The things always happen that you really believe in; and the belief in a thing makes it happen.
C. REVIEW OF LITERATURE

Cardiovascular diseases are still the primary cause of death in most industrialized countries. Effective prevention includes treatment of a series of risk factors: smoking, hypertension, diabetes, obesity, and dyslipidemia, which include elevated triglycerides, total and low-density-lipoprotein (LDL) cholesterol levels, as well as lowered high-density-lipoprotein (HDL) cholesterol (NCEP guidelines).

Atherosclerosis is the major source of morbidity and mortality in the developed world. The magnitude of this problem is profound, as atherosclerosis claims more lives than all types of cancer combined and the economic costs are considerable. Although currently a problem of the developed world, the World Health Organization predicts that global economic prosperity could lead to an epidemic of atherosclerosis as developing countries acquire Western habits.

Atherosclerosis is characterized by the accumulation of cholesterol deposits in macrophages in large- and medium-sized arteries. This deposition leads to a proliferation of certain cell types within the arterial wall that gradually impinge on the vessel lumen and impede blood flow. This process may be quite insidious lasting for decades until an atherosclerotic lesion, through physical forces from blood flow, becomes disrupted and deep arterial wall components are exposed to flowing blood, leading to thrombosis and compromised oxygen supply to target organs such as the heart and brain. The loss of heart and brain function as a result of reduced blood flow is termed
heart attack and stroke, respectively, and these two clinical manifestations of atherosclerosis are often referred to as coronary artery disease and cerebrovascular disease. To simplify any discussion of the underlying pathology in these clinical syndromes, coronary artery disease and cerebrovascular disease are commonly referred to by the collective term *cardiovascular disease*. Over the last 40 years, a number of clinical and laboratory variables have proven predictive of the incidence of cardiovascular disease and thus qualify as cardiovascular disease risk factors. With respect to the underlying pathology of atherosclerosis, there are a number of environmental and genetic "cardiovascular risk" factors that warrant consideration.

C.1 Epidemiology and Risk Factors

1) Age

Although it is not subject to modification, age is among the most important risk factors for predicting incident cardiovascular disease. This concept is, perhaps, best illustrated if one considers the risk of developing cardiovascular disease over a 10-year period. Based on experience in the United States, the average risk of developing cardiovascular disease for a 30- to 34-year-old male is ~ 3%. This number raises some sevenfold to 21% for a comparable individual aged 60-64 yr. Prediction of coronary heart disease uses risk factor categories (Wilson et al., 1998). The exact magnitude of age-related risk compared with other cardiovascular disease risk factors is illustrated by work from the Framingham Heart Study that has
resulted in a 14-point scoring system to predict incident 10-yr cardiovascular disease. In this system, increasing risk is characterized by a higher score, and up to 7 points can be attributed to age alone. Thus age is an overriding risk factor for incident cardiovascular disease.

2) **Gender**

Numerous observational studies have indicated that males exhibit excess risk for cardiovascular disease compared with age-matched women (Barrett et al., 1991). There has been considerable speculation that estrogens offer a "protective" effect to women, as cardiovascular disease accelerates in women after menopause. However, this speculation has been difficult to substantiate, as the treatment with estrogen has not reduced the incidence of cardiovascular disease of postmenopausal women (Hulley et al., 1998). Alternatively, some of this apparent protection could be due to the fact that women exhibit relatively higher concentrations of HDL-C than do age-matched men. Nevertheless, incident cardiovascular disease is less common in premenopausal women than their age-matched male counterparts.

3) **Obesity**

There is a growing appreciation that obesity, defined as an excess body weight with an abnormal high preponderance of body fat, is a condition that increases the incident risk of cardiovascular disease. The exact mechanism(s) to explain this phenomenon, however, are controversial. A number of other risk factors for
cardiovascular disease, such as hypertension, low HDL cholesterol, and diabetes mellitus, often coexist with obesity (Wilson et al., 1999). This relation between obesity and cardiovascular disease has become of considerable concern as the prevalence of obesity in the developed world is increasing at an alarming rate.

4) **Cigarette Smoking**

In the Framingham heart study, Cardiovascular mortality increases by 18% in men and 31% in women for each 10 cigarette smoked per day (Kannel and Higgins et al., 1990). The carbon monoxide of cigarette smoke is especially injurious to lining of blood vessels and triggers atherosclerotic changes. The main metabolites of cigarette smoke are Allylamine and the end products Acrolein and reactive oxygen species (ROS). Smoking also increases platelet aggregation and the plasma concentration of the fibrinogen, both of which contribute to the occlusion of arteries (Glynn et al., 1966). It is thought that oxidative stress reduces the level of antioxidants available causing reduced ability to inhibit lipid peroxidation, endothelial dysfunction in particular subendothelial oedema and mitochondrial swelling (Zimmerman and McGeachie et al., 1987).

5) **Hypertension (HT)**

Hypertension induces endothelial dysfunction by reducing the Nitric Oxide (NO) mediated vasodilatation and increased vascular resistance (Radu et al., 1999). This is mediated through increased Ca\(^{++}\) either by reduced Nitric Oxide Synthase (NOS) or excess production of oxygen derived free radicals which inhibits NO production and may
lead to endothelial injury, which trigger atherosclerotic events. High blood pressure increases the heart workload causing the heart to enlarge and weaken overtime. Concentration of angiotensin II, the principal product of Renin Angiotensin System (RAS), is often elevated in patients with hypertension; angiotensin II is a potent vasoconstrictor. In addition to causing hypertension, it can contribute to atherogenesis by stimulating the growth of smooth muscle (Chobanian et al., 1996). Angiotensin II binds to specific receptors on smooth muscle, resulting in the activation of phospholipase C, which can lead to increase in intracellular Ca++ concentrations and in smooth muscle contraction. It also increases smooth muscle lipoxygenase activity, which can increase inflammation and the oxidation of LDL. Hypertension also has proinflammatory actions increasing the formation of hydrogen peroxide and free radicals such as superoxide anion and hydroxyl radicals in plasma (Griendling et al., 1997; Lacy et al., 1998). These substances reduce the formation of NO by the endothelium (Vanhoutte et al., 1995), increase leukocyte adhesion and increase peripheral resistance.

6) Diabetes

Hyperglycemia contributes to the interaction between endothelial functions producing abnormal responses to acetylcholine (Ach), increase production of thromboxane and prostaglandins (PGs), raised intracellular Ca++, all of which contributes to the release of endothelial vasoconstricting agents such as Ach and endothelin-I (ET-I). It also increases the production of free radicals causing
glycoxidation, and glycative stress within the cell, raises the quantity of glycated LDL and the atherogenic potential of LDL. The shunt in glucose to sorbitol via aldose reductase produces fructose. This sorbitol enhances cell damage by augmenting cell swelling. Endothelium derived aldose reductase contributes to highly abnormal cellular functioning and oxidative stress (Kawamura et al., 1994).

7) **Autoimmunity**

Atherosclerosis that was formerly considered an "inert" process is gradually perceived as a dynamic disorder in which immune system components appear to play a major role. Thus, T lymphocytes, macrophages as well as major histocompatibility complex (MHC) expressing cells (endothelial and smooth muscle cells) are present and functionally operate in the vicinity of atherosclerotic plaque. These findings, combined with the ones showing the presence of other humoral mediators (cytokines, chemokines and growth factors) with the atheroma have led to the conceptual paradigm of atherosclerosis as an inflammatory condition.

A further extension of this concept holds that autoimmunity to plaque components/molecule is capable of altering the "fate" of plaque. This may occur through classical mechanism such as altered self or molecular mimicry. Modified forms of host lipoproteins constitute the initial example of implementation of autoimmune concept to the evolution of plaque. Thus, when self-lipoproteins are being modified by processes such as oxidation, they result in tolerance breakdown by the host and a subsequent establishment of
autoimmunity (altered self). Anti-oxidized LDL (anti-OxLDL) antibodies are produced and clinical trials have shown their association with atherosclerosis and their positive predictive value with respect to development of atherosclerotic complications. Induction of anti-OxLDL antibodies in atherosclerotic prone animals by active immunization with OxLDL have been shown to be associated with reduced plaque formation. This suggests that anti-OxLDL antibodies may stand as active participants rather than innocent bystanders (Frostegard et al., 2005).

