prostate cancers that would not have surfaced during the lifetime of the patients. An important challenge is to identify aggressive cancers that need to be treated while avoiding diagnosing patients, who do not benefit from being diagnosed. Therefore, there is an urgent need for better methods to discriminate between prostate cancers that need and do not need to be cured.

3. Prostate-Specific Antigen

PSA was first purified from prostatic tissue and was shown to be prostate specific in 1979 [26]. One year later, it was found to be present in serum of patients with prostate cancer [27, 28]. Although PSA was later shown to be produced also in other tissues, it is in practice a specific marker for prostate cancer and prostatic diseases.

3.1. Biochemistry and Structure of PSA

PSA (also called hK3) is a 33-kDa serine protease with chymotrypsin-like enzymatic activity [29–31]. It is encoded by a gene clustered in chromosomal region 19q13.4, together with hK2, tissue kallikrein (hK1), and 12 other characterized structurally related human kallikrein genes [30–33]. The genes of the human kallikrein family are numbered KLK1–15, and the corresponding proteins named hK1–15. All members of the kallikrein family have five exons and display considerable (40–79%) sequence similarities at the DNA and amino acid levels [29, 30, 32–34]. The best-known kallikreins are hK1, hK2, and PSA.

PSA and hK2 are the most closely related, with 79% identity in amino acid sequence, while hK1 displays 62% identity with PSA [34].

PSA is a single-chain glycoprotein. The structure deduced from the cDNA sequence shows that PSA is synthesized as a 261 amino acid preproenzyme, comprising a 17-amino acid signal peptide, followed by a 7-amino acid propeptide and a 237-amino acid mature, enzymatically active protein [35, 36]. The signal peptide is removed during synthesis. The secreted proenzyme, called proPSA, is enzymatically inactive and can be activated into the mature form by proteolytic cleavage with trypsin, hK2, hK4, and prostin [37–41]. Trypsin is the most efficient activator, followed by hK4, prostin, and hK2 [37, 40–42]. PSA also contains a carbohydrate chain linked to Asn-45 (numbering according to active PSA). The average molecular weight of mature PSA determined by mass spectrometry is 28430 [43], and the relative molecular mass determined by SDS-PAGE and gel filtration is about 33 kDa [43–45].

PSA contains a catalytic triad that is characteristic to serine proteases. Residues His-41, Asp-96, and Ser-189 are located in typical sites of the active pocket [46, 47]. Ser-183, which is located at the bottom of the active site, is crucial for the specificity of PSA [48]. The enzymatic activity of PSA is chymotrypsin-like, but very restricted, and it preferentially hydrolyzes peptide bonds at the carboxy-terminus of the hydrophobic residues tyrosine and leucine [49, 50]. Several residues surrounding these preferred P1 residues play an important role in determining the substrate specificity [50–52].

3.2. Expression of PSA

3.2.1. *Tissue Expression of PSA*

PSA is secreted by normal prostatic epithelial cells, benign prostatic hyperplasia, and prostate cancer cells, and its expression is stimulated by androgens through androgen receptor mediated transcriptional activation [26, 44, 53–55]. In LNCaP cells, which are the most widely used model for studying PSA expression, dihydrotestosterone is the most potent inducer of PSA synthesis [56]. Several growth factors and other factors have also been suggested to affect PSA expression [57, 58].

In the prostate, the secretion of PSA is directed into the prostatic ducts, and PSA occurs at very high concentrations, 0.5–2 mg/ml, in human seminal fluid [45]. In seminal fluid, most of the PSA occurs in an intact, enzymatically active form, while 35–40% is internally cleaved and inactive (nicked PSA) [49, 59]. ProPSA has not been detected in seminal fluid [60].

PSA is quite organ specific, but it has been shown to be weakly expressed in some other human tissues, for example, in the periurethral glands, the anal gland, and breast tissue of males and females [61–64]. Low expression of PSA

has also been detected in the gastrointestinal tract [65]. PSA can occasionally be found in other cancers such as adrenal, kidney, lung, and colon cancers, and it has also been used as a prognostic marker for breast cancer [66–69]. With ultrasensitive immunoassays, PSA can be detected at very low concentrations in female serum and other body fluids, including amniotic fluid, breast fluid, cyst fluid, and nipple aspirate fluid [70–72].

The expression of PSA is higher in benign prostatic tissue than in cancer [73], but normal epithelial cells of the prostate secrete PSA into the glandular ducts, and it reaches circulation only by leaking into interstitial space and then diffusing into circulation [74]. Thus, the serum concentrations are normally about 1 million-fold lower than those in seminal fluid. In prostate cancer, however, the tissue architecture of the prostate is deranged, and when the tumor loses connection with the prostatic ducts PSA is released directly into the interstitial space and circulation [74, 75]. This explains why a prostate cancer produces about 30-fold higher serum concentrations of PSA per gram tissue than the normal prostate [2]. The PSA released from prostate cancer is thought to be more active than the PSA leaking out from benign prostatic tissue. This probably explains why the proportion of PSA occurring in complex with ACT is increased in serum from prostate cancer patients [12, 75].

3.2.2. *PSA-Producing Cell Lines*

Several of the prostate cancer cell lines used in research produce PSA [76, 77]. Although the amount and activity of the PSA produced by these cell lines are much lower than in normal prostate tissue or primary human prostate cancers [78], these cell lines are valuable for studying the regulation and function of PSA. The LNCaP cell line, isolated from a needle biopsy of a lymph node metastatic lesion, is the most widely used PSA-producing prostate cancer cell line [76, 77, 79]. When grown in a medium containing fetal bovine serum, LNCaP cells mainly secrete PSA as the inactive proenzyme [42, 80]. The amount of enzymatically active PSA increases when the cells are grown in a medium without serum [80]. LNCaP cells express androgen and estrogen receptors, but the androgen receptor contains a mutation that enables the receptor to also bind some other steroids [81]. In addition to the parental line, there are also over 50 LNCaP cell sublines with variable steroid responses.

3.3. Isoforms of PSA from Seminal Fluid

Most of the PSA purified from seminal fluid is in an intact, enzymatically active form, while 35–40% is internally cleaved and inactive (also called nicked PSA) [29, 49, 59, 60]. Purified PSA can be further subfractionated

by reverse phase [29] and anion exchange chromatography [59]. By using anion exchange chromatography, purified PSA from seminal fluid can be separated into five isoforms (A, B, C, D, and E). Isoforms PSA-A and -B are intact enzymatically active forms, which differ in glycosylation, while PSA-C, -D, and -E are internally cleaved forms [59]. The internal cleavage sites in the peptide backbone are at Arg-85, Lys-145, and Lys-182. The cleavages disrupt the conformation of PSA, causing loss of catalytic activity [29, 49, 59]. The fragments in cleaved PSA molecules are held together by disulfide bonds, but they can be separated in SDS-PAGE under reducing conditions [59].

Intact PSA forms complexes with A2M, pregnancy zone protein, ACT, protein C inhibitor, and API when incubated with these protease inhibitors *in vitro* [14, 49, 59, 82]. When added to human serum, PSA preferentially forms complexes with A2M and ACT [83], but it also forms a complex with API [14, 84]. The complex between PSA and protein C inhibitor has only been found in human seminal plasma [82, 85]. The complexes of PSA-ACT and PSA-API dissociate gradually during storage in solution. The released PSA is enzymatically active and can form a complex with A2M [14, 83].

Nicked isoforms of PSA (PSA-C, -D, and -E) are very inefficient in forming complexes with ACT and API as compared to intact PSA, but 40–80% of these nicked forms react with A2M, though the rate is slow [59, 83]. The binding of PSA with A2M requires enzymatic cleavage of the bait region of A2M, resulting in a conformational change and encapsulation of the protease by A2M. Thus, the ability of nicked PSA to form a complex with A2M is surprising.

3.4. Biological Functions of PSA

The main biological function of PSA is liquefaction of the seminal gel formed after ejaculation by proteolytic cleavage of semenogelin I and II that are physiological substrates of PSA and major proteins in seminal fluid [86]. Some studies have suggested that PSA plays a role both in inhibition and promotion of prostate cancer invasion and metastasis. PSA may inhibit cell growth and angiogenesis by generating angiostatin-like fragments from plasminogen [87–89], and it may also induce apoptosis [90]. Other experiments suggest that PSA activates the urokinase-type plasminogen activator that is thought to be involved in tumor invasion and metastasis [91]. PSA might also affect tumor spread by proteolytic modulation of cell adhesion receptors [92]. Furthermore, PSA has been found to cleave insulin-like growth factor binding protein-3 and -4 (IGFBP-3 and -4), causing local release of active insulin-like growth factor-I (IGF-I) that could enhance tumor growth [93, 94]. However, prostate cancer patients with high concentrations of PSA in serum do not have an increased proportion of cleaved IGFBP-3

[95]. PSA also cleaves fibronectin and laminin [96], and activates latent TGF-β [92]. By these latter mechanisms PSA may mediate progression of prostate cancer. It is not clear, which function of PSA is most important or even physiologically relevant, but based on the association between low-tissue concentrations of PSA and adverse prognosis [97], it is conceivable that PSA prevents rather than promotes progression of prostate cancer.

4. Measurement of PSA in Circulation

PSA was first detected in the serum of prostate cancer patients in 1980 [27]. After the clinical study by Stamey *et al.* serum PSA became a widely used marker for early detection, screening, and monitoring of prostate cancer [2, 9]. However, PSA is not tumor specific but rather organ specific. Thus, prostate cancer, BPH, and prostatitis can all cause increased PSA concentrations in circulation. Manipulation of the prostate, for example, cystoscopy and prostate biopsy, also increase the concentrations of serum PSA [98, 99].

A problem in the clinical use of PSA is that various immunoassays may give different results [100]. Initially this was due to lack of a common standard and differences in the recognition of free PSA and PSA-ACT by the antibodies used [101–103]. This problem has been reduced by the preparation of international standards for free PSA and PSA-ACT [104]. These have made a significant reduction in intermethod variation in proficiency testing programs [104, 105].

4.1. Epitope Mapping

Epitope mapping of 83 monoclonal antibodies to PSA have revealed six partially overlapping antigenic regions on the PSA molecule [106, 107]. One of the six PSA epitopes is completely covered when PSA is complexed with ACT. This epitope contains amino acids 86–91, and constitutes a part of the kallikrein loop of PSA surrounding the active site [108]. Antibodies recognizing this epitope are specific for free, that is, noncomplexed PSA, they inhibit the enzymatic activity of PSA and display no cross-reaction with the structurally similar protein, hK2 [109, 110]. As most antibodies specific for free PSA display no or weak reactivity to reduced PSA on Western blotting, the corresponding epitopes appear to be conformation dependent [110]. The other five epitope-regions are exposed on both free PSA and PSA-ACT, and antibodies binding to these regions react with both forms and are thus called total PSA specific. Some of these antibodies bind with free PSA and PSA-ACT fairly equally, whereas other antibodies bind free PSA preferentially

TABLE 1
AGE-SPECIFIC REFERENCE RANGES FOR SERUM PSA

Age range (years)	Total PSA concentration (μg/liter)	Free PSA concentration (μg/liter)
40–49	<2.0	<0.5
50–59	<3.0	<0.7
60–69	<4.0	<1.0
70–79	<5.5	<1.2

[106]. Several antibodies specific for total PSA display cross-reaction with hK2 [107, 111].

4.2. REFERENCE VALUES FOR SERUM PSA

Traditionally, 4 μg/liter has been used as a cut-off level for total PSA, but this value does not correspond to any properly determined reference limit. Among apparently healthy men over 50 years of age, the serum concentration of PSA exceeds 4 μg/liter in about 10%, about 8% have concentrations of 4–10 μg/liter, and 2% have levels over 10 μg/liter [112, 113]. Among men with a serum PSA in the range 4–10 μg/liter about 25–30% has a prostate cancer, which is detectable by biopsy, while those with PSA levels above 10 μg/liter have a 40–50% likelihood of prostate cancer [9, 114]. However, prostate cancer can also be detected by biopsy in 20–30% of men with a PSA level of 3–4 μg/liter [112, 115–117].