8) **Infection**

Atherosclerosis is an inflammatory disease. Infectious agents play a role in the etiology of CAD. Certain infectious agents have been isolated from the atheromatous plaque such as

- Chlamydia Pneumonia
- H. Pylori
- Human chorionic virus (HCV) & cytomegalo virus (CMV)

C. Pneumoniae appears to exhibit strongest association. Possible mechanisms by which infectious agents exert their effects (Kuo et al., 1993) may include,

a) Local effects on the endothelium, Smooth Muscle Cells (SMCs), or macrophages or,

b) Systemic effects by generating cytokines, stimulating monocytes, and promoting hypercoagulability.
9) **Physical inactivity**

Regular physical activities have been shown to reduce risk for CAD events in a number of observational epidemiological studies (Arnold et al., 1985). Exercise increases caloric expenditures and also benefits the hearts by increasing the efficiency. Inactive patients tend to have higher serum lipid levels and they have more LDL than HDL.

10) **Race**

The incidence, prevalence, and presentation of CAD vary significantly with the race, as does the response to therapy. African Americans appear to have higher morbidity and mortality rates, even when corrected for educational and socio-economic status, than whites. Asian Indians exhibit 2-3 fold higher incidence of CAD than whites in the United States. People in Mediterranean areas have a lower incidence of CAD (Budoff et al., 2006).

11) **Isoprostanes**

Isoprostanes are non-enzymatic, free-radical catalyzed isomers of cyclooxygenase-derived enzymatic products of arachidonic acid (Pratico et al., 2004; Morrow et al., 2005). In contrast to enzymatically generated prostaglandins, which are generated from free arachidonic acid, isoprostanes can be generated on intact cholesteryl esters and phospholipids, which are major components of lipoprotein particles and cell membranes: Following generation, isoprostanes are released by a phospholipase activity, circulate in plasma, and are ultimately excreted in urine. F2 isoprostanes are stable, specific, and unique
end-products (up to 64 species can be generated) of lipoprotein metabolism and can be measured with high sensitivity and specificity with gas chromatography/mass spectrometry. However, measurement of F2 isoprostanes does not necessarily reflect LDL oxidation as they are also generated basally at low levels under normal physiological functions and are also elevated in most inflammatory disorders. Elevated F2 isoprostane levels have been documented in patients with hypercholesterolemia, diabetes mellitus, smoking, renovascular hypertension, and hyperhomocysteinemia (Pratico et al., 2004; Morrow et al., 2005). Evidence of enhanced presence of F2 isoprostane has been detected within carotid atherosclerotic plaques. In addition, they colocalize with foam cells immunocytochemically (Pratico et al., 1997), and their levels correlate with unstable carotid plaques (Mallat et al., 1999). Urinary F2 isoprostane levels correlate with plasma LDL-C levels and LDL-associated isoprostanes. Interestingly, unlike in mouse models, vitamin E does not seem to affect urinary isoprostane levels in humans (De Caterina et al., 2002). In addition, isoprostanes are elevated in unstable angina and in the coronary sinus and urine of patients undergoing percutaneous coronary intervention (PCI) or thrombolysis and reperfusion during acute myocardial infarction. Measurement of F2 isoprostanes has become the gold standard in measuring oxidative stress in vivo. However, despite the promising clinical data, no prognostic information is currently available. In addition, the relative sophistication and expense required to perform
isoprostane assays in specialized laboratories in an efficient and cost-effective manner inhibits widespread use.

12) **Low Density Lipoprotein Cholesterol (LDL-C)**

A predominance of small, dense low-density lipoproteins (LDL) has been accepted as an emerging cardiovascular risk factor by the National Cholesterol Education Program (NCEP) Adult Treatment Panel III. LDL size seems to be an important predictor of cardiovascular events and progression of coronary heart disease and evidences suggests that both quality (particularly small, dense LDL) and quantity may increase cardiovascular risk. However, some researchers have suggested that LDL size measurement does not add information beyond that obtained by measuring LDL concentration, triglyceride levels and HDL concentrations. Therefore, it remains debatable whether to measure LDL particle size in cardiovascular risk assessment and, if so, in which categories of patient.

The therapeutic modulation of distinct LDL subspecies is also of great benefit in reducing the risk of cardiovascular events (Berneis et al., 2004). The peak size of LDL in humans shows a bimodal (rather than a normal) distribution, and can be separated into two phenotypes that differ in size, density, physicochemical composition, metabolic behaviour and atherogenicity. These phenotypes have been called 'pattern A' (larger, more buoyant LDL) and 'pattern B' (smaller, denser LDL predominate) (Packard et al., 1997; Berneis et al., 2002). LDL size correlates positively with plasma HDL levels and negatively with plasma triglyceride concentrations, and the combination of small,
dense LDL, decreased HDL cholesterol and increased triglycerides has been called the ‘atherogenic lipoprotein phenotype’ (Austin et al., 1990). This partly heritable trait is a feature of the metabolic syndrome, and is associated with increased cardiovascular risk.

The prevalence of the pattern B phenotype is approximately 30% in adult men, 5–10% in young men and women <20 years, and approximately 15–25% in post-menopausal women (Barrett et al., 1991; Wilson et al., 1998). LDL size is genetically influenced, with a heritability ranging from 35–45%, based on an autosomal dominant or codominant model with varying additive and polygenic effects (Austin et al., 1992). Clearly, non-genetic and environmental factors influence the expression of this phenotype, and abdominal adiposity and oral contraceptive use are both associated with an increase in small, dense LDL (Terry et al., 1989; De Graaf et al., 1993; Rizzo et al., 2003).

Dietary factors are also important. A very low-fat high-carbohydrate diet can induce pattern B in people who are genetically predisposed to this phenotype (Dreon et al., 1997). In addition, the predominance of small, dense LDL is commonly found in conjunction with familial disorders of lipoprotein metabolism that are associated with increased risk of premature coronary artery disease, including familial combined hyperlipidaemia, hyper-beta-lipoproteinaemia and hypo-alpha-lipoproteinaemia (Berneis et al., 2004). Several reasons have been suggested for the atherogenicity of small, dense LDL. Smaller, denser LDL are more easily taken up by arterial tissue than
are larger LDL (Bjornheden et al., 1996), suggesting greater transendothelial transport of smaller particles. Oxidative susceptibility increases and antioxidant concentrations decrease with decreasing LDL size (Tribble et al., 1992). The altered properties of the surface lipid layer associated with a reduced content of free cholesterol (Tribble et al., 1992) and increased content of polyunsaturated fatty acids (PUFA) (De Graaf et al., 1993) might also contribute to the enhanced oxidative susceptibility of small, dense LDL.

According to the National Cholesterol Education Program Adult Treatment Panel III, clinical forms of non-coronary atherosclerosis carry a risk for Coronary Heart Disease (CHD) equal to those with established CHD. These conditions include peripheral arterial disease, symptomatic (transient ischaemic attack or stroke of carotid origin) and asymptomatic (>50% stenosis on angiography or ultrasound) carotid artery disease and abdominal aortic aneurysm.

Smaller, denser LDL particles are a risk factor for peripheral arterial disease, whether in the absence or presence of diabetes. Common features of peripheral arterial disease are represented by increased triglyceride levels and lower HDL cholesterol concentrations (O’Neal et al., 1998), and patients with such lipid abnormalities mostly have atherogenic small, dense LDL particles (Austin et al., 1990; Rizzo et al., 2005).
Hypolipidaemic treatment can alter LDL subclass distribution, and statins and fibrates are currently the most widely used lipid-lowering agents. Statins are potent inhibitors of hydroxy-methylglutaryl-coenzyme A (HMG Co A) reductase, the rate-limiting enzyme in hepatic cholesterol synthesis, and are the primary drugs of choice for the treatment of elevated plasma LDL cholesterol concentrations (Lamarche et al., 1999). Fibrates have a major impact on triglyceride metabolism, mediated by peroxisome proliferation activator receptors (PPAR) and through stimulation of lipoprotein lipase (Marais et al., 2000). Fibrates seem to have more effect than statins on LDL size. Therapy with fenofibrate, benzafibrate and gemfibrozil usually results in a beneficial effect (Lamieux et al., 2002).

In brief, Genetic and environmental factors influence the expression of small, dense LDL, which is not completely independent of traditional lipids, correlating negatively with plasma HDL concentrations and positively with plasma triglyceride levels. Small, dense LDL is associated with the metabolic syndrome, and with increased risk for cardiovascular disease and diabetes mellitus. LDL size also seems to be an important predictor of cardiovascular events, and progression of coronary artery disease and a predominance of small, dense LDL has been accepted as an emerging cardiovascular risk factor by the National Cholesterol Education Program Adult Treatment Panel III.
ATHEROSCLEROSIS (AS):

An exceedingly common disease of blood vessels characterized by the accumulation of lipids in the innermost layer of the large blood vessels, resulting in narrowing of the blood passageway and loss of the elasticity and weakening of the wall.

The interaction between the vulnerable atherosclerotic plaque and thrombus formation, a process referred to as Atherothrombosis, is the stone of acute coronary syndromes.

C.2 Theories of Genesis

Several theories have been given by different scientists

1) The Encrustation Theory

It is proposed by Rokitansky in 1851, suggested that AS begins in the intima with the deposition of thrombus and its subsequent organization by infiltration of fibroblasts and secondary lipid deposition.

2) The Lipid Theory

It is proposed by Virchow in 1856, suggested that AS starts with the lipid transudation into the arterial wall and interaction with cellular and extracellular elements, causing "Intimal proliferation ".

3) The Response to Endothelial Injury Theory

It is proposed by Ross in 1999, suggested the response-to-injury. It postulates that the beginning of AS with endothelial
injury, making it susceptible to accumulation of lipids and deposition of thrombus.

The currently accepted response-to-vascular injury theory

Over the past decade, Fuster and colleagues (1992) have proposed that vascular injury starts the atherosclerotic process. The effect of such vascular injury can be classified as follows.

a) Type-I
Vascular injury involving functional changes in the endothelium with minimal structural changes (i.e. increased lipoprotein permeability and WBC (White Blood Cell) adhesion)

b) Type-II
Vascular injury involving endothelial disruption with minimal thrombosis.

c) Type-III
Vascular injury involving damage to media, which may stimulate severe thrombosis, resulting in unstable coronary syndromes.

So, according to the response-to-vascular injury theory, injury to the endothelium by local disturbances of blood flow at angulated or branch points, along with systemic risk factors, such as hyperglycemia, dyslipidemia, cigarette smoking, and, possibly, infection, perpetuates a series of events that culminate in development of atherosclerotic plaque.

Atherosclerosis can be considered to be a form of chronic inflammation resulting from interaction between modified lipoproteins,
monocyte-derived macrophages, T-cells, and the normal cellular elements of the arterial wall. This inflammation process can ultimately lead to the development of complex lesions, or plaques, that protrude into the arterial lumen. Plaque rupture and thrombosis results in the acute complications of myocardial infarction and stroke (Navab et al., 1996).

**Lipoproteins and Atherosclerosis**

Serum cholesterol is carried by several lipoprotein particles that perform the complex physiologic tasks of transporting dietary and endogenously produced lipids. Chylomicrons provide the primary means of transport of dietary lipids, while very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) function to transport endogenous lipids. Triglyceride (TG) rich VLDL particles containing apolipoprotein B-100 and Apo-E are synthesized by the liver and function to transport fatty acids to adipose tissue and muscle. After triglyceride removal in peripheral tissues, a portion of the remaining VLDL remnants are metabolized to LDL particles by further removal of core triglyceride and dissociation of apolipoproteins other than Apo B-100. The majority of serum cholesterol is carried by LDL particles in human.

While LDL has an essential physiological role as a vehicle for the delivery of cholesterol to periphery tissues, increased LDL-C levels are associated with increased risk of cardiovascular disease. The expression of LDL receptors is subjected to feedback control by intracellular cholesterol levels. Low levels of intracellular cholesterol
leads to activation of the SREBP (sterol regulating element binding protein) transcription factors, which stimulate transcription of the LDL receptor gene and other genes involved in cholesterol biosynthesis (Brown and Goldstein, 1986).

Development of atherosclerosis in murine models, as in virtually all animal models of atherosclerosis is driven by extreme elevations in circulating cholesterol levels that result in the formation of extensive lesions over a time scale of weeks to months. In contrast, the development of atherosclerosis in human evolves over decades and advanced lesions are typically less cellular than those observed in animal models.

C.3 Process of Atherosclerosis

1) Initiating Events- LDL modification

Atherosclerotic lesions begin as fatty streaks underlying the endothelium of large arteries. Recruitment of macrophages and their subsequent uptake of LDL derived cholesterol are the major cellular events contributing to fatty streak formation. Many lines of evidence suggest that oxidative modifications in the lipid and apolipoprotein B components of LDL drive the initial formation of fatty streaks (Navab et al., 1996).

The specific properties of oxidized LDL (ox-LDL), usually studied following oxidation of native LDL in-vitro, depend on the extent of modification. This can range from "minimal" modification (mm LDL) in which the LDL particle can still be recognized by LDL receptors
(Navab et al., 1996), to extensive oxidation, in which the apo B component is fragmented and lysine residues are covalently modified with reactive breakdown products of oxidized lipids. Such particles are not bound by the LDL receptor, but rather by several so-called scavengers receptors expressed as macrophages and smooth muscle cells. While LDL is protected from oxidation in the plasma compartment, it is thought to become susceptible to enzymatic & nonenzymatic modifications when retained by extracellular matrix proteins in the artery wall. (Schwenke and carew, 1989).

A number of potential oxidant generating system have been investigated that could directly or indirectly target LDL lipids, including myeloperoxidase (MPO), nitric oxide synthase (NOS) & 15-lipoxygenase (15-LO) (Heinecke et al., 1998).

2) Monocyte / Macrophage Recruitment

Although the recruitment of monocytes to the arterial wall and their subsequent differentiation into macrophages may initially serve a protective function by removing cytotoxic and proinflammatory oxLDL particles or apoptotic cells, progressive accumulation of macrophages and their uptake of oxLDL ultimately lead to the development of atherosclerotic lesions. A lesion-prone site of large arteries is regulated by cell adhesion molecules that are expressed on the surface of endothelial cells in response to inflammatory stimuli.

Several cell adhesion molecules have been suggested to play roles in macrophage recruitment. One of the first to be implicated was vascular cell adhesion molecule-1 (VCAM-1), based on its increased
expression on endothelial cells over lesion-prone areas, its preferential recruitment of monocytes, and its pattern of regulation by proinflammatory stimuli (Cybulski and Gimbrone, 1991).

Migration of monocytes into the artery wall is likely to be stimulated in part by oxLDL, which can directly attract monocytes and can also induce the expression of monocyte chemotactic protein-1 (MCP-1). Intriguingly, monocyte expression of CCR2, the receptor for MCP-1, is stimulated by hypercholesterolemia and monocytes derived from hypercholesterolemic patients exhibit increased chemotactic responses to MCP-1 (Han et al., 1999). Disruption of the MCP-1 or CCR2 genes markedly reduces the development of atherosclerosis in apo E(-) or apo-B overexpressing mice respectively (Boring et al., 1998). IL-8, which is present in human atherosclerosis lesions, may also play a role in monocyte-macrophage trafficking.
Fig. C.3.1 Initiating events in the development of a fatty streak lesion.

LDL is subject to oxidative modifications in the subendothelial space, progressing from minimally modified LDL (mmLDL) to extensively oxidized LDL (oxLDL). Monocytes attach to endothelial cells that have been induced to express cell adhesion molecules by mmLDL and inflammatory cytokines. Adherent monocytes migrate into the subendothelial space and differentiate into macrophages. Uptake of oxLDL via scavenger receptors leads to foam cell formation. OxLDL cholesterol taken up by scavenger receptors is subject to esterification and storage in lipid droplets, is converted to more soluble forms, or is exported to extracellular HDL acceptors via cholesterol transporters, such as ABC-AT.
3) Foam cell formation

The development of macrophage “foam cells” that contain massive amounts of cholesterol esters is a hallmark of both early and late atherosclerotic lesions. Cholesterol accumulation in these cells is thought to be mediated by uptake of modified forms of LDL via so-called scavenger receptors (Yamada et al., 1998). Several proteins may contribute to this overall process, scavenger receptors A (SR-A) and CD-36 have been demonstrated to play quantitatively significant roles.

oxLDL derived cholesterol brought into the macrophage via scavenger receptors consists of free cholesterol as well as cholesterol esters that are hydrolysed in lysosomes. Free cholesterol has a number of potential metabolic fates, including esterification by Acyl coA : cholesterol acyltransferase-1 (ACAT-1) and storage in the lipid droplets that characterize foam cells. Cholesterol esters within these lipid droplets can in turn be hydrolysed by hormone-sensitive lipase, generating free cholesterol for incorporation into membranes and transport out of cell. Disruption of ACAT-1 results in marked systemic abnormalities in lipid homeostasis in hypercholesterolemic apo-E deficient and LDL-R deficient mice, leading to extensive deposition of free cholesterol in skin and brain (Accad et al., 2000).