The serum concentration of PSA increases with age, and therefore properly determined reference value is age dependent [118, 119]. The age-specific upper reference limit based on the 95th percentile of serum PSA among healthy men in various age groups are shown in Table 1 [119]. The upper reference limit for total PSA increases from 2.0 μg/liter in men below 50 years of age to 5.5 μg/liter in those above 70 years of age. The increase in serum PSA with age is partially dependent on increasing prevalence of BPH [120] and partially on the increasing prevalence of occult prostate cancer. Thus, the probability of finding prostate cancer by biopsy is about the same in men of various ages with similar PSA values. This may explain why many clinicians prefer using fixed cut-off levels rather than age-specific reference ranges.

4.3. MEASUREMENT OF PSA-INHIBITOR COMPLEXES

Specific measurement of the various complexed forms of PSA in circulation can be used to improve the detection of prostate cancer (Fig. 1). The complex PSA-ACT comprises 60–95% of total PSA while 1–2% of PSA is

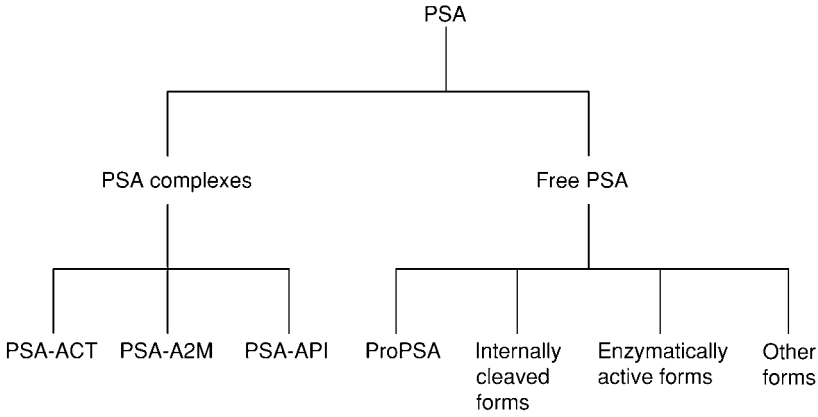

FIG. 1. Different forms of circulating PSA.

bound to API [12, 15, 84]. The complex with A2M is not measured by assays for total PSA, but its concentration is in the range 1–10% of total PSA.

4.3.1. *PSA-ACT*

The proportion of PSA-ACT is higher in serum from patients with prostate cancer than in healthy men and patients with BPH [12, 13]. Measurement of PSA-ACT or the ratio of PSA-ACT to total PSA improves the detection of prostate cancer [12, 13, 121–124]. About 20–30% of the false-positive results caused by BPH can be avoided by assaying the proportion of either PSA-ACT or free PSA in relation to total PSA [121].

Immunoassays for PSA-ACT using a polyclonal anti-ACT antibody as a tracer [12, 106, 121, 125] were hampered by a variable background caused by nonspecific binding of free ACT to the solid phase. The interference could be reduced by adding heparin and detergent to the assay buffer [106, 126], or by addition of milk casein [127], a polyclonal anticathepsin G antibody or latex microparticles [128]. Monoclonal antibodies with increased specificity for the PSA-ACT complex have been produced by immunizing mice with PSA-ACT, but the use of these have not improved the sensitivity and specificity substantially [129, 130]. Zhu *et al.* [131] developed a dual-label immunoassays for simultaneous measurement of PSA-ACT and free PSA, and PSA-ACT and total PSA. These assays utilized a novel monoclonal antibody specific to complexed ACT in combination with monoclonal antibodies to free and total PSA. In these assays, concentrations of PSA-ACT and free PSA or PSA-ACT and total PSA can be measured simultaneously by using tracer antibodies labeled with different fluorophores. With these assays the

clinical performance of the proportion of PSA-ACT and free PSA in relation to total PSA were equal [131].

4.3.2. *PSA-API*

The PSA-API complex can be detected in serum from patients with prostate cancer and BPH by using a monoclonal antibody to capture PSA and a polyclonal antibody to API as a tracer [12, 14, 84]. The sensitivity of the assay was limited by a high nonspecific background, caused by the huge excess of free API present in serum [84]. To overcome this problem, the nonspecific background and PSA-API were measured in separate assays and the net concentration of PSA-API was estimated by subtracting the background signal from PSA-API signal [19]. Interestingly, the proportion of PSA-API was higher in BPH than in cancer sera, and preliminary results suggest that measurement of PSA-API can improve the clinical validity of PSA testing [18, 19].

4.3.3. *Complexed PSA Assay*

An assay measuring two forms of complexed PSA, PSA-ACT and PSA-API, called complexed PSA (cPSA), has been developed by blocking the reactivity of free PSA using an anti-free PSA antibody. The assay measures the remaining PSA comprising PSA-ACT and PSA-API, but not PSA-A2M. Some investigations show that cPSA improves detection of prostate cancer [132–134], but this has not been confirmed in other studies [135].

4.3.4. *PSA-A2M*

When added to serum, PSA rapidly forms a complex with A2M [49, 83, 136]. Because A2M encapsulates PSA, complex formation hinders access of anti-PSA antibodies to PSA [137]. Therefore, the PSA-A2M complex cannot be detected by conventional immunoassays [13, 83]. However, it can be detected by immunoblotting with antibody specific to PSA, indicating that denaturation of A2M by SDS reveals epitopes in PSA [101]. Utilizing this observation, we developed a dissociation assay for the detection of PSA-A2M in serum [15]. Initially PSA-ACT, PSA-API, and free PSA are removed from the serum sample by immunoadsorption. Thereafter, PSA-A2M is denatured at high pH and neutralized. PSA released from PSA-A2M or still bound to denatured A2M is measured by an immunoassay specific to total PSA. Using this assay, PSA-A2M in serum containing clinically relevant concentrations of PSA could be quantified. PSA-A2M represents 1–10% of total PSA in serum, and the proportion of PSA-A2M of total PSA is higher in patients with BPH than those with prostate cancer [15]. This could be explained by the recent finding of increased expression of A2M in stromal tissue in BPH [138]. Determination of PSA-A2M alone or in combination with free PSA in serum improves the cancer specificity of the PSA test [15, 17].

Some novel monoclonal antibodies for PSA with potency to recognize PSA bound to A2M have been produced [139]. These antibodies might facilitate direct measurement of PSA-A2M complex in serum.

4.4. Analysis of Subfractions of Free PSA

Free PSA represents 5–35% of the total PSA in circulation, and the proportion of free PSA is higher in patients with benign prostatic hyperplasia (BPH) than in those with prostate cancer [12]. Several studies have suggested that various forms of free PSA in serum may provide additional clinical information [140–143]. Free PSA in serum consists of proenzyme forms (proPSA), internally cleaved forms [20, 144–146], and an enzymatically active form of PSA (Fig. 1) [22].

4.4.1. *ProPSA*

Mikolajczyk *et al*. [144] identified proPSA in serum from prostate cancer patients, and showed that approximately 25% of free PSA in prostate cancer sera consists of proPSA. ProPSA lacks enzymatic activity and does not form complexes with protease inhibitors [38, 80]. Peter *et al*. [147] purified serum PSA by immunoaffinity chromatography and used mass spectrometry to show that proPSA contained various isoforms including intact (with 7 amino acid propeptide), and truncated forms containing 5, 4, 2, and 1 amino acids of the propeptide. These proPSA forms also occur in serum from healthy men and patients with benign prostatic hyperplasia, but the proportion is lower than in prostate cancer sera [21, 25, 148].

Assays detecting subforms of proPSA have been shown to improve the accuracy of prostate cancer detection, proPSA being more cancer specific than free PSA [148]. Mikolajczyk *et al*. [141] have developed monoclonal antibodies to various truncated forms of proPSA and used these to develop specific immunoassays. They found that truncated proPSA containing two amino acids of the propeptide, called (-2)proPSA, is the predominant subfraction of proPSA in serum of prostate cancer patients [21, 140]. By immunohistochemical staining with antibodies to (-2)proPSA, cancer tissue stained more strongly than benign tissues [21, 149]. Clinical studies suggest that measurement of various isoforms of proPSA in serum can improve the detection of prostate cancer in men with total serum PSA concentration between 2 and 10 μg/liter [140, 141, 143, 150, 151]. Furthermore, (-2)proPSA appears to be associated with aggressive cancer [140].

4.4.2. *Internally Cleaved Forms of Free PSA*

Chen *et al*. [152] characterized free PSA purified from BPH tissues by gel electrophoresis and N-terminal sequence analysis, and showed that it

contained a large proportion of internally cleaved PSA. Free PSA in sera from BPH and prostate cancer patients also contains internally cleaved PSA, the proportion of which is higher in BPH patients than in cancer patients [20, 145, 146, 153]. A subfraction of free PSA that is internally cleaved after Lys-145 and Lys-182 has been called BPSA [154, 155]. This form has been identified in BPH tissue and also shown to be significantly elevated in the serum from BPH patients [154–156]. An assay that specifically detects internally cleaved PSA after Lys-182 has been reported to increase the specificity for BPH [155], and specific determination of this form has been suggested to improve differentiation between prostate cancer and BPH [142, 143].

Using monoclonal antibodies to intact and internally cleaved forms of PSA, an assay that does not recognize PSA cleaved after Lys-145 has been developed [24, 157]. With this method the proportion of intact free PSA was shown to be larger in prostate cancer compared to BPH sera [24].

4.4.3. *Enzymatically Active Free PSA*

Niemelä *et al.* have described a method for measurement of enzymatically active PSA [22]. In this assay, PSA is captured by a monoclonal antibody, and its enzymatic activity is measured using a fluorescent PSA substrate [50, 51, 158]. This assay was used to show that about 1–3% of PSA in serum from prostate cancer patients occurs in an enzymatically active form [22].

5. New Approaches for Detection of PSA

5.1. Identification of Novel PSA-Binding Ligands

Immunoassays are very sensitive and specific facilitating accurate measurement of individual proteins in the presence of more than 1 million-fold excess of other proteins [159]. The use of monoclonal antibodies has made it possible to develop assays that differentiate between variants of the same protein, but apparently because antibodies recognize epitopes on the surface of proteins, recognition of differences in internal structures that are not well exposed is hard to achieve with antibodies. In contrast, small peptides are able to recognize differences in such structures and peptides with specific binding properties can be identified with phage display libraries [160, 161].

Phage display is a molecular diversity technology and a powerful approach for selection of novel ligands for target molecules, such as enzymes [162–164], antibodies [165] and receptors [166, 167]. Foreign peptides or proteins are

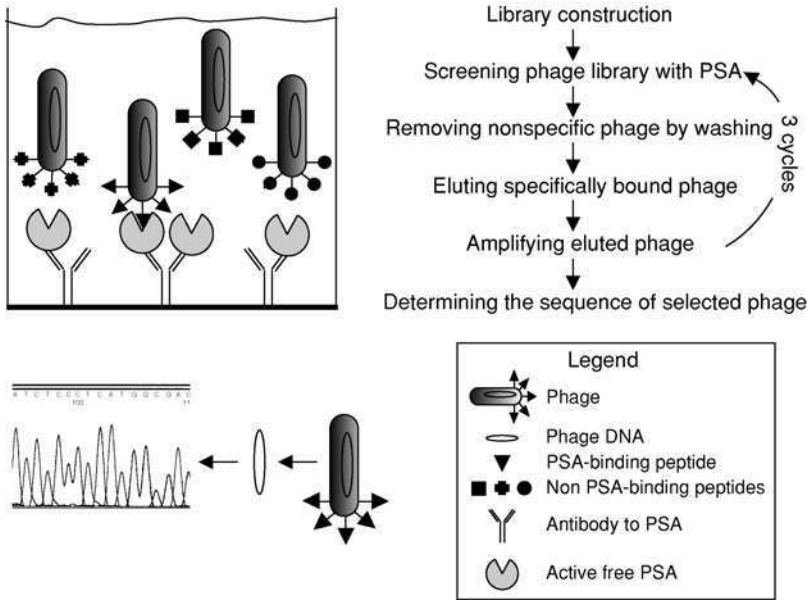

FIG. 2. Schematic illustration of selection of PSA-binding peptides by phage display.