ACAT-1 deficiency did not present development of atherosclerosis in these models, but reduced the lipid and macrophage content of lesions. The macrophage has two potential mechanisms for disposing of excess cholesterol,
a) Enzymatic modification to more soluble forms and efflux via membrane transporters

b) The major mechanism for cholesterol efflux is likely to be via membrane transporters, with HDL serving as the primary extracellular acceptor. This role of HDL is thought to be critical for physiologic “reverse cholesterol transport” and to at least partially explain why risk of atherosclerosis is inversely correlated with HDL-C levels (Tall et al., 2000).

In Tangier disease, there is a null mutation in ABC A-1. This ABC A-1 mediates transport of cholesterol from cells to HDL acceptors. In the absence of proper lipidation, HDL particles are rapidly cleared, suggesting a probable explanation for the extremely low HDL-C levels in Tangier patients.

Once the free cholesterol has been taken up from peripheral cells by HDL, it is esterified to cholesterol esters by lecithin-cholesterol acyltransferase (LCAT). HDL can subsequently exchange cholesterol esters for TG carried by other lipoproteins via cholesterol ester transfer protein (CETP). Alternatively, HDL can selectively deliver cholesterol esters to the liver for excretion by binding to the HDL receptor SR-B1.

4) Lesion progression and immunologic responses

The transition from the relatively simple fatty streak to the more complex lesion is characterized by the migration of smooth muscle cells from the medial layer of the artery wall past the internal elastic lamina and into the intimal, or subendothelial space. Intimal smooth muscle cells may proliferate and take up modified lipoproteins,
contributing to foam cell formation, and synthesize extracellular matrix proteins that lead to the development of fibrous cap (Ross et al., 1999).

This phase of lesion development is influenced by interactions between monocyte/macrophages and T cells that result in a broad range of cellular and humoral responses and the acquisition of many features of a chronic inflammatory state. Lesional T cells appear to be activated, expressing both T-helper cells (Th) 1 and Th 2 cytokines (Hansson, 1997). Similarly, macrophages, endothelial cells, and smooth muscle cells appear to be activated based on their expression of MHC class II molecules and numerous inflammatory products such as tumor necrosis factor-α (TNF-α), IL-6, MCP-1. Lymphocytes do not appear to be required for the development of atherosclerosis.
Interactions between macrophage foam cells, Th 1 and Th 2 cells establish a chronic inflammatory process. Cytokines secreted by lymphocytes and macrophages exert both pro- and antiatherogenic effects on each of the cellular elements of the vessel wall. Smooth muscle cells from the medial portion of the arterial wall, proliferate and secrete extracellular matrix proteins that form a fibrous plaque.

Some data implied that once early lesions develop, immune responses modulate progression. Immune responses appear to exert both atherogenic and antiatherogenic, e.g. the potent Th 1 derived cytokine, interferon-α (IFN-α), reduces scavenger receptor expression on macrophages, decreases collagen synthesis and inhibits smooth muscle cell proliferation, all potentially antiatherogenic effects. On the
other hand, IFN-α also stimulates macrophage production of proinflammatory cytokines and increases expression of MHC class II molecules. Th II derived cytokines also appear to have complex effects on lesion development. IL-4 exerts a number of effects that are predicted to be antiatherogenic, including antagonistic effects on IFN-α activity in macrophages and inhibition of Th I cell function. However, IL-4 is also a potent inducer of 15-LO, which promotes LDL oxidation and development of atherosclerosis in mice.

IL-10, which cross regulates Th I cells, has potent deactivating properties in macrophages and modulates several other cellular processes that may interfere with the development and stability of the atherosclerotic plaque. These observations indicate that immune activation is ongoing in atherosclerotic lesions. The most significant antigens responsible for immune activation are not known with certainty. There are now many reports in mice and human of a strong correlation between autoantibody titers to epitopes of oxLDL and extent of atherosclerosis (Horokko, 2000). It is not yet clear whether such autoantibodies are simply markers for the extent of disease, or if they have any physiological or pathological role. These observations suggest that it might be possible to modulate the development of atherosclerosis by immunization or other immune-based interventions.

Matrix metalloproteinases secreted by macrophages have been detected in regions of plaque rupture and are suggested to influence plaque stability by degrading extracellular matrix proteins (Galis et al.,
1994). Thus methods to alter the expression or activities of metalloproteins would be of potential clinical benefit.

**Fig. C.3.3 Plaque rupture and Thrombosis**

Necrosis of macrophage and smooth muscle cell-derived foam cells leads to the formation of a necrotic core and accumulation of extracellular cholesterol. Macrophage secretion of matrix metalloproteinases and neovascularization contribute to weakening of the fibrous plaque. Plaque rupture exposes blood components to tissue factor, initiating coagulation, the recruitment of platelets, and the formation of a thrombus.
Neovascularization is prevalent in human atherosclerotic lesions associated with plaque rupture, hemorrhage or unstable angina. Angiogenesis occurs in association with remodeling and protease activation in surrounding tissues, suggesting that neovascularization could contribute to plaque instability and rupture.
C.4 Free Radicals

Exogenous sources, such as radiation, air pollution, smoking and some endogenous sources like stress, some abnormal levels of enzymes, can generate free radicals. Free radicals are reactive chemical species that contain one or more unpaired electrons in the outer orbit. They are generally unstable in nature (Gilbert et al., 2000). Examples of free radicals are superoxide (O$_2^-$), hydroxyl (OH$^-$), peroxyl (RO$_2^-$), alkoxyl (RO$^-$), and hydroperoxyl (HO$_2^-$) radicals. Nitric oxide and nitrogen dioxide (NO$_2$) are two nitrogen free radicals. Oxygen and nitrogen free radicals can be converted to other non-radicals reactive species, such as hydrogen peroxide, hypochlorous acid (HOCl), hypobromous acid (HOBr), and peroxynitrite (ONOO$^-$). Reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive chlorine species are produced in human and animals under physiologic and pathologic conditions (Evans and Halliwell, 2001). Thus, ROS & RNS include radical and non radical species.

C.4.1 Types of free radicals in the body

a) Superoxide radical (O$_2^-$)

It is formed when oxygen is reduced by the transfer of a single electron to its outer shells (Cheeseman & Slater, 1993) produced by phagocytic cells to inactivate viruses and bacteria. Its main significance is that it is a main source for generation of H$_2$O$_2$ which forms lethal hydroxyl radical. Superoxide is generated by different pathways: autooxidation of catecholamines, tetrahydrofolates, and
reaction of flavins with molecular oxygen and electron leak from the mitochondrial electron transport chain (ETC) (Halliwel et al., 1994). This radical is an important intracellular signaling molecule which participates in growth regulation and in defense mechanisms by activated phagocytes. Excessive release of vascular O$_2^-$ has been implicated in the development of atherosclerosis (White et al., 1994) and has been regarded as a major factor leading to the development of hypertension. In an organic medium, unlike the aqueous medium, O$_2^-$ is a highly reactive entity since it rapidly reacts with different metabolic enzymes (Gardner et al., 1992). Its reaction with cations such as iron and copper produces the most reactive species, i.e. hydroxyl radical (Halliwel et al., 1989).

b) Hydrogen peroxide (H$_2$O$_2$)

It's not a free radical but is a ROS and the main source of formation of hydroxyl radicals in the presence of transition metal ions. It is also involved in the production of HOCl by neutrophils.

\[ 2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \]

The above reaction is called as Dismutation reaction as the radical reactants produce nonradical products H$_2$O$_2$, which is easily diffusible within and between cells. Hydrogen peroxide is also formed by some oxidases such as amino acid oxidase, xanthine oxidase and NADPH oxidase. Some of the functions of H$_2$O$_2$ include the upregulation of genes especially those controlled by nuclear factor-kB.
(NF-kB) transcription factor and the induction of intracellular Ca++ overload in cardiomyocytes for the occurrence of heart dysfunction. At this point, it is important to mention that H$_2$O$_2$ also a precursor of the OH$^*$ radical and hypochlorous acid (HOCl). In activated neutrophils, the NADPH-dependent oxidase reduces molecular oxygen to O$_2$$^-$ and H$_2$O$_2$. In the presence of myeloperoxidase and chloride ion, H$_2$O$_2$ forms HOCl both inside and outside the cell followed by the formation of other non-radical oxidants such as singlet oxygen (O$_2$) in human neutrophils.

\[
\text{H}_2\text{O}_2 + \text{H}^+ + \text{Cl}^- \rightarrow \text{H}_2\text{O} + \text{HOCl}
\]

\[
\text{H}_2\text{O}_2 + \text{HOCl} \rightarrow \text{H}_2\text{O} + \text{H}^+ + \text{Cl}^- + \text{O}_2
\]

c) Hydroxyl radical (OH$^*$)

It is an extremely reactive oxidising radical that reacts to most biomolecules. It is highly important in radiobiological damage and is much more reactive towards cellular constituents than superoxide radicals.

\[
\text{O}_2^+ + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{H}_2\text{O} + \text{OH}^*
\]

This formula is known as Haber-Weiss reaction. In the body the pool of free iron that is available to catalyse the reaction from H$_2$O$_2$ to the hydroxyl ion is, under normal conditions, extremely limited. Red blood cells contain much of the iron in the body. Fortunately, several iron transporters are available to prevent inadvertent release and therefore limit the availability of free iron to catalyse the Haber-Weiss reaction.
When generated, OH* induces significant damage in the cell especially with limited diffusion capacity. The harmful effect of OH* occurs on the cellular proteins, carbohydrates, lipids and DNA; it is also capable of initiating a free radical chain reaction by initiating lipid peroxidation (Halliwel et al., 1989).

d) Hypochlorous acid (HOCl)

It is generated by the action of Myeloperoxidase on chloride ions in the presence of H$_2$O$_2$

$$H_2O_2 + Cl^- \rightarrow HOCl + OH^-$$

This reaction occurs in the neutrophils phagocytic vacuoles after fusion with the myeloperoxidase containing lysosomal vesicles. HOCl can initiate lipid peroxidation (Panasenko et al., 1995) and damage DNA and DNA repair processes (Vanrensburg et al., 1992). There are also some other free radicals are involved in atherosclerosis, are HOCl, peroxynitrite, singlet oxygen etc.

O$_2^-$ inactivates catalase by converting the resting ferric enzyme to the poorly active ferro-oxy form. Superoxide dismutase (SOD) protects catalase and peroxidase against this inactivation. At the same time, H$_2$O$_2$ inactivates two of the three known types of superoxide dismutases, and catalases and peroxidases prevent this. Superoxide dismutases plus catalases and peroxidases thus constitute a mutually supportive team.
C.4.2 Hypotheses of free radicals

The cytotoxic effect of free radicals is deleterious to mammalian cells and mediates the pathogenesis of many chronic diseases, but is responsible for the killing of pathogens by activated macrophages and other phagocytes in the immune system (McCord et al., 2001). Thus, there are "two faces" of free radicals in biology in that they serve as signaling and regulatory molecules at physiologic levels but as highly deleterious and cytotoxic oxidants at pathologic levels.

The mitochondrial electron transport system is a source of superoxide (Freidowich et al., 1999). Because NADH, NADPH, & FADH$_2$ are produced almost exclusively via the aerobic metabolism of protein, fat, and glucose, an increase in dietary energy intake enhances mitochondrial free radical production, which results in oxidative stress. Thus, calorie restriction reduces the generation of free radical species and retards ageing in animals.

Under physiologic conditions, approximately 1% to 3% of the O$_2$ consumed by the body is converted into superoxide and other ROS. Throughout the life cycle, any person may be at a risk of oxidative stress, induced by high rates of oxygen use (e.g. strenuous work and competitive sports), prolonged exposure to free radicals, even at a low concentration, may result in the damage of biologically important molecules and potentially lead to DNA mutation, tissue injury, and disease. Thus, although molecular oxygen is absolutely essential for aerobic life, it can be toxic under certain conditions. This phenomenon has been termed the Oxygen Paradox.
C.4.3 Oxidative stress and free radicals

Over the past five decades, a great deal of research work has been carried out to seek understanding of the role of oxygen toxicity as well as reactive oxygen species (ROS) in cardiac dysfunction under a wide variety of pathophysiological conditions (Kukreja et al., 1992). Likewise, a large body of information is available in the literature which implicates ROS in the genesis of vascular abnormalities. The increased formation of ROS is generally associated with oxidative stress and subsequent cardiovascular tissue injury. Particular attention has been paid to discuss the role of oxidative stress in various cardiovascular diseases such as atherosclerosis, ischemic heart disease (IHD), hypertension, cardiomyopathy, cardiac hypertrophy and congestive heart failure (CHF).

Free radicals are a chemical species that possess an unpaired electron in the outer shell of the molecule and they are highly reactive. The highly reactive means that they have low chemical specificity i.e. they can react with most molecules in their vicinity includes proteins, lipids, carbohydrates & deoxy-nucleic acid (DNA). It gains stability by capturing the needed electron; they don't survive in their original state for very long and quickly react with their surroundings. Hence, free radicals attack the nearest stable molecule, “stealing” its electron. When the “attacked” molecule loses its electron, it becomes a free radical itself and begins a chain reaction and finally leads to the disruption of cell.
C.4.4  Role of free radicals in Cardiovascular Diseases

Although oxidative stress is known to occur in different types of cardiovascular disease, direct cause and effect relationships have not been clearly delineated. The increase in the generation of ROS under several pathophysiological conditions, that seem to be related to inflammatory processes, is poorly understood; this may be due to difficulties in defining their site of origin. Impaired mitochondrial reduction of molecular oxygen may be an intracellular source, whereas secretion by phagocytic white blood cells, dysfunctional endothelial cells, or the auto-oxidation of catecholamines may be the extracellular sources.

ROS may also result from cellular injury due to exposure to ionizing radiation, ultraviolet rays, cigarette smoking or other air pollutants. The stimulus for such alterations may vary from one disease to another and the mechanisms for such changes are far from understood. Nonetheless, vascular defects leading to contractile dysfunction and dysrrhythmias are associated with oxidative stress. Oxidative stress can be seen to promote the entry of Ca++ into vascular myocytes and thus may stimulate neointimal hyperplasia for the occurrence of atherosclerosis as well as vasoconstriction for the development of hypertension.

Excessive increase in the intracellular concentration of Ca++ would result in Ca++ overload and thus produce myocardial cell damage as seen in cardiomyopathic or ischemic-reperfused hearts. The intracellular Ca++ overload as a consequence of oxidative stress
may also play a crucial role in the transition of cardiac hypertrophy to heart failure. On the other hand, it can be argued that oxidative stress may be a result of the cardiovascular disease and is thus associated with some secondary effects of the disease process. While it is a difficult question to settle on the basis of information available in the literature, some of the evidence supporting the role of oxidative stress in different types of cardiovascular diseases.

C.4.4.1 **Role of free radicals in Atherosclerosis**

Although a high level of plasma cholesterol is considered to trigger atherosclerosis, the oxidation of cholesterol seems to be a necessary step. In fact, uptake of oxLDL was shown to be an early event leading to the development of atherosclerosis. oxLDL and oxidized lipoprotein (a) have been reported to stimulate $O_2^-$ formation leading to apoptosis of cells in the umbilical vascular wall; this was prevented by treatment with antioxidants SOD and catalase (Galle et al., 1999). In cultured human coronary artery smooth muscle cells, low levels of oxLDL stimulate the extracellular matrix synthesis indicating the involvement of oxidative stress in the pathogenesis of atherosclerosis (Bachem et al., 1999). High levels of oxLDL were apoptotic implicating the additive role of ROS in increased plaque vulnerability; this effect was reduced by probucol and catalase. Patients with atherosclerosis and hypercholesterolemia showed higher susceptibility of LDL to oxidation in comparison to patients treated with lipid-lowering agents such as lovastatin and probucol (Anderson
et al., 1996); the protection was reflected by improved vasodilator response to acetylcholine.