displayed on the surface of the phage by inserting the displayed genes (DNA fragments or synthetic oligonucleotides) between the DNA encoding the signal peptide and the structural gene encoding major (pVIII) or minor (pIII) coat proteins [168]. This facilitates linking the phenotype of the phage to its genotype [169]. Phage libraries can display over 10^9 different peptides expressed as a fusion with phage surface proteins and offer a means of searching for ligands possessing unique binding specificities. By the phage display approach, we have identified various peptides binding to PSA (Fig. 2) [160]. A monoclonal antibody to PSA, which does not block the active site of PSA, was used to capture PSA on the solid phase. By this technique, peptides binding specifically to enzymatically active free PSA, but not to internally cleaved or pro forms of free PSA were identified [160, 161]. The peptides do not bind to chymotrypsin or cathepsin G that have an enzymatic specificity similar to that of PSA. They did not bind to hK2, which is structurally closely related to PSA, showing 79% identity at the amino acid level [36]. In addition, the peptides did not bind to PSA-serine protease inhibitor (serpin) complexes occurring in serum, that is, PSA-ACT and PSA-API, in which the serpins

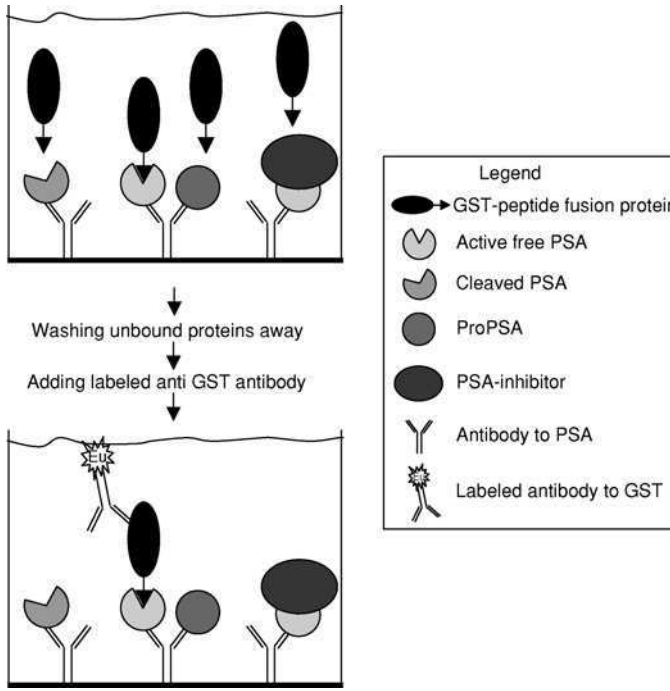

FIG. 3. Principle of immunopeptidometric assay (IPMA) for active free PSA.

cover the active site of PSA. The binding of peptides to PSA was also blocked by antibodies that react with free PSA through epitopes covered in PSA-serpin complexes; these antibodies inhibit the enzymatic activity of PSA [107]. Thus, the peptides are specific for enzymatically active free PSA.

5.2. IMMUNOPEPTIDOMETRIC ASSAY FOR ACTIVE FREE PSA

Immunopeptidometric assay (IPMA) is a new assay principle based on the use of PSA-binding peptides as a tracer in combination with a monoclonal capture antibody. In the assay all forms of PSA are captured by the immobilized monoclonal antibody, but only enzymatically active free PSA is recognized by the PSA-binding peptide (Fig. 3). The IPMA detects enzymatically active PSA, but not internally cleaved PSA or proPSA, which are enzymatically inactive. Between 1% and 10% of free PSA in serum from prostate cancer patients is detected by this assay [170]. The finding of active

PSA in patient samples also indicates that the reaction between PSA and inhibitors leaves part of the active PSA free. The IPMA offers a potential approach for detection of cancer-associated forms of PSA, but the sensitivity of the assay needs to be improved in order to detect active PSA at clinically important concentrations, that is, 2–10 μg/liter of total PSA.

5.3. Detection of Protein Analytes by a Nanoparticle-Based Bio-Bar-Code Approach

Nam *et al.* [171] have reported a nanoparticle-based bio-bar-code method to detect PSA at very low concentrations [171]. This method uses two types of probes, one is a magnetic microparticle coupled to a monoclonal PSA antibody, and the other one is a gold nanoparticle that carries multiple copies of a unique DNA and a polyclonal antibody to PSA. In the assay, PSA is first captured by magnetic microparticle probes, then gold nanoparticle probes are added. The probes and PSA form a complex that can be separated with a magnet. The PSA concentration is measured by detecting the oligonucleotide in the nanoparticle probe. The nanoparticle probe carries a large number of oligonucleotides per PSA molecule and this amplifies the signal. The signal can be further amplified by the polymerase chain reaction. By this approach, PSA could be detected at very low concentrations, that is, 3 attomol/liter, which is about six orders of magnitude more sensitive than that of conventional immunoassays. The method is also suitable for simultaneous detection of multiple analytes. If applicable to analysis of serum samples, this assay might be useful for simultaneous detection of different cancer-associated forms of PSA that are present at very low levels in sera and are not readily detectable by conventional immunoassays.

5.4. Detection of Subfractions of Free PSA by Two-Dimensional Gel Electrophoresis

Charrier *et al.* [20, 146] have studied free PSA in serum from men with prostate cancer and BPH by two-dimensional gel electrophoresis and showed that BPH sera contain more low-molecular-weight forms of free PSA than prostate cancer sera. Jung *et al.* [172] reported a method, in which PSA was captured by immunoadsorption on streptavidin-coated magnetic beads with a biotinylated monoclonal antibody to PSA and separated by two-dimensional electrophoresis followed by detection of PSA by Western blotting. They separated and quantified free PSA isoforms in men with total PSA concentrations of 2–20 μg/liter and found 15 forms of free PSA differing with respect to molecular weight and isoelectric point.

6. Detection of Circulating Prostate Cancer Cells by Measurement of PSA mRNA

Amplification of organ-specific mRNA by reverse transcription-polymerase chain reaction (RT-PCR) is a powerful method for detection of circulating prostate cancer cells. Many groups have reported detection of PSA mRNA in circulating cells by measurement of PSA mRNA by RT-PCR [173] and this approach is potentially useful for evaluating the prognosis of prostate cancer. It has been suggested to improve preoperative staging and prognosis of prostate cancer [174, 175] by identifying preoperatively organ-confined and extracapsular cancers, respectively [176–178]. However, at present it is not clear whether PSA mRNA levels correlate with tumor stage and grade [179]. By RT-PCR even one circulating PSA expressing cell among more than 100 million other cells may be detected [180, 181]. However, the method is prone to methodological problems and false-positive results have been found to be caused by low background expression of prostate-specific genes in normal hematopoietic tissues and blood cells [182, 183]. Therefore, the clinical utility of this method remains to be determined.

7. Enhancing Utility of PSA by Multivariate Methods

PSA is a sensitive diagnostic marker for prostate cancer, but its use is hampered by a high rate of falsely elevated values. Approximately 75% of men with a PSA of 4–10 μg/liter do not have prostate cancer in biopsy. This causes unnecessary anxiety and costs. The proportion of free PSA in serum can be used to reduce the number of unnecessary biopsies by about 20–30% [184, 185]. Many other variables, such as prostate volume, digital rectal examination findings and different molecular forms of PSA have been evaluated in order to reduce the number of unnecessary biopsies. However, for the clinician it is difficult to make an accurate biopsy decision based on many variables. With multivariate methods, that is, by using regression and neural network techniques, the utilization of several variables can be optimized and made easier. PSA-based algorithms have also been developed for staging and prognosis of prostate cancer.

7.1. Multivariate Modeling Techniques

Multivariate algorithms or models can be established using patient data on diagnostic variables and outcome of interest. The goal is to find the best fitting, yet biologically reasonable model to explain the relationship between

the diagnostic variables and the outcome variable. Logistic regression has been used extensively for statistical modeling in medicine. The method is similar to linear regression, but the output value is binary [186]. An advantage with logistic regression is that the effect of the diagnostic variables is easy to understand and quantify. This is usually more difficult with neural network techniques [187]. On the other hand, a drawback with logistic regression is that complex relationships between variables are difficult to demonstrate. The type of correlation between the diagnostic variables and the outcome variable must be selected manually by transforming the diagnostic variables. Interactions between variables must also be inserted by hand. For utilizing more complex information the multilayer perceptron (MLP) is better suited. This is an artificial neural network method that can be considered as an extension of logistic regression. About 90% of all artificial neural networks published in clinical medicine are MLPs.

7.2. Application of PSA Based Multivariate Algorithms

7.2.1. *Diagnosis of Prostate Cancer*

The most common type of multivariate PSA algorithms predicts the outcome of prostate biopsy [188–194] (Table 2). The PSA range is often limited to 4–10 μg/liter, as this is considered a diagnostic gray zone, but there are also algorithms for the PSA range 2–4 μg/liter and higher than 10 μg/liter. In most studies, logistic regression was reported to perform as well as artificial neural networks. We have tested algorithms in an external testing group that was derived chronologically in a prospective manner from the study population [194]. Our results indicate that the neural network, despite the use of efficient methods to avoid overtraining, had adapted too well to the training set and did not recognize patients in the testing set very effectively. This is a potential problem for the practical use of neural networks.

7.2.2. *Staging of Prostate Cancer*

Correct prediction of the spread of prostate cancer on the basis of preoperative variables would be important, as unnecessary lymph node dissections and radical prostatectomies could be avoided. Tewari *et al.* [195] constructed MLP models for prediction of margin, seminal vesicle, and lymph node positivity using information on serum PSA, age, race, DRE findings, tumor size, Gleason score, bilaterality, and number of positive biopsy cores. The models had sensitivities of 81–100% at 72–75% specificity. Batuello *et al.* [196] used information on 6454 patients with clinically localized prostate cancer to train and test an MLP model for prediction of lymph node spread. Clinical stage, Gleason score, and serum PSA were

TABLE 2
MULTIVARIATE ALGORITHMS FOR PREDICTION OF PROSTATE BIOPSY RESULTS

First author	PSA range (μg/liter)	Number of subjects	Age range (years)	Sensitivity (%)	Specificity (%)			Prevalence of prostate cancer
					ANN	LR	FT	
Carlson [188]	4–20	4298	>45	95		34	23	0.33
Virtanen [189]	3–10	212	55–66	85–90	26	41	26	0.25
Finne [190]	4–10	656	55–67	95	33	24	19	0.23
Djavan [191]	2.5–4	272	31–89	95	59	33	33	0.24
	4–10	974	31–89	95	67	60	40	0.35
Stephan [192]	2–4	129	40–86	95	63		20	0.42
	4.1–10	504	40–86	95	44		27	0.65
	10.1–20	295	40–86	95	37		27	0.77
Stephan [193]	2–4	219	40–89	95	65		10	0.33
	4.1–10	606	40–89	95	48		13	0.62
	10.1–20	363	40–89	95	55		31	0.76
Finne [194]	4–10	1775	55–67	95	19	22	17	0.23

Abbreviations: ANN = artificial neural network; LR = logistic regression; FT = proportion of free PSA.

input variables. At 44–64% sensitivity 81% specificity was reached. Using essentially the same patient material, Han *et al.* [197] constructed an MLP model for prediction of organ confinement and lymph node spread using as input variables Gleason score, serum PSA, clinical TNM, and age. At 90% specificity, the MLP model could detect 34% of the patients with organ-confined cancer and 59% of those with lymph node spread that was more accurate than nomograms based on logistic regression.

7.2.3. *Prognosis of Prostate Cancer*

Some authors have constructed algorithms for prediction of clinical and biochemical recurrence of prostate cancer. Snow *et al.* [198] predicted post-operative prostate cancer recurrence using age, clinical stage, tumor grade, serum PSA, and race as input variables in the MLP model that was constructed using information on 938 patients. At 67% sensitivity all men not going to develop a recurrence could be identified. Han *et al.* [199] developed an MLP algorithm to predict a rise in serum PSA (biochemical recurrence) 3 and 5 years after radical prostatectomy among patients with a Gleason score of 7. Age, race, serum PSA, clinical stage, type of Gleason score 7 ($4 + 3$ or $3 + 4$), and some other pathological features were used as input variables. At 37% sensitivity, 90% of those not going to have a recurrence at 3 years could be recognized. Zupan *et al.* [200] discussed the problem of handling survival data with standard neural network techniques – the eventual outcome of patients not developing a recurrence is unknown if the follow-up time is short. The author presented a new way of handling censored data with neural networks and compared this approach to Cox regression that is a regression technique for modeling of censored data. Recurrence after radical prostatectomy was predicted using either preoperative (serum PSA, Gleason score, and clinical stage) or postoperative (serum PSA, Gleason score, and pathological information on tumor spread) information. There was no difference in classification accuracy between the neural network and the Cox regression equation.

7.3. Usefulness and Limitations of Multivariate Models

Very few PSA based multivariate algorithms have been taken into clinical use. The algorithms presented so far appear promising, adding accuracy to that provided by individual variables such as serum PSA and the proportion of free PSA. But almost none of these algorithms have been strictly validated using data from external test groups of patients. In most cases, the test set has been separated from a larger data set by randomization. Randomized division into training and test sets is acceptable for initial evaluation of the algorithm performance, but it produces similar distributions of all variables

in both data sets making the prediction easier than in a real clinical setting. With more differences between training and test data the accuracy is expected to decrease. Therefore, it is important that algorithms are used in similar settings (age, geography, and ethnicity) as where they were constructed. When clinical practice, for example, biopsy technique, develops with time, algorithms will require updating. Schwarzer *et al.* [201] stated that most artificial neural networks for diagnosis and prognosis of prostate cancer suffer from methodological deficiencies, the most common problem being overoptimistic assessment of the performance. Another problem was that the designs of the neural networks were not reported in enough detail. It seems that the methodological deficiencies have become somewhat less frequent in recent years. More patient data are accumulating with time and this will enable extensive validation of existing algorithms. This may possibly bring some of the algorithms into broader clinical use.

8. Conclusions

Assay of PSA in serum is a useful method for monitoring of patients with prostate cancer. It is also widely used for early detection and screening, although it is not yet known whether population-based screening reduces prostate cancer mortality. In addition, PSA based screening causes a large number of false-positive results leading to unnecessary further examination by prostate biopsy. As about one of three biopsies is positive, the positive predictive value is only around 30%. The cancer specificity can be improved by measurement of the two major molecular forms of PSA in circulation, that is, PSA-ACT and free PSA, and the ratio between these or the proportion of free PSA. Further improvement has been achieved by measurement of minor subfractions, that is, PSA-A2M, PSA-API, proPSA, BPSA, and enzymatically active PSA. These methods are not generally available, but assays for free and total PSA are used widely. A proper use of these can reduce the rate of false-positive results and the need to perform biopsy to confirm the diagnosis. Multivariate algorithms may become useful for optimal combination of several diagnostic variables.

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ADVANCES IN PRION DISEASE SURVEILLANCE

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1. Abstract

This chapter provides an updated review on the ongoing surveillance and diagnosis of prion disease, also termed transmissible spongiform encephalopathy. While prion disease occurs in both animals and humans, this review focuses on human prion disease surveillance. It includes an overview of human prion disease, current diagnostic criteria and emerging technologies, surveillance centers around the world, especially the United States, epidemiology, and clinical and pathological features of cases examined in recent years.

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2. Introduction

2.1. Human Prion Disease

Prion disease is a group of neurodegenerative disorders characterized by the presence of the abnormal or scrapie form of prion protein (PrP^{Sc}) in affected brains [1]. The disease includes scrapie in sheep and goats, chronic wasting disease (CWD) in mule deer and elk, and bovine spongiform encephalopathy (BSE) in cattle, and Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker syndrome (GSS), and fatal familial insomnia (FFI) in humans. GSS and FFI are genetic prion diseases affecting people who carry mutations in *PRNP* on chromosome 20, the gene that encodes PrP protein. CJD occurs in three different forms. Sporadic CJD (sCJD) is a spontaneous disease that accounts for a majority of CJD cases. Familial CJD occurs due to genetic mutations in the PrP gene. Iatrogenic CJD (iCJD) is the result of accidental transmission during medical treatments. A newly emerged CJD phenotype, commonly called variant CJD (vCJD), has occurred in the UK since 1995. vCJD has a unique disease profile different from conventional CJD and may have arisen from consumption of BSE contaminated meat products. It occurred mainly in young people with initial mental and sensory disturbance preceding other neurological symptoms. Therefore, human prion disease manifests as three etiologically different groups that could occur spontaneously (sCJD), through inheritance (familial CJD, GSS, and FFI), and by infection (iCJD and vCJD) (Table 1).

At variance with other neurodegenerative diseases, such as Alzheimer's disease and Parkinsons disease, the biggest challenge in the diagnosis and surveillance of human prion disease is its prominent heterogeneity in clinical and histopathological phenotypes. It is likely that different etiologies contribute to this phenomenon of phenotypic heterogeneity. However, this does not explain a wide spectrum of disease phenotypes with the same disease

TABLE 1
Human Prion Disease

Sporadic form
CJD
Familial (inherited) form
Familial CJD
FFI
GSS
Acquired (transmitted) form
iCJD
vCJD

group such as sCJD. Studies have revealed two additional factors that greatly influence the disease phenotypes [2]. These two factors are the particular subtypes of PrP^{Sc} and the genotype at polymorphic codon 129 that encodes either methionine (M) or valine (V) on the two alleles of *PRNP* gene. There are two distinct PrP^{Sc} subtypes that can be readily distinguished on Western blots based on the size of the PrP^{Sc} domain resistant to digestion by proteinase K (PK) [3, 4]. In PrP^{Sc} type 1 the PK-resistant domain has a mobility of about 21 kDa. In PrP^{Sc} type 2, the corresponding PK-resistant domain migrates to approximately 19 kDa (Fig. 1). The size difference between PrP^{Sc} type 1 and type 2 is due to differential cleavage by PK, most likely a result of different conformation [5]. The disease phenotypes of the CJD patients associated with PrP^{Sc} type 1 often are different from the phenotypes associated with PrP^{Sc} type 2.

The common M/V polymorphism at codon 129 of the human *PRNP* gene is another modifier of the disease phenotypes (Fig. 2). In the Caucasian population, about 36% of individuals are M homozygous (MM), 52% are heterozygous (MV), and 12% are V homozygous (VV). Susceptibility to CJD is largely influenced by the homozygosity of codon 129 [6, 7]. Moreover, the codon 129 M/V status often dictates the defining features of clinical and pathological profiles of the disease [3, 8].

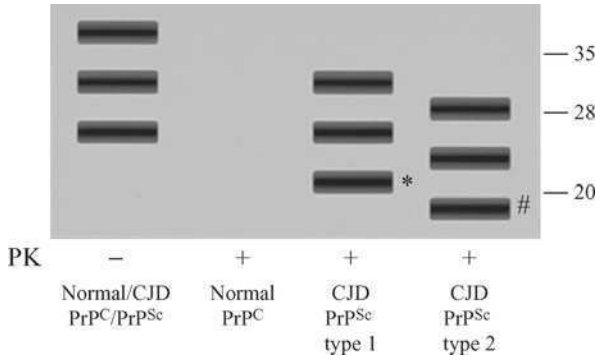

FIG. 1. Diagram illustrating the detection of two PrP^{Sc} subtypes in CJD. On Western blots, the normal cellular PrP (PrP^{C}) and the disease-associated isoform, PrP^{Sc}, migrate as three bands (lane 1, from bottom to top) representing unglycosylated, monoglycosylated, and diglycosylated forms, respectively. Upon digestion by PK (PK+), normal PrP^{C} is completely degraded hence is not detected (lane 2). PrP^{Sc} from brains of CJD is resistant to digestion by PK with the PK-resistant core domain being readily detectable (lanes 3 and 4). Two distinct PrP^{Sc} subtypes are recognized in different CJD phenotypes. The unglycosylated band of PrP^{Sc} type 1 (*) migrates at about 21 kDa (lane 3), while that of PrP^{Sc} type 2 (#) has a mobility of about 19 kDa (lane 4). The position of molecular weight makers (in kDa) is indicated on the right.

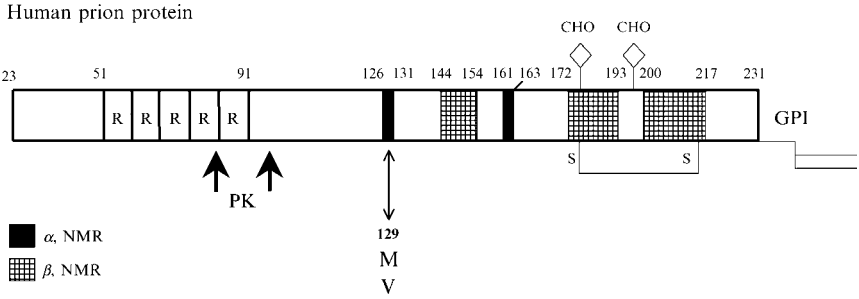

FIG. 2. Human prion protein. The structure of human prion protein is presented as a linear diagram depicting the octapeptide repeats (R), α-helical and β-pleated secondary structures, and co- or post-translational modifications including a disulfide bond (S-S), N-linked glycosylation sites (CHO), and glycophosphatidyl inositol (GPI) linkage. PK cleavage sites and codon 129 polymorphic M/V residues are indicated. The secondary structures of PrP are derived from the NMR determination of recombinant human PrP [72].

Based on the disease phenotype, the PrP^{Sc} subtype and genotype at codon 129 on *PRNP* alleles, the diverse phenotypes of sCJD can be subdivided into six subtypes: sCJDMM1, sCJDMV1, sCJDVV2, sCJDMV2, sCJDMM2, and sCJDVV1. These six sCJD groups have different rate of occurrence, age of disease onset, disease duration, and clinical and pathological features (Table 2). Genetic CJD, also known as familial CJD, can also be classified into different groups according to the *PRNP* mutation and codon 129 M/V genotype. All neurologically confirmed cases of vCJD are carriers of homozygous MM genotype at codon 129 with the presence of a specific PrP^{Sc} subtype. Therefore, the recognition of the disease-modifying role of PrP^{Sc} subtype and codon 129 genotype on *PRNP* has lead to a more rational and practical classification that is expected to facilitate the diagnosis and surveillance of human prion disease.

3. Prion Diagnostics

3.1. Current Diagnostic Methods

Definitive diagnosis of prion disease is accomplished through the detection of the abnormal form of the prion protein, PrP^{Sc}, in brain tissue. The brain tissue is acquired most commonly at autopsy, although it is possible to perform a biopsy of the brain if CJD is suspected. However, due to the limited treatment options, and the risks associated with brain surgery, the World Health Organization does not recommend a biopsy for the diagnosis of a suspected case of vCJD or sCJD [9].

TABLE 2
sCJD Subtypes with Corresponding Phenotypic Features

Subtype	Short description	Other features	Onset (years)/ duration (months)	Percentage of cases[a] (number of cases)
MM1 MV1	Classical	Rapid progression (<4 months), PSWCs on EEG, prominent and early myoclonus, "synaptic" pattern of immunostaining	63.2/3.9	56 (335)
VV1	Early onset	Progressive dementia, no PSWCs on EEG, younger patients (~46 years)	46.0/15.3	3 (18)
MM2	Slowly progressive	Progressive dementia, no PSWCs on EEG, coarse spongiosis, long duration (>15 months), cortical pathology, weak "synaptic" immunostaining	60.3/15.7	11 (64)
MV2	Ataxic with kuru plaques	Ataxia at onset, no PSWCs on EEG, long duration (>15 months), kuru plaques in cerebellum, subcortical pathology	60.3/17.0	14 (81)
VV2	Ataxic with short duration	As MV2 but no kuru plaques, short duration (<7 months), subcortical pathology, plaque-like immunostaining	60.3/6.6	16 (96)

[a] Data collected at NPDPSC.
Adapted from [17], by permission of Lippincott Williams & Wilkins.

The most convenient method currently used for the detection of PrP^{Sc} is the Western blot [10]. This method involves pretreatment of the brain homogenate with PK (enzyme concentration of 50–200 μg/ml) for 1 h at 37°C. PK digestion is performed because PrP^{Sc} is partially PK resistant, whereas the normal cellular form of PrP (PrP^{C}) is completely PK sensitive. PK digestion of PrP^{Sc} produces a C-terminal core fragment called PrP27-30 [11], which can be detected using one of many commercially available antibodies against PrP, such as 3F4. Western blotting shows a high level of sensitivity and is relatively easy to interpret. Another advantage of this method is that it does not require a large amount of tissue. In addition, this method allows the differentiation between subtypes of PrP^{Sc}, and can be

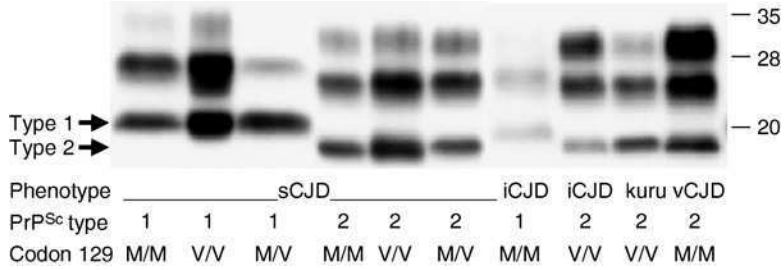

FIG. 3. Western blot detection of PK-resistant PrP^{Sc} in brains of CJD patients. Brain homogenates of sCJD, iCJD, kuru, and vCJD were digested with PK followed by Western blotting with mAb 3F4. PrP^{Sc} subtypes (type 1 or 2) and codon 129 polymorphic residues were indicated. The position of molecular weight makers (in kDa) is indicated on the right. Adapted from [5], by permission of National Academy of Sciences, USA.

extremely useful in the diagnosis of different variants of human prion disease when used in combination with genetic studies of the *PRNP* genotype, to determine the codon 129 polymorphism, and to check for mutations, and with histopathological lesions (Fig. 3) [10].