In the atherosclerotic lesion produced in the rabbit aorta, significant increases in the iron content were observed suggesting that iron-catalysed free radical reactions may be associated with the development of atherosclerosis. The occurrence of intracellular Ca++ overload has been proposed as a mechanism of injury due to oxidative stress because human endothelial cells subjected to oxidative stress showed an increase in the level of intracellular Ca ++ and plasma membrane blebbing. Endothelial dysfunction may play an important role in the atherosclerotic process because in patients with atherosclerosis, antioxidants, probucol and ascorbic acid, improved the endothelium-dependent relaxation suggesting the involvement of ROS in endothelial dysfunction (Anderson et al., 1995). Increased production of O$_2^*$ has been implicated in the impaired endothelium-dependent relaxation in cholesterol fed rabbits and was suggested to be an early event in the hypercholesterolemic atherosclerotic process (Ohara et al., 1993).

Oxidative inactivation of NO$^*$ by superoxide has been proposed as plausible explanation for endothelial dysfunction (Harrison et al., 1997). When exposed together, O$_2^*$ and NO$^*$ react with each other three times faster than the reaction rate of O$_2^*$ with either Mn$^{++}$ and Cu$^{++}$/Zn$^{++}$-SOD (Thomson et al., 1995). Therefore, O$_2^*$ would preferentially react with NO$^*$ rather than SOD and cause inactivation of NO$^*$. in human atherosclerotic arteries, the production of
endothelial nitric oxide synthase as well as NO• has been shown to be depressed. SOD was shown to protect the inactivation of NO• in the canine coronary artery. The generation of O$_2$•$^-$ was thought to be due to the activation of the vascular and endothelial enzyme NADH/NADPH oxidase. Moreover, an increase in NADH/NADPH oxidase-dependent vascular O$_2$•$^-$ was reported in hypercholesterolemic rabbits. Oxidation of NO• by O$_2$•$^-$ results in the formation of peroxynitrite which could initiate lipid peroxidation or play a role in the oxidation of lipoproteins (Backman et al., 1990; White et al., 1994). Both of the above may be important steps in the development of atherosclerosis.

It should be noted that cholesterolemic rabbits fed with a flax seed diet (which is the richest source of ω-3 fatty acid and lignans) have shown reduced levels of aortic atherosclerosis and reduced production of ROS by polymorphonuclear leukocytes (Prasad et al., 1997). These data suggest that dietary supplements with antioxidant properties can prevent hypercholesterolemic atherosclerosis, although the beneficial effects of these supplements have been demonstrated in age-related diseases (Meydani et al., 1998).

They are generated as an ordinary process of oxygen metabolism and that a wide range of conditions are associated with either an excess generation of free radicals or an inadequate antioxidant defense system. If free radicals not quenched by an antioxidant, these compound will react with the nearest fat, protein, carbohydrate, RNA, or DNA molecule, altering its structure and function.
Such interactions may also generate additional free radical centers, especially in polyunsaturated fatty acids (PUFA). The nuclear DNA in every human cell receives an estimated 10,000 oxidative “hits “per day, indicating that cells are under constant bombardment from reactive oxygen species.

Short term oxidative stress may occur in tissues injured by trauma, infection, heat, radiation, hyperoxia, toxins, and excessive exercise. These injured tissues also produce increased radical-generating enzymes (e.g. xanthine oxidase, lipoxygenase, cycloxygenase), activation of phagocytes, release of free iron and copper ions, or a disruption of the electron transport chains of oxidative phosphorylation, producing excess ROS. According to this hypothesis, the result is tissue damage, as seen in rheumatoid arthritis, adult respiratory distress syndrome, ethanol and iron overload-induced disease of the ulcer, ischemia or reperfusion injury and a number of other injuries. Long term oxidative stress has been linked to cardiovascular disease because oxLDL appear to be a prerequisite for foam cell formation and atherogenesis. ROS have also been implicated in the induction of diabetes mellitus, age-related eye disease, and parkinsons’ disease.
C.4.4.2 Detection of free radicals

Direct detection of free radicals has been performed using electron spin resonance and spin trapping techniques. Although the electron spin resonance technique is suitable for detecting free radicals in solution chemistry, it has limited application to biological tissues owing to their usually high content of water. However, their problem can be overcome by the use of the spin trapping technique, which involves the conversion of highly reactive free radicals to relatively inert radicals, followed by electron spin resonance analysis (Jackson et al., 1999).

C.5 Antioxidant defense mechanism

These are the substances that protect other chemicals of the body from damaging oxidation reaction by reacting with free radicals and other ROS within the body. So, it protects the body from free radicals.

The body has developed several endogenous antioxidant systems to combat ROS. These include, enzymatic and nonenzymatic.

Enzymatic includes,

a) superoxide dismutase (SOD)
b) Catalase
c) Glutathione peroxidase
d) Malondialdehyde (MDA)
Nonenzymatic includes,

a) Vitamin A  
b) Vitamin E  
c) Vitamin C  
d) GSH

Mechanism of action of antioxidants

1) Chain breaking reactions e.g. α-tocopherol
2) Reducing the concentration of ROS e.g. Glutathione
3) Scavenging initiating radicals e.g. SOD
4) Chelating the transition metal catalysts

C.5.1 Antioxidant enzymes

1) Superoxide dismutase (SOD)

It is an endogenously produced enzyme present in every cell in the body. It appears in three forms,

a) Cu-Zn SOD – in cytoplasm
b) Mn-SOD – in mitochondrion  
c) Cu-SOD – extracellularly

\[2\text{O}_2 + 2\text{H} \xrightarrow{\text{SOD}} \text{H}_2\text{O}_2 + \text{O}_2\]

SOD is considered fundamental in the process of eliminating ROS by reducing (adding an electron to) superoxide to form \(\text{H}_2\text{O}_2\). SOD also exhibits antioxidant activity by reducing \(\text{O}_2\) that would lead to the reduction of \(\text{Fe}^{3+}\) to \(\text{Fe}^{2+}\) and thereby promote \(\text{OH}\) formation. It is
increased during stress condition. So, after giving high cholesterol diet, SOD activity is significantly increased.

Excessive superoxide inhibits glutathione peroxidase and catalase to modulate the equation from $H_2O_2$ to $H_2O$. Likewise, increased $H_2O_2$ slowly inactivates Cu-Zn-SOD. Meanwhile, catalase and glutathione peroxidase, by reducing $H_2O_2$, conserve SOD; and SOD, by reducing superoxide, conserves catalase and glutathione peroxidase.

2) Catalase

Present in most aerobic cells in animal tissues, especially concentrated in liver and erythrocytes

**M/A:** $2H_2O_2 \rightarrow 2H_2O + O_2$

An increase in the production of SOD without subsequent elevation of catalase or glutathione peroxidase leads to the accumulation of hydrogen peroxide, which gets converted into the hydroxyl radical.

3) Glutathione peroxidase

It has 4 atoms of selenium bound as seleno-cysteine moieties that confers the catalytic activity

$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$

$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$

Glutathione peroxidase reduces $H_2O_2$ to $H_2O$ by oxidizing glutathione (GSH). Re-reduction of the oxidized form of the glutathione (GSSG) is then catalysed by glutathione reductase. These enzymes also require trace metal co-factors for maximal efficiency, including Se
for glutathione peroxidase; copper, zinc, or manganese for SOD; and iron for catalase.

C.5.2 Effects of nutrients on free radical production and removal

C.5.2.1 Proteins

Amino acids are building blocks for the synthesis of proteins, including antioxidant enzymes. Some amino acids (e.g. arginine, citrulline, glycine, taurine, and histidine), small peptides (e.g. GSH and carnosine), and nitrogenous metabolites (e.g. creatinine and uric acid) directly scavenge oxygen free radicals. In addition, available evidence has shown that taurine and taurine chloramines inhibit inducible nitric oxide synthase (iNOS) expression and inducible NO synthesis in various cell types, including hepatocytes, macrophages, and glial cells (Wu and Meininger, 2002). Thus, a dietary deficiency of protein not only impairs the synthesis of antioxidant enzymes but also reduces tissue concentration of antioxidants, thereby resulting in a compromised antioxidant status (Sies et al., 1999).

On the contrary, high protein diets may lead to oxidative stress in human on the basis of the following considerations

i) Homocysteine, an independent risk factor for cardiovascular disease, increases endothelial superoxide production and induces oxidative stress in the vasculature

ii) Increasing protein intake has recently been shown to stimulate the generation of ROS and lipid peroxidation in human...
polymorphonuclear leukocytes and mononuclear cells (Mohanty et al., 2002)

iii) Increasing dietary protein intake increases whole body NO production by constitutive and inducible NOS in rats.

C.5.2 Lipids

Polyunsaturated fatty acids (PUFA) are prone to be oxidized by free radicals and other ROS (Henning et al., 2001). Thus, high intake of PUFA may render the organism more susceptible to lipid peroxidation, which may be alleviated by dietary supplementation of antioxidants such as Vitamin C, Vitamin E, and carotenoids.

Increasing extracellular concentration of fatty acids and LDL induces iNOS expression in many cell types including pancreatic β cells, VSMCs, and macrophages. Similarly, feeding a high saturated fat diet to rats increases iNOS activity in liver and colon (Wan et al., 2000) and stimulates free radical production and oxidative damage in skeletal muscle mitochondria and the whole body (Sreekumar et al., 2002).

Epidemiologic studies have shown that consumption of fish oil, rich in ω-3 PUFA, reduces the risk of cardiovascular disease in human (Brown et al., 2001).

This effect of fish oil results in part from an inhibition of lipogenesis and stimulation of fatty acid oxidation in the liver. Interestingly, like other PUFAs, fish oil can be easily peroxidized to form hydroperoxides and would increase oxidative stress. To
understand this apparent paradox of fish oil, recent work has been shown that, in contrast to \( \omega-6 \) PUFAs, \( \omega-3 \) PUFAs are inhibitors of free radical generation (Takahashi et al., 2002). Takahashi et al (2002) reported that long term feeding of a diet rich in fish oil increases the expression of antioxidant genes in mouse liver and upregulate the expression of lipid catabolism genes. In addition, \( \omega-3 \) PUFAs inhibit iNOS expression and inducible NO synthesis by cytokine activated macrophages (Khair-Eldin et al., 1996). Thus, \( \omega-3 \) PUFA exerts its beneficial effect on cardiovascular function through two mechanisms

- a) By decreasing plasma triacylglycerol concentration
- b) By inhibiting free radical production

C.5.2.3 Vitamins

Many vitamins inhibit NO production by iNOS, in support of their known antiatherogenic and anti-neuroinflammatory roles e.g. Vitamin A inhibits iNOS gene transcription in VSMCs, endothelial cells (Grosjean et al., 2001), cardiac myocyte, and mesangial cells (Datta et al., 1999). So, by reducing NO generation by iNOS, these vitamins play an important role in preventing radical induced cytotoxicity.

Vitamins also directly scavenge ROS and upregulate the activities of antioxidant enzymes. Among them, Vitamin E inhibits ROS, induced generation of lipid peroxyl radicals, thereby protecting cells from peroxidation of PUFA in membrane phospholipids, from oxidative damage of plasma VLDL, cellular proteins, DNA and from membrane degeneration.
Consequently, a dietary deficiency of Vitamin E reduces the activities of hepatic catalase, GSHPx and glutathione reductase (Chow et al., 1969) induces liver lipid peroxidation and causes neurologic and cardiovascular disorders (Carr et al., 2000) all of which can be reversed by dietary Vitamin E supplementation. In support of the critical antioxidant role of Vitamin E, Yokota et al., (2001) demonstrated that increase in brain lipid peroxidation and neurodegeneration in mice with a deficiency of α-tocopherol transfer protein.

By serving as components of NADP+/NADPH, NAD+/NADH, and FAD/FADH2−, nicotinamide and riboflavin play an important roles in protecting organisms from oxidative stress (Aruoma et al., 1998). NADPH and FAD are cofactors for glutathione reductase, the major enzyme responsible for the regeneration of GSH from GS-SG. In addition, NADPH is tightly bound to catalase and is thus necessary to maintain the enzyme function. Further, NADPH is required for the production of endothelial NO, (Wu et al., 2001) and physiologic concentration of NO inhibit superoxide production by vascular endothelial cells.

C.5.2.4 Minerals

The role of minerals in enzyme function has been studied extensively in nutrition and biochemistry e.g. Mg++ is a cofactor for glucose 6-phosphate dehydrogenase (G6PD) and 6 phosphogluconate dehydrogenase, two pentose cycle enzymes catalyzing the production
of NADPH from NADP⁺. Thus, a deficiency of dietary Mg⁺⁺ reduces glutathione reductase activity and results in radical induced protein oxidation and marked lesions in tissues (e.g. skeletal muscle, brain and kidney) (Mickel et al., 1993; Rock et al., 1995).

Iron is the most abundant trace element in the body, and almost all iron occurs bound to proteins. Fe⁺⁺ participates in the generation of free radicals. Thus, an increase in extracellular or intracellular iron concentration, which can result from dietary protein deficiency, dietary iron loading, low concentration of iron-binding proteins, or cell injury, promotes ROS production, lipid peroxidation and oxidative stress. Increased extracellular concentration of non heme iron also enhances iNOS protein expression and inducible NO synthesis in many cell types.

Cu and Zn and Mn are indispensable metals for the activities of Cu, Zn-SOD & Mn-SOD respectively. Therefore, a dietary deficiency of these minerals markedly reduces tissue Cu, Zn-SOD & Mn-SOD activities and result in peroxidative damage and mitochondrial dysfunction. A deficiency of Cu or Zn in rats also enhances Cyp450 activity in microsomes of liver and lung, stimulates ROS generation, and increased intestinal iNOS expression (Hammermueller et al., 1984; Wepnir et al., 2000).

Since the discovery of GSHPx as a selenium-dependent enzyme, selenium has been identified as an essential cofactor for selenoprotein P and other selenoproteins. Strikingly, a dietary deficiency of selenium
markedly reduces tissue GSHPx activity by 90% and result in peroxidative damage and mitochondrial dysfunction.

C.6 Implications of Homocysteine in Coronary Artery Disease (CAD)

Homocystein is a nonprotein-forming, thiol containing amino acid formed by demethylation of methionine. It is metabolized by remethylation to methionine or by transsulfuration to cysteine. An elevated plasma homocysteine level may occur as a result of inherited disorders, which alter enzyme activity in the transsulfuration and remethylation pathways. Alternatively, nutritional deficiencies of essential cofactors or enzyme substrates, including cobalamin (vitamin B\textsubscript{12}), folate, or pyridoxine (vitamin B\textsubscript{6}), can result in blockade of homocysteine metabolic pathways. An elevated plasma homocysteine level has been established as an independent risk factor for thrombosis and vascular disease (Clarke et al., 1991; Boushey et al., 1995; Motulski et al., 1996; Wald et al., 1998).

If homocystinuria is untreated, about 50 percent of patients have thromboembolic events, and mortality is about 20 percent before the age of 30 years (Hong et al., 1997). Observations in patients with homocystinuria (Gerritson et al., 1962) led to the idea that homocysteine may be involved in the pathogenesis of arteriosclerosis (McCully et al., 1969) and prompted a large number of epidemiologic
studies of the relation between moderately elevated homocysteine levels and vascular disease.

The prevailing view of the pathogenesis of coronary heart disease involves a slow progression of coronary atherosclerosis, followed by unstable angina, myocardial infarction, or sudden death. The acute event is frequently due to rupture or erosion of an atherosclerotic plaque with associated thrombus formation (Fuster 1992). There is increasing evidence that homocysteine may affect the coagulation system and the resistance of the endothelium to thrombosis (Malinow 1994) and that it may interfere with the vasodilator and antithrombotic functions of nitric oxide (Stamler et al., 1996).