While Western blotting provides adequate sensitivity for PrP^{Sc} detection, immunohistochemistry provides better anatomical resolution. This method provides information about the distribution of PrP^{Sc} among various brain structures, and does not require fresh tissue. Its sensitivity is low compared to Western blotting, and there can also be problems with background reactivity from PrP^{C}. This can be overcome to a large extent with special processing steps, such as treatment of the tissue section with formic acid and hydrolytic autoclaving [10].

Because PrP^{Sc} is currently the only known component of the transmissible agent causing prion diseases, strict precautions should be undertaken to minimize a potential risk of exposure to the pathogen. While routine patient contact does not pose a risk to healthcare personnel, there is some risk in handling the brain tissue [10]. In general, brain tissues from suspected cases of CJD should be handled in a Class II Biological Safety Cabinet in a specialized Biosafety Level II facility for sporadic or genetic forms of the disease, or a Level III facility for suspected cases of vCJD. Individuals performing these procedures should wear protective clothing, including protective gowns, gloves, and goggles. All protective equipment must be disposable and equipment must be disposed of according to appropriate biohazardous waste procedures [10].

3.1.1. *Premortem Diagnosis*

Although definitive diagnosis of CJD is only possible by tissue examination, there are several tests that may be undertaken while the patient is still alive, which may lead to a probable diagnosis. For each form of the disease,

the tests are slightly different. In the case of sCJD, a diagnosis may be made based upon results from cerebrospinal fluid (CSF) tests, electroencephalograms (EEG) and magnetic resonance imaging (MRI) [12]. However, in cases of genetic CJD, these tests may be negative. In addition, the affected individual may not be aware of any family history, so analysis of the *PRNP* gene is essential to diagnose these cases [9]. For vCJD, the only noninvasive test to show any sign of the disease is the MRI. The diagnosis of vCJD may potentially be made using a biopsy of the tonsils and other lymphatic tissue [13, 14].

3.1.1.1. *The 14-3-3 Proteins.* In 1986, it was discovered that there were proteins in the CSF of patients with CJD that did not appear in unaffected patients with high sensitivity and specificity [15]. These proteins were later identified to belong to the 14-3-3 protein family. The 14-3-3 family of proteins is a highly conserved family of multifunctional proteins that have high levels of expression in the neurons [16]. Its presence in the CSF is indicative of acute neuronal damage. Assays for 14-3-3 have been developed, and are routinely used in the premortem diagnosis of CJD. Initial studies suggested that the sensitivity was as high as 95% [15]. The most common assay used for the detection of 14-3-3 from clinical samples is the Western blot (Fig. 4) [9]. So far, the Western blot for 14-3-3 has been found to be a better method than other assay formats based on 2D electrophoresis and Enzyme-Linked Immunosorbent Assay (ELISA). An ELISA has the potential for greater sensitivity than a Western blot, but the correct antibody has been difficult to obtain, as 14-3-3 is a heterogeneous protein, usually in the form of a dimer that may be made of a combination of two of several different isotypes [16]. Together with

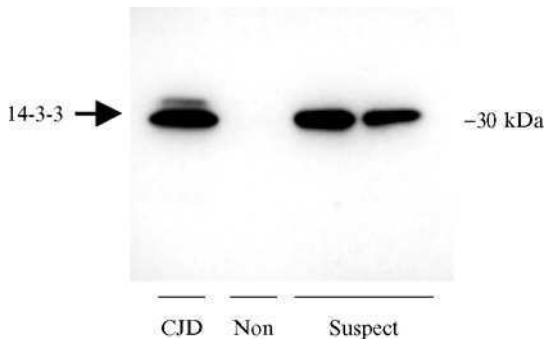

FIG. 4. The 14-3-3 assay of CSF on Western blots. CSF samples from a confirmed CJD case (CJD, lane 1), a non-CJD subject (Non, lane 2), and a subject suspected of CJD (Suspect, duplicated samples, lanes 3 and 4) were subjected to Western blotting with an antibody against the 14-3-3 β protein (Santa Cruz). The test subject was positive on the CSF 14-3-3 assay.

clinical symptoms, a positive test for 14-3-3 identifies a case of probable CJD [9]. Other studies have found the sensitivity of 14-3-3 as a surrogate marker for CJD to be quite variable depending on the subtype of sCJD [17]. The overall sensitivity, when all six subtypes of sCJD are accounted for, is 87%. The highest sensitivities for this test are for subtypes sCJDMM1, sCJDMV1, and sCJDVV1 at 94%, 100%, and 100%, respectively [17]. The lowest sensitivities are 57% and 70% for sCJDMV2 and sCJDMM2, respectively [17]. Therefore, if the test is negative, and there are other symptoms of the disease present, CJD cannot be ruled out [18].

While detection of 14-3-3 protein in the CSF is highly specific and generally sensitive for sCJD and certain types of genetic CJD (E200K and V210I), it is a less reliable marker for other forms of prion disease. 14-3-3 protein is consistently absent in cases of FFI, and is rare in GSS [12]. It has a much lower sensitivity for iatrogenic cases of CJD (60%), and is only found in 48% of vCJD cases [19]. Therefore, its use as a diagnostic marker is largely limited to sCJD. If elevated levels of 14-3-3 are detected in the CSF, CJD may not be the only cause. Because the protein is found in the CSF as a result of neuronal damage, it is also found in other neurological conditions, such as stroke, hemorrhage, paraneoplastic disorders, cerebral neoplasia, and is also present shortly after an epileptic seizure. Many of these disorders can be readily distinguished from CJD by clinical presentation and other neurological tests. It should be noted that among the neurodegenerative diseases, only rare non-CJD cases are positive for 14-3-3, although elevated 14-3-3 levels have been detected in multiple sclerosis. There is only one case reported in which 14-3-3 was found to be elevated in Alzheimer's disease, the most common differential diagnosis for CJD [15]. This emphasizes that an elevated level of 14-3-3 is suggestive, but not diagnostic, of CJD, and the results must be interpreted in the proper context.

Attempts were made to detect PrP^{Sc}, as a more reliable marker, in the CSF of CJD patients, but this was unsuccessful. Both Western blotting and an ELISA assay were used [20]. However, it is possible that PrP^{Sc} is present in such a small amount, that it is not possible to detect in this manner. Therefore, further study in the area of sensitive detection of PrP^{Sc} in the CSF is required.

3.1.1.2. *Tonsillar Biopsy.* vCJD, as mentioned previously, is thought to arise from the consumption of contaminated beef from cattle affected by BSE [21]. In animal prion diseases, as well as in murine models of the disease, prion replication is known to occur in the spleen and other lymphoreticular tissues [22]. Replication in these tissues precedes prion invasion of the central nervous system by a significant period of time, such that PrP^{Sc} may be found in the tonsils of animal models of scrapie in one-third to half of the normal incubation period [22]. Similarly, PrP^{Sc} was found in the tonsils in autopsy

samples of individuals who had died of vCJD, both by Western blot and immunohistochemistry [23]. It is important to note that PrP^{Sc} is not found in the tonsils of patients with sporadic or inherited prion diseases, so the presence of PrP^{Sc} in the tonsil represents a way to differentiate vCJD from other forms of human prion disease [13]. The biopsy procedure itself is not without risk, although it is certainly much less risky than a brain biopsy. The patients whose tonsils were biopsied while they were still living made uneventful recoveries, and resumed a normal diet, without the need for pain medication, by the first postoperative day [13]. This recovery compares favorably with that from a full tonsillectomy procedure. The possible disadvantages of this method as a widely used screening tool are that not all individuals have sufficient amounts of tissue to perform an adequate biopsy. Tonsils are often removed surgically, and some individuals experience tonsillar atrophy [13].

Because PrP^{Sc} is found in the tonsils and other lymphatic tissue in cases of vCJD, prions can be found in individuals who have not yet developed clinical disease. It is through testing of the spleen at autopsy that the discovery was made of a presymptomatic individual, who had died of another cause [24]. This finding was remarkable because, for the first time, the presence of PrP^{Sc} was found in an individual with possible vCJD who was heterozygous at codon 129. Although this individual did not exhibit any symptoms of the disease, it was known that the patient was exposed to a vCJD-contaminated blood transfusion 5 years prior to death [24]. The finding of PrP^{Sc} in a codon 129 heterozygous individual has significant implications for the surveillance of vCJD, as this is the largest genetic subgroup in the UK [24]. While it is not known whether this individual would have ever developed the disease, it raises concern for iatrogenic transmission via nonneural tissue, especially as prion infectivity in this case may not have reached the brain or spinal cord. While awareness exists of potential risks from prion contamination of neurosurgical instruments, the risk of transmission from surgical instruments on the spleen or lymphatic tissue from asymptomatic individuals with vCJD may not be as obvious [25]. A second possibility is that codon 129 MV heterozygous individuals are susceptible to vCJD, but only after a more extended incubation period than MM homozygotes. In either case, the importance of continued surveillance is apparent.

3.1.1.3. *MRI and EEG.* There are currently fewer premortem diagnostic tests available for the diagnosis of vCJD than for sCJD. Fortunately, the MRI in vCJD differs from that seen in sCJD, thus, providing a unique tool in differential diagnosis [9]. In cases of vCJD, the most pronounced signal enhancements occur in the posterior thalamus, known as the pulvinar sign [26]. Because this is seen in 78% of vCJD cases, it has been included in the diagnostic criteria for the disease [9]. In addition, it represents an important

distinction between sporadic and vCJD, which is essential from a public health perspective. There is no signal enhancement in the posterior thalamus in patients with sCJD. Instead, atrophy is sometimes seen in sCJD, but this is not a reliable indication. Hyperintensites are found in the basal ganglia 60–80% of the time, in cases of sCJD in which they are observed. These types of signals are seen most often using fluid-attenuated inversion recovery (FLAIR) or diffusion weighted imaging techniques [12].

The EEG has been used since the 1950s in the diagnosis of CJD. The characteristic pattern in sCJD is periodic sharp wave complexes (PSWCs) [9]. This pattern may be seen as early as 3 weeks after disease onset in some cases. PSWCs are seen some time during the course of disease in 60–70% of sCJD cases and in genetic cases resulting from mutations in codon 200 and 210 [9]. The presence or absence of the characteristic EEG pattern in sCJD may be a result of the genotype at codon 129, and whether type 1 or type 2 PrP^{Sc} is responsible [17]. The two most prevalent subtypes of sCJD, MM1 and MV1, show PSWCs, but other subtypes, such as VV2, MM2, MV2, and VV1, do not [17]. There is no diagnostic EEG pattern in GSS, iCJD, or vCJD [12]. Therefore, the EEG may be another tool to help distinguish between sporadic and vCJD. In FFI, EEG polysomnography associated with an appropriate clinical syndrome may be diagnostic if it demonstrates the typical changes of this disease.

3.1.1.4. *Clinical Diagnosis.* sCJD typically affects individuals in the seventh decade of life, with an incidence of approximately one case per million persons per year [9]. There is no evidence of geographical clustering, or that any factor, such as diet, occupation, or previous surgery, increases the risk of developing the disease. The classic symptoms of the typical sCJD, also identified as sCJD MM1 and MV1, are rapidly progressive dementia, myoclonus, and in some cases ataxia [9]. The phenotype of sCJD varies in the other subtypes (Table 2). At disease onset, there are often no specific symptoms and in a small percentage of cases, patients may exhibit psychiatric symptoms, including hallucinations and delusions [12]. As the disease progresses, multifocal central nervous system failure occurs, resulting in rigidity, cortical blindness, dysphagia, and possibly Cheyne–Stokes respiration at the terminal stage [9]. The mean duration of the disease is approximately 8 months, with less than 4% of patients surviving longer than 2 years [9]. The most important differential diagnoses include Alzheimer's disease, vascular dementia, diffuse Lewy body disease, brain tumors, and cerebellar degeneration [9].

There are some important differences between the clinical presentation of sCJD and that of vCJD (Table 3). vCJD has a longer disease course than sCJD, with a median of 14 months, compared to approximately 8 months for sCJD [9]. In addition, much younger patients are affected by vCJD with a median age at onset of 28 years [9]. However, vCJD has been found in older

TABLE 3
DIFFERENCES BETWEEN DIAGNOSIS OF vCJD AND sCJD

Phenotype	sCJD	vCJD
Mean age at onset	65 years	28 years
Median duration	4.5 months	14 months
Common presenting signs	Progressive dementia, myoclonus, ataxia	Sensory and psychiatric disturbances
14-3-3 assay	Usually positive	Usually negative
EEG	PSWCs present in the majority of cases	PSWCs absent
MRI	Hyperintensities in the basal ganglia	Pulvinar sign

patients including a 74-year-old man, so this diagnosis should not be eliminated solely on the basis of age [27]. A distinct feature of vCJD is that the initial symptoms are almost always psychiatric [28]. The symptoms include depression, anxiety, withdrawal, and in some cases suicidal ideation or psychosis [9]. Of the first 14 histopathologically confirmed cases of vCJD in the UK, four patients initially presented with sensory symptoms. These included foot pain, pain below the knees, hand and face dysesthesia, and the feeling of cold feet [9]. Neurological dysfunction is often not seen early in the disease. Such dysfunction, usually in the form of ataxia, is seen after a median time of 6 months from disease onset [9]. Once neurological symptoms are seen, the progression is rapid, with global cognitive impairment, involuntary movements, leading to akinetic mutism, occasional cortical blindness, and dysphagia in the terminal stages [9].

Upon autopsy, the examination of brain tissue by standard histology, and immunohistochemistry is used to confirm a diagnosis of CJD [29]. The general features of prion disease neuropathology common to all subtypes are reactive gliosis, neuronal loss, and spongiform change. However, there are some features that are useful for distinguishing the subtypes of prion disease, including sporadic and vCJD. For example, in certain forms of prion disease, PrP^{Sc} can be deposited into amyloid plaques [29]. Such ''kuru'' plaques are found in kuru in the MV2 subtype of sCJD and in GSS. A different but characteristic plaque-type PrP^{Sc} deposition is found in vCJD, where it forms part of what is known as a ''florid plaque.'' A florid plaque contains a dense core of PrP^{Sc} immunopositive deposition, surrounded by a halo of spongiform change (Fig. 5) [30]. In addition, the neuropathology of vCJD may also include multiple small plaques not associated with spongiform change. The large numbers of florid plaques found in

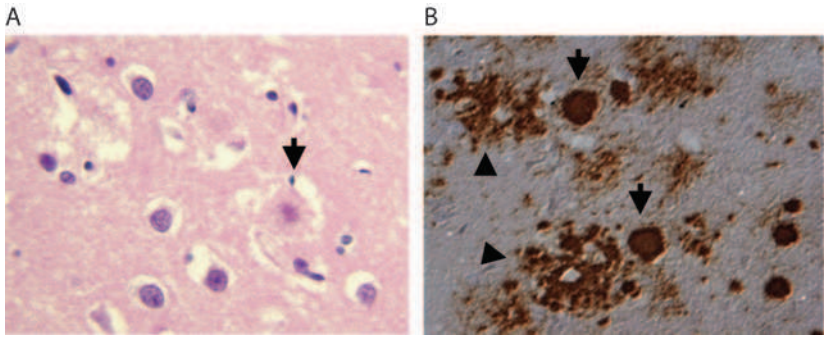

FIG. 5. Neuropathological features of vCJD. (A) Histology of a section of frontal cortex stained with hematoxylin and eosin. Typical "florid plaques" (arrow) is present along with spongiform degeneration. (B) Immunohistochemistry of PrP^{Sc}. Very intense immunostaining of PrP in the form of round dense aggregates probably corresponding to plaques (arrow) and irregular deposits (arrowhead). Specimens were provided by Dr James Ironside, Western General Hospital, Edinburgh, UK.

vCJD are a useful distinguishing feature, which allows differentiation from sCJD [30].

3.2. Limitations of Current Methods

The most significant limitation of the current diagnostic methods is that prion disease cannot be diagnosed conclusively until autopsy. While there are many clinical signs and the presence of the 14-3-3 protein as a surrogate marker, the disease can still be present in the absence of many of these signs. In addition, the EEG and MRI signs can also be absent in the presence of a histopathologically confirmed case of CJD [18]. Therefore, despite the progress in developing premortem tests for the diagnosis in CJD, confirmation of the presence of PrP^{Sc} in the brain tissue remains the only method by which the diagnosis can be confirmed. If the detection of PrP^{Sc} is the only way to definitively diagnose prion diseases, then it is important that future efforts in this area focus on novel ways to detect the protein premortem, or ideally, presymptomatically. Because PrP^{Sc} has not yet been found in CSF, this eliminates a CSF test for the detection of PrP^{Sc}. The least invasive ways to accomplish this in a living patient is through testing of blood and urine. At this time, the detection of PrP^{Sc} in blood and urine is still in the experimental stage, and no diagnostic test has been introduced that is based on blood or urine.

3.2.1. *PrP^{Sc} Detection in Blood*

With the documentation of cases of vCJD resulting from blood transfusions, it is apparent that the blood contains infectivity [31]. It is thought that the lymphoreticular system plays an important role in the pathogenesis of the disease because PrP^{Sc} has been found in the tonsils of individuals with vCJD [13]. Therefore, the lymphocyte fraction of the blood is thought to contain the infectivity. Finding evidence of PrP^{Sc} in blood has been difficult, as it is not detectable by Western blot, but PrP^{C} has been found using the highly sensitive dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) technique [32]. In this case, it suggests that the majority of the PrP^{Sc} would be found in the lymphocyte and platelet fractions of the blood, once the conformational change had taken place. In sheep, naturally affected by scrapie, an increase in beta sheet content of the membranous fraction was detected using Raman spectroscopy [33]. This is not a definitive finding of PrP^{Sc}, but it is suggestive of its presence. Although vCJD has been transmitted through blood transfusions, there have been no other cases of iCJD that have arisen in this manner [24]. The infectivity of the blood may be specific to vCJD due to differences in the pathogenesis of this disease compared to other human prion diseases [13]. The presence of infectivity in the blood suggests that there is PrP^{Sc} present, which should be able to be directly detected, given a sensitive and specific enough assay. A blood test for vCJD would be minimally invasive and amenable for use as a screening tool, which would be especially helpful in cases in which known exposure to BSE has occurred. Based on the assumption that the majority of prion infectivity is contained in the leukocyte fraction, blood used for transfusion in the UK now undergoes leukocyte depletion [34]. However, this procedure is estimated to remove only 42% of the infectivity of the blood, indicating that it does exist in other fractions [34]. Although there is a large proportion of PrP^{C} found in the platelet fraction of blood, platelets are not found to be associated with infectivity in experimentally infected hamsters [32, 35].

3.2.2. *PrP^{Sc} Detection in Urine*

There have been no reported incidences of CJD infection arising from contact with urine. However, as an easily accessible peripheral body fluid, the possibility exists that a diagnostic test can be developed based on the discovery of PrP^{Sc} in urine. This has been a controversial area with no definitive findings of PK-resistant PrP^{Sc} in the urine of infected animals. Shaked *et al.* [36] reported finding "a protease resistant PrP" in the urine of scrape infected hamsters using 3F4 antibody. However, the PrP they recovered from urine was not infectious upon intracerebral injection. Later studies demonstrated that contamination by bacterial membrane proteins [37] or the presence of

immunoglobulins [38] can be a source of confusion when using 3F4 for the detection, and in either case, these proteins may have been misidentified as PrP in the initial study by Shaked *et al*. Despite this setback, our recent work has demonstrated that PrP^{C} is readily detectable in urine using anti-C antibody raised against PrP at an epitope different than that of 3F4 [39]. This renews the interest that it may be possible to find PrP^{Sc} in urine under the right conditions, although this has yet to be confirmed. However, as a precaution proposed by some investigators, urinary derived gonadotropins are not recommended for use in reproductive procedures, such as *in vitro* fertilization [40]. Like a blood test, a urine test for CJD would provide a minimally invasive method on which to base a diagnostic test.

3.3. The Role of Effective Diagnosis in Prion Disease Surveillance

With the recent discovery of BSE among cattle in the United States and Canada, surveillance of human prion diseases is now especially important. Although vCJD has not so far affected a large segment of the British population, the potential for the disease to be transmitted by blood transfusion raises important public health issues. Because one individual who was found upon splenic biopsy to be positive for PrP^{Sc}, carried MV at codon 129 of *PRNP*, this raises the possibility of more asymptomatic carriers of vCJD than previously realized [24]. A second possibility is that individuals who are not MM homozygous at codon 129 of *PRNP* may have longer incubation periods, before they become symptomatic [24]. In either case, it may be possible for asymptomatic individuals to still transmit the disease through blood donations. This allows for spread of the disease beyond cases that result from direct contact with BSE contaminated beef, and allow a vCJD outbreak to continue beyond containment of any BSE outbreak.

It is for this reason that effective surveillance and adequate diagnostic methods are necessary. If a case of vCJD was to appear in North America, it needs to be recognized. In addition, cases of sCJD also need to be recognized to prevent any possibility of iatrogenic transmission of the disease. CJD has been transmitted in the past through cadaver-derived growth hormone, corneal and dura mater transplants, and contaminated neurosurgical instruments [25, 41–43].

3.4. Emerging Diagnostic Technologies

3.4.1. *Nasal Biopsy*

Highly sensitive studies of tissue distribution of PrP^{Sc} in cases of sCJD revealed that the eye and the optic nerve were PrP^{Sc}-positive in vCJD [44]. This prompted Zanusso *et al*. [45] to examine the olfactory sensory pathway

for PrP^{Sc} in tissue derived from sCJD cases. Initially, the studies took place using autopsy samples from individuals who have neuropathologically confirmed CJD. PrP^{Sc} was found in the olfactory epithelium of these individuals using Western blotting [45]. The olfactory epithelium potentially represents a novel biopsy site for prion disease, especially sCJD. This is significant, because the tonsillar biopsy previously described reliably detects only PrP^{Sc} from cases of vCJD [13]. A biopsy from this site does not carry the same risk as a brain biopsy, and the presence of PrP^{Sc} would give a definitive diagnosis of the condition.

The nasal biopsy has also been performed on a patient presenting with suspected CJD, premortem, 45 days after the onset of symptoms [46]. The procedure can be performed under local anesthesia. PrP^{Sc} was detected in this case using immunohistochemistry but not Western blotting [46]. PrP^{C} is also present in the olfactory epithelium, and this was readily detected using Western blotting in samples untreated with PK. The ability to detect PrP^{Sc} in a premortem patient indicates that the olfactory biopsy is a promising candidate for confirmation of a CJD diagnosis [46]. Although this method requires only minor surgery, a possible disadvantage is that, since so much tissue is required to detect the PrP^{Sc} on a Western blot, it does not provide a method by which to differentiate between different forms of prion disease.

3.4.2. *Conformation Dependent Immunoassay*

The conformation dependent immunoassay is one method by which PrP^{Sc} may be differentiated from PrP^{C}. This method has been increasing in sensitivity with the adaptation of new, highly sensitive DELFIA techniques [47]. An ELISA method is also amenable to high throughput screening, which is necessary if a large population of individuals is to be tested, for example for vCJD in the United Kingdom. The conformation dependent immunoassay attempts to differentiate between PrP^{Sc} and PrP^{C} based on their conformation. It does not require antibodies specific to either species. One method of performing a conformation dependent immunoassay involves denaturing the protein in increasing concentrations of guanidine HCl [48]. Because of its conformation, PrP^{Sc} is resistant to denaturation, especially at lower concentrations of guanidine. Denaturation produces two fractions, a soluble fraction and an insoluble fraction. Because native PrP^{Sc} is highly insoluble, the highest concentration is thought to be contained in the insoluble fraction, whereas PrP^{C}, whether denatured or not, is soluble [48]. Therefore, it is possible to detect PrP^{Sc} in an ELISA-based format from the insoluble fraction of a guanidine-based conformation dependent immunoassay. Experimentally, the presence of PrP^{Sc} can be confirmed using PK digestion of the samples.

A second technique that can be used for this assay with a high degree of sensitivity is precipitation of PrP^{Sc} using the sodium salt of phosphotungstic

acid. This method is known to concentrate PrP^{Sc} but not PrP^{C} [49]. This type of assay in combination with a DELFIA has found to be extremely sensitive when compared to immunohistochemistry [47]. PrP^{Sc} was detected in brain sections that were negative when tested using immunohistochemical methods [47]. This type of sensitivity is promising, if it could be applied to the premortem diagnosis of prion disease. However, this method has only been attempted using brain tissue, so its limitations are similar to those of immunohistochemistry and Western blotting.

3.4.3. *Techniques to Enhance Sensitivity*

As mentioned previously, one of the biggest barriers preventing the detection of PrP^{Sc} in peripheral body fluids is the lack of sensitivity of the available methods. There have been techniques developed that potentially enable the small amount of PrP^{Sc} thought to be present in blood, for example, to be amplified to within the detection limits of a Western blot or an ELISA. One such method has been reported, in which minute quantities of PrP^{Sc} present in infected brain homogenates could be amplified using a sonication method [50]. The substrate for conversion was reported to be the PrP^{C} naturally present in the brain homogenate. Using this method, PrP^{Sc} could be detected in the brains of experimentally infected hamsters and cattle, in the presymptomatic stage of the disease. Prior to amplification, PrP^{Sc} was not detectable by Western blot [50]. If a method such as this became widely available, it could aid in the development of a highly sensitive test for PrP^{Sc}, which could detect the disease in the presymptomatic stage. Moreover, if this method could be applied to peripheral body fluids, such as blood, then it is possible that PrP^{Sc} present in blood could be detectable. If a conventional Western blot is used, PK digestion can be used to differentiate various forms of prion disease.

4. Human Prion Disease Surveillance

4.1. History of Prion Disease Transmission

The first evidence that the infectious agent of the transmissible spongiform encephalopathy, commonly called prion disease, could be naturally transmitted through the ingestion of contaminated food stuff dramatically came from the observation that a neurodegenerative disease called kuru selectively affected a tribe of New Guinea that practiced ritualistic endogenous cannibalism [51]. Tribe members were likely exposed to contaminated flesh from a subject affected by a naturally occurring prion disease, probably CJD. The disease transmission was subsequently amplified by the continuous practice of cannibalism to reach an epidemic proportion [51].

A second important event, demonstrating the transmission of prion disease within the same species through food ingestion, came when a disease epidemic of cattle, affecting the nervous system in the mid-1980s in the United Kingdom, namely mad cow disease, was identified as a prion disease now known as BSE [52]. Strong evidence indicated that BSE was acquired by human-induced bovine cannibalism. The epidemic that began simultaneously at many geographic locations was traced to contamination of meat and bone meal (MBM), a dietary supplement prepared from rendering of slaughterhouse offal. The epidemic was likely initiated by the presence of PrP^{Sc}, the only identified component of the prion disease infectious agent, in the MBM from sheep affected by scrapie (a long-standing prion disease of sheep). It is likely that scrapie was first transmitted to cattle in the early 1980s when most rendering plants abandoned the use of organic solvents in preparing MBM. In the United Kingdom, the BSE epidemic peaked in 1993 and has decreased progressively since that time, likely as the result of the prohibition on feeding ruminant-derived protein to cattle, a measure introduced in the United Kingdom in July 1988. However, over 180,000 cattle have since developed the disease in the United Kingdom, and 1–3 million are likely to have been infected with the BSE agent, most of which were slaughtered for human consumption before developing signs of the disease. Furthermore, BSE has been reported in most major countries that have established a BSE surveillance system. As of April 2005, a total of 5281 heads of affected cattles have been reported in 25 countries excluding the United Kingdom [53].

The third important event occurred in 1995 when a new type of CJD called vCJD was identified, and evidence gathered since that time links vCJD causally to BSE [54, 55]. The exact mechanism of BSE transmission to the human population has not been identified, but dietary exposure to BSE contaminated beef products remains the most likely hypothesis [54]. This chain of events has established in a dramatic way the ability of the prion infection to cross species barriers.

4.2. Human Prion Disease Surveillance Outside of North America

The obvious threat of the potential human exposure posed by BSE led the United Kingdom to establish a national surveillance center for human prion disease, the National CJD Surveillance Unit, in Edinburgh Scotland in 1990 [56] with the primary aim of identifying any changes in the characteristics of CJD that might be linked to BSE. A coordinated prion disease surveillance in member and associated countries of the European Union was established by the European Commission under the label of EUROCJD in 1993 [57]. EUROCJD includes the national prion disease registries of Australia,

Austria, Canada, France, Germany, Italy, the Netherlands, Slovakia, Spain, Switzerland, the United States, and the United Kingdom. The EUROCJD principal goal is to determine whether the incidence of CJD is similar throughout the European Union, and if there is any major difference between putative risk factors in various countries. This harmonization of the surveillance centers has provided a unique framework to study putative risk factors for CJD and other prion diseases, especially the occurrence of vCJD. In 1996, the European Union Council recommended that the epidemiological surveillance of CJD be extended to all member states. Thus, prion surveillance in Belgium, Denmark, Finland, Greece, Iceland, Ireland, Israel, Norway, Portugal, and Sweden is coordinated under the program called NEUROCJD. All major Eastern European countries and China were also included in 2001. In 1997, a Prion Disease Surveillance Center was established in Argentina under the auspices of the World Health Organization (Taratuto, A. L., personal communication). Through March 2005, the national surveillance centers of the 21 countries included in the EUROCJD and NEUROCJD programs combined have reported 6164 cases of possible and probable prion disease [57]. The mean prion disease-related mortality rates per million population over the 2002–2004 period range widely from 0.68 in Portugal to 2.43 in Switzerland. While high mortality rates in some small countries, such as Israel, are attributed to a large number of genetic CJD cases, the high Swiss mortality rate remains to date unexplained [58]. During the same period, a total of 169 cases of the iatrogenic form of CJD have been reported across the two programs, all but two of which were identified in France and the United Kingdom [59]. From April 1999 through March 2003, the CJD Surveillance Committee under the Prion Disease and Slow Viral Infection Research Committee sponsored by the Ministry of Health, Labor, and Welfare of Japan has examined a total of 409 patients with prion disease, including 97 cases of iCJD all due to dura mater implant [60]. The percentage of definite cases of prion disease, that is, the number of cases confirmed by tissue examination, also varies considerably from country to country. In the EUROCJD program participants, the percentage of the cases confirmed by tissue examination between 1999 and 2002 varies from about 50% of Germany to 100% of Slovakia, with most of the major countries having a rate between 60% and 75%.

The intense, widespread prion surveillance has resulted in the detection of cases of vCJD in several countries. Through April 2005, a total of 180 cases of probable and proven vCJD have been reported. The cases observed in the United Kingdom including probable and confirmed cases are 156 [59]. France listed 13 cases; three cases were observed in Ireland and one case each in Italy, Netherlands, Portugal, Spain, and Saudi Arabia, respectively (Will, R., personal communication). Canada, the United States, and Japan

have also reported one case each, but these vCJD cases were likely acquired during a stay in the United Kingdom [61]. Furthermore, two cases of vCJD in recipients of transfusion of blood from presymptomatic blood donors incubating vCJD have also been reported in the United Kingdom [62].

4.3. Human Prion Disease Surveillance in the United States

The National Prion Disease Pathology Surveillance Center (NPDPSC) was established in 1997 with the endorsement of the American Association of Neuropathologists and the support of the Centers for Disease Control and Prevention (CDC). Since 2001, the United States Congress has been making appropriations to CDC earmarked for the NPDPSC. The purpose of the NPDPSC is to examine, diagnose, classify, and store as many prion disease cases as possible. However, the real challenge of the NPDPSC is the timely detection of any human case of acquired prion disease that indicates the presence of a new source of prion infection. To date, BSE appears to be fairly contained in North America. Four BSE-affected cattle have been identified in Canada, and one in the US, but in this case the affected animal was apparently imported from Canada [53]. However, BSE surveillance in the United States, although expanded, remains significantly less stringent than that in European countries and Japan. All commercially slaughtered cattle older than 24 or 30 months are tested in Continental Europe, and all slaughtered cattle regardless of age are tested in Japan. In comparison, the United States testing is limited to ''high risk'' cattle (nonambulatory cattle, cattle with signs of central nervous system impairment or other BSE signs, and dead cattle) over 30 months old and to 20,000 randomly selected ''normal'' cattle over the age of 30 months. Approximately 176,500 heads of cattles were tested in 2004, while about 182,250 have already been tested as of May 10, 2005 out of a commercial cattle slaughter that in 2002 was 35.7 million [63].

A second potential source of human prion infection is CWD, a prion disease that affects free-ranging and captive elk and deer. First reported in 1967, CWD was once considered a rare and geographically contained disease. However, data support the presence of CWD among free-ranging and captive cervids in at least 13 states in the United States and two provinces in Canada with a prevalence of up to 20% in some endemic areas (Fig. 6) [64].

This finding along with other considerations, such as the high United States cervid population, estimated at 22 million, the several million big game hunters and the widespread consumption of elk and deer meat, underscore the possible risk of CWD exposure. Along with the recognition that the outbreak of BSE led to the emergence of vCJD, these considerations heightened concerns about possible direct-contact and food-borne CWD transmission to humans. In fact, about 130 people are known to have been

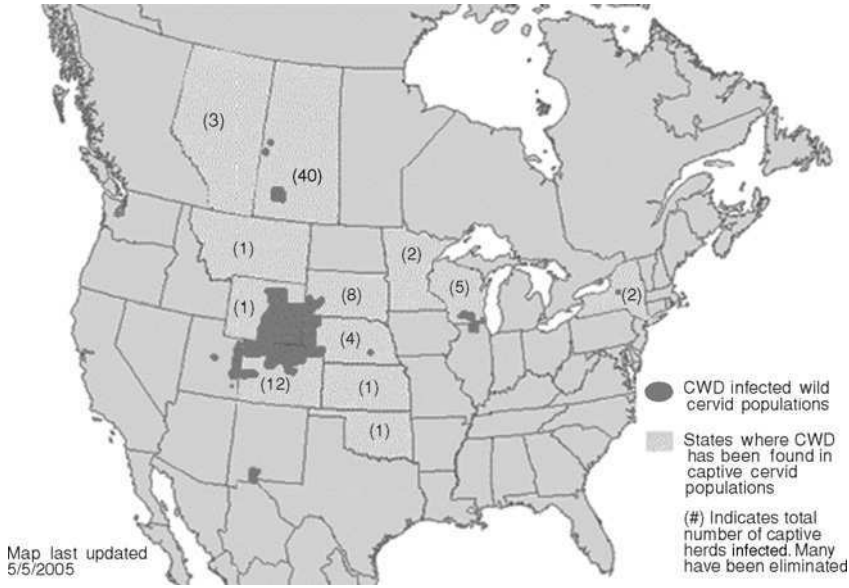

FIG. 6. CWD in North America. Map of areas affected by CWD. Reproduced from [71], by permission of Chronic Wasting Disease Alliance.

exposed to meat from elk subsequently proven to have CWD. Furthermore, 27 CJD patients who regularly consumed elk and deer meat were reported to the NPDPSC [65–67]. None of these cases appeared to have a novel form of prion disease. However, human disease acquired from CWD might have an unusual phenotype or a phenotype that is difficult to distinguish from sCJD. This uncertainty is a serious public health concern in the United States.

To promptly detect cases of human prion disease in which the disease is acquired from prion contaminated meat or other sources of prion infection, the NPDPSC was designed not only to identify cases but also characterize the positive cases in detail and establish the type of prion disease. The neurohistopathology and the immunostaining pattern of PrP^{Sc} are analyzed whenever possible in order to establish the disease phenotype. Western blot is used to establish the characteristics of PrP^{Sc}, while the *PRNP* genotype is determined from brain tissue- or blood-extracted DNA to detect the presence of mutations and identify polymorphisms, especially the common M/V polymorphism at codon 129 [2]. The presence of a pathogenic mutation in the open reading frame of the *PRNP* gene establishes whether the prion disease is genetic or sporadic. Western blotting reveals a number of PrP^{Sc} characteristics based on the gel mobility and glycoform ratio of PrP^{Sc} bands.

4.3.1. *PrP^{Sc} Type*

Based on the size of the PK-resistant PrP^{Sc} fragment, which in turn determines the gel mobility of the fragment, PrP^{Sc} is separated into two types: type 1 (in which the unglycosylated form migrates approximately to 21 kDa), and type 2 (the same form of which migrates to 19 kDa) [2]. *PRNP* genotype at polymorphic codon 129 distinguishes prion disease patients without mutation into three groups: MM, MV, and VV. Since both the PrP^{Sc} type and the PrP genotype at codon 129 are determinants of the disease phenotype in sporadic prion diseases, they have been used to classify these diseases into six distinct phenotypes identified by the codon 129 genotype and PrP^{Sc} subtype as shown in Table 2 [2]. iCJD cases are identified based on detailed examination of the clinical history searching for risk factors. Similarly, travel history to BSE affected countries and consumption of cervid meat are also investigated in suspected cases. When appropriate, CDC carries out a field investigation on these cases. Additional analyses of PrP^{Sc} including two-dimensional immunoblot, conformation dependent immunoassay, enrichment by sodium phosphotungstate precipitation or immunoprecipitation with specific antibodies [49, 68] as well as bioassays with transgenic mice are carried out to further characterize or detect very small amounts of PrP^{Sc} in special cases.

4.4. Epidemiological Data of Prion Disease in the United States

From its inception in 1997 through April 2005, NPDPSC examined nearly 1800 tissue samples from cases of suspected CJD and identified over 1000 cases of confirmed CJD (Table 4).

The data obtained for year 2005 appear consistent with 2004, and outreach efforts are currently underway to further increase the cases examined in an attempt to meet the goal of detecting at least 80% of the expected cases in the United States. A trend analysis shows that the number of positive cases examined has risen steadily, approaching 70% of the expected with about 287 cases per year in the United States, a number of proven cases which is comparable to other surveillance centers in other countries of similar size (Fig. 7) [69].

About 80% of the cases referred to the NPDPSC that are positive for prion disease are fully characterized by analysis of PrP^{Sc} subtype and *PRNP* genotype using Western blotting and gene sequencing, respectively (Tables 2 and 5). Because these assays are performed using frozen tissue, significant efforts have been made to inform clinicians, pathologists, neurologists, and state health officials about NPDPSC tissue collection protocols and specifically the need for frozen tissue. The protocols are made available from NPDPSC upon request, via the NPDPSC website (http://www.cjdsurveillance.com) and

TABLE 4
CASES REFERRED AND DIAGNOSED ON TISSUE AT NPDPSC (1997–APRIL 2005)

Year	Referrals	Prion disease total	Sporadic	Familial	Iatrogenic	vCJD
1997	104	60	54	6	0	0
1998	94	51	44	6	1	0
1999	114	74	65	9	0	0
2000	169	111	97	12	2	0
2001	247	154	138	16	0	0
2002	265[a]	151	127	22	1	1[b]
2003	284[c]	191[d]	142	45	1	0
2004	357[e]	200[f]	163	21	0	0
2005	113[g]	54[h]	21	12	0	0
Total	1747	1036	851	149	5	1

[a] Includes two inconclusive cases.
[b] Acquired in the UK.
[c] Includes one inconclusive case.
[d] Includes three cases with unknown type.
[e] Includes four pending cases.
[f] Includes seven cases with unknown type, six cases with type pending.
[g] Includes 19 pending cases.
[h] Includes 21 cases with type pending.

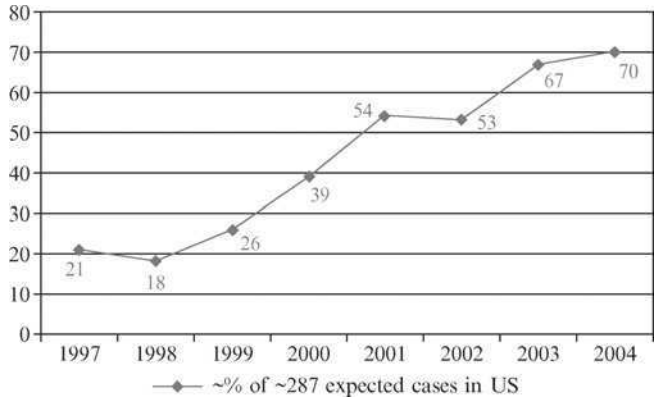

FIG. 7. Cases examined by NPDPSC as a percentage of cases expected. Cases expected are calculated based on the estimated incidence of one case per million population.

from participating state health departments. In cases where frozen tissue is unavailable, NPDPSC staff contact the submitting physicians to inform them on the need for frozen tissue for any subsequent cases [70]. These outreach

TABLE 5
CASES CHARACTERIZED AT NPDPSC AS CLASSIFIED BY PrP^{Sc} SUBTYPE AND *PRNP* GENOTYPE

Year	Total sporadic	Classified	Percentage of total
1997	54	42	78
1998	44	32	73
1999	65	38	58
2000	97	66	68
2001	138	78	57
2002	127	89	70
2003	142	122	86
2004	163	127	78
Total	830	594	72

TABLE 6
14-3-3 CSF EXAMINATIONS BY YEAR

Year	Total referrals	Positive (%)	Ambiguous (%)	Negative (%)
1997	13	38	0	62
1998	34	32	3	65
1999	75	36	4	60
2000	132	48	16	36
2001	204	46	34	20
2002	309	37	36	27
2003	292	28	46	26
2004	497	24	38	38
2005*	535	21	35	43
Total	2091	28	37	35

*Through April 2005.

efforts have resulted in the NPDPSC's ability to fully classify more cases than other prion surveillance centers combined [69].

The NPDPSC has also made significant progress in testing for 14-3-3, a surrogate marker for sCJD present in the CSF. The NPDPSC is now one of the very few facilities in the United States that perform the 14-3-3 test, and the number of samples received on a monthly basis has quintupled since 2004. If properly interpreted, the 14-3-3 provides valuable diagnostic information to clinicians (Table 6). As mentioned previously, the sensitivity of the 14-3-3 test varies according to the form of prion disease (Table 7) [17]. 14-3-3 sensitivity is high in sCJD MM1, the "typical" sCJD, but lower in the less common subtypes of sCJD and is not helpful for vCJD. Unfortunately, the

TABLE 7
CASES OF sCJD IN THE SIX MOLECULAR SUBTYPES WITH THE CORRESPONDING SENSITIVITY OF THE 14-3-3 PROTEIN TEST

sCJD and subtypes	*N* (total)	14-3-3 sensitivity (%)	*n* positive/*N* (total)
sCJD (total)	90	87	78/90
MM1	48	94	45/48
MV1	3	100	3/3
VV1	3	100	3/3
MM2	10	70	7/10
MV2	7	57	4/7
VV2	19	84	16/19

From [17], by permission of Lippincott Williams & Wilkins.

autopsy rate of 14-3-3 positive cases remains relatively low: tissue submitted to the NPDPSC accounted for only 28% and 37% of the 14-3-3 positive cases in 2003 and 2004, respectively.

The experience of the NPDPSC has been that PK-resistant PrP^{Sc} may be detectable only in some brain regions, and its characteristics may change from one region to another. These features increase the possibility that cases, which might pose serious threats to public health are missed, providing compelling argument for the use of a multiassay approach. A major effort of the NPDPSC has been devoted to perform and report each of the aforementioned tests with the shortest turnaround-time possible. This is very important to patients' families and caregivers, and, for cases of public health concern, to CDC and the health departments of the individual states.

4.4.1. *Autopsy Coordination Program*

The NPDPSC autopsy coordination program was developed to overcome the common obstacles reported by families in obtaining an autopsy in cases of suspected CJD or other forms of prion disease. Due to concerns about infectivity, families often have problems locating a pathologist and/or funeral home willing to assist with the autopsy. In order to resolve this issue, NPDPSC provides autopsy coordination services to families and/or clinicians upon request for cases of suspected CJD, including pathologist selection, transportation and storage arrangements, funeral home consultation, and payment for costs associated with the autopsy (Fig. 8). This endeavor has resulted in a more comprehensive surveillance with a higher percentage of autopsies performed on suspected cases.

In an effort to continually improve the network of providers willing to perform autopsies in cases of suspected CJD, NPDPSC monitors all cases submitted and approaches new pathologists and neuropathologists who

FIG. 8. NPDPSC autopsy network. The black stars represent facilities that perform autopsies for NPDPSC on a regular basis. The gray stars represent pathologists who are willing to take cases from outside their local area. These pathologists will travel or transport the patient to their facility for autopsy. All areas not covered by a black star are served by one or more gray stars.

submit outside cases for consultation to determine if they would like to join the network. Backup pathologists are also identified in the event that a preferred provider is not available when needed. NPDPSC also maintains a listing of funeral homes that may be willing to assist families with final arrangement, if a referral is needed. Outreach efforts related to the autopsy network focus on educating stakeholders about the program. Commonly, cases are referred to NPDPSC by the following individuals: family members, neurologists, pathologists or neuropathologists, and state health departments. NPDPSC reaches out to family members through advocacy organizations such as the CJD Foundation. Neurologists, pathologists, and neuropathologists are contacted through their professional organizations on a yearly basis. State health departments are encouraged to develop submission agreements with NPDPSC, wherein they refer cases of suspected CJD to the center and receive copies of all reports generated for patients from their state. This results in a more comprehensive surveillance not only in terms of the number of autopsies completed but also the information available to the states for planning purposes. As of May 2005, a total of 20 states have established submission agreements with NPDPSC.

4.4.2. *Brain Banking Services and Related Research*

NPDPSC regularly provides samples to researchers throughout the United States and around the world. Brain tissue, blood, CSF, and urine are each available for research into improved diagnostic testing, transmissibility, and potential treatments. Research can be further targeted by the form (sporadic, genetic, etc.) and subtype (sCJD MM1, MM2, etc.) of CJD as well as a variety of demographics such as age and duration of illness. Test results are also provided to submitting clinicians for the purpose of presenting cases in grand rounds or other venues.

5. Conclusions

The surveillance of human prion disease has been established worldwide, largely in response to the outbreak of BSE that may have been transmitted to humans to cause vCJD. The fact that CJD manifests as three different forms (sporadic, genetic, and acquired) with a wide spectrum of phenotypic heterogeneity presents a formidable challenge to diagnosis and surveillance. Studies on the role of the *PRNP* genotype at codon 129 and PrP^{Sc} subtype in the expression of disease phenotypes of human prion disease have led to a more rational and practical disease classification. As a result, considerable improvement has been made in the diagnosis of clinical and neuropathological variants of human prion disease. The recognition of well-defined phenotypes of sCJD will facilitate the surveillance of human prion disease in an effort to monitor any changes in CJD prevalence and demographics and promptly detect newly acquired (transmitted) disease variants (such as vCJD and iCJD).

For molecular diagnostics, definitive diagnosis is currently accomplished through the detection of PrP^{Sc} following digestion by PK. Limited sensitivity of this methodology may have weaken our ability to detect PrP^{Sc} in peripheral tissues and body fluids, the sites critical for premortem diagnosis. Emerging technologies that enrich PrP^{Sc} or distinguish it from normal PrP without the use of protease have been reported. The widely used surrogate marker, the 14-3-3 protein, has been useful in the CSF assay, but specificity varies depending on the disease phenotype. It is likely that future improvements in early diagnosis will benefit greatly from the ultrasensitive detection of PrP^{Sc} or new and specific surrogate markers in body fluids such as blood or urine.

The success of human prion disease surveillance will also depend on the infrastructure that will enable timely detection and diagnosis of every case of human prion disease. Many countries have established surveillance centers for human prion disease, and a global alliance for prion disease surveillance

has been formed. A wealth of data on epidemiology, clinical, and molecular pathology of human prion disease has emerged from these surveillance programs in recent years. There is no doubt that surveillance on prion disease should be further strengthened to protect public from the risk of disease transmission with the concerted efforts from physicians, basic scientists, clinical chemists, government officials and policy makers, and affected families and communities at large.

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