Homocystinuric children are known to develop premature vascular disease involving all major blood vessels (Carson et al., 1965; Schimke et al., 1965). McCully first drew attention to a possible link between elevated plasma homocysteine and vascular disease, making the seminal observation that extensive arterial thrombosis and atherosclerosis commonly occurs in children with homocystinuria. Boers et al., (1985) highlighted the association between accelerated vascular disease and moderate elevation in plasma homocysteine, without the other manifestations of homocystinuria. Since then, there has been considerable interest in mild hyperhomocysteine as a risk factor for coronary artery disease, stroke, and peripheral vascular disease.
Fig. C.6 Homocysteine formation and metabolism

C.6.1 Formation of Homocysteine

Methionine, a protein, is converted to homocysteine through intermediates viz: S-adenosyl-methionine and S-adenosyl-homocystein. Homocystein irreversibly condenses with serine to form cystathione. This reaction is catalyzed by cystathione β synthase (CBS) and is also dependent on pyridoxal-5'-phosphate, which is an active metabolite of vitamin B₆. Cystathione is metabolized to cysteine by the enzyme cystathionase, which is also a vitamin B₆ dependent reaction.
TABLE C.6 Causes of elevated plasma homocysteine levels

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<th>Nutritional deficiencies</th>
<th>Medications</th>
<th>Disease states</th>
<th>Genetic</th>
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<td>Folate</td>
<td>Methotrexate</td>
<td>Chronic renal failure</td>
<td>Mutation in MTHFR</td>
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<td>Vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>Phenytoin</td>
<td>Acute lymphoblastic leukemia</td>
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<td>and carbamazepine</td>
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<td>Vitamin B&lt;sub&gt;6&lt;/sub&gt;</td>
<td>Nitrous oxide</td>
<td>Malignancies</td>
<td>Defective vitamin B&lt;sub&gt;12&lt;/sub&gt; transport</td>
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<td>Colestipol</td>
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<td>Theophylline</td>
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Lussier-Cacan et al., (1996) studied a large number of healthy men and women, excluding individuals with major and common disorders. They determined that gender was a major determinant of fasting plasma homocysteine concentration and that woman had a 21% lower concentration than men. The gender difference in homocysteine concentrations between men and women persist in elderly persons, although postmenopausal women have higher concentrations than premenopausal women. Plasma homocysteine
concentrations increase with age and remain an independent risk factor for vascular disease in the elderly (von Eckardstein et al., 1994). The marginal folate and other vitamin deficiencies known to be common in the elderly are likely to be contributing factors to hyperhomocysteinemia (Selhub et al. 1993; Joosten et al., 1993). There are significant negative correlations between plasma homocysteine and serum folate and vitamin \textit{B}_{12} concentrations. Plasma homocysteine was also highest in individuals in the lowest quartile of serum pyridoxal-5-phosphate, although this active metabolite of vitamin \textit{B}_{6} is more important in determining postmethionine load plasma homocysteine than fasting homocysteine (Lussier-Caccan et al., 1996).

Positive correlations have also been found between plasma homocysteine and uric acid and creatinine concentrations that may be related to the links between homocysteine metabolism with those of creatinine and uric acid (Jacobsen et al., 1996). Plasma albumin concentration also correlates with plasma homocysteine and may reflect an increase in protein-bound homocysteine.

In the Hordaland homocysteine study, elevated plasma homocysteine was associated with male gender, increasing age, smoking, hypertension, elevated cholesterol, and lack of exercise (Nygard et al., 1995). In a multivariate analysis, Malinow et al., 1995 demonstrated that systolic blood pressure, plasma uric acid, and hematocrit were predictors of concentrations of plasma homocysteine in men who did not have a history of atherosclerotic disease. It is
possible that homocysteine in plasma is an "acute phase reactant," rising after vascular injury. Plasma homocysteine concentrations rise acutely after a stroke and then decrease over several weeks (Lindgren et al., 1995). In contrast, plasma homocysteine concentrations tend to be lower immediately after a myocardial infarction (MI) than 6 weeks later (Landgren et al., 1995). The reason for this discrepancy is not clear. Several other disease states and medications also cause elevations in plasma homocysteine. The role of genetic mutations, acute events, nutritional status, and hormonal effects are discussed below.

**Folate**

There is controversy about the exact amount of supplemental folic acid that is required to reduce plasma homocysteine. Shimakawa et al., (1997) reported that people who use multivitamin supplements have significantly lower plasma homocysteine concentrations than nonusers. The recommended dietary allowance for folate in the United States is 200 mg/day. It has been suggested that an intake of 400 mg of folic acid above the dietary level will prevent birth defects. Such an increased supplementation will also significantly decrease plasma homocysteine concentrations in most of the population (Garg et al., 1997).

**Vitamin B\textsubscript{12}**

The importance of vitamin B\textsubscript{12} in the remethylation of homocysteine to methionine is well recognized, and hyperhomocysteinemia is a feature of vitamin B\textsubscript{12} deficiency (Joosten
et al., 1993; Ma et al., 1996). However, the relationship between vitamin B$_{12}$ intake/plasma levels and hyperhomocysteinemia related cardiovascular disease is less well defined. In the Framingham study, plasma homocysteine exhibited a strong inverse association with plasma folate but a weaker association with plasma vitamin B$_{12}$. Subjects in the lowest decile of plasma B$_{12}$ had significantly higher plasma homocysteine when compared with those in the highest decile (Selhub et al., 1993). Homocysteine was also inversely associated with intakes of folate and vitamin B$_6$, but not vitamin B$_{12}$. Many of the case control studies of hyperhomocysteinemia in patients with vascular disease have excluded subjects with vitamin B$_{12}$ deficiency. Treatment of patients with hyperhomocysteinemia with vitamin B$_{12}$ seems to have very little impact on plasma homocysteine.

**Estrogen**

Several investigators have examined the effect of menopausal status on plasma homocysteine. Wouters et al., (1995) measured fasting and post methionine plasma homocysteine concentrations in premenopausal and postmenopausal healthy women without a history of vascular disease. Fasting and post methionine plasma homocysteine was significantly higher in postmenopausal women as compared with premenopausal women. The difference appears to be too large to be explained as an effect of age alone [the increase in plasma homocysteine over a decade of life is modest] and is more likely to be related to hormonal status.
The rise in homocysteine levels after menopause may partly explain the sharp rise in cardiovascular disease that occurs in this age group and its attenuation by hormone replacement therapy (HRT). However, many cardiovascular risk factors improve with estrogen and further investigation is required to determine whether the lower incidence of vascular disease in premenopausal women and in women taking HRT may be related to the lower concentrations of plasma. In a prospective study, van der Mooren et al (1994) measured fasting serum homocysteine during HRT in postmenopausal women. The mean serum homocysteine decreased by approximately 11% with a greater decrease (17%) in those women who had a high homocysteine level before treatment with very little change in those who had a low level. There are well recognized mechanisms to explain the beneficial effect of HRT on cardiovascular risk, including effects on lipids and fibrinolysis. However, it is possible that a lowering of plasma homocysteine also contributes to this benefit.

Oral contraceptive agents, in contrast to HRT, do not affect biochemical folate indices and homocysteine concentrations in young women (Green et al., 1998). Tamoxifen, an estrogen antagonist with partial agonist activity, decreased plasma homocysteine by a mean of 30% after 9–12 months treatment in postmenopausal women with breast cancer (Anker et al., 1995). These changes were independent of the tumor burden. These data, in combination with the effect of estrogen, suggest that there is an estrogen receptor-mediated homocysteine-lowering effect. In addition, there may be an indirect
effect related to a modest elevation in plasma folate concentrations with tamoxifen (Lien et al., 1997).

C.6.2 Genetic Defects in Homocysteine Metabolism

Elevations in plasma homocysteine are typically caused either by genetic defects in the enzymes involved in homocysteine metabolism or by nutritional deficiencies in vitamin cofactors. Homocystinuria and severe hyperhomocysteinemia are caused by rare inborn errors of metabolism resulting in marked elevations of plasma and urine homocysteine concentrations. Cystathionine $\beta$-synthase (CBS) deficiency is the most common genetic cause of severe hyperhomocysteinemia. The homozygous form of this disease — congenital homocystinuria — can be associated with plasma homocysteine concentrations of up to 400 $\mu$mol per liter during fasting (Mudd et al., 1995). A homozygous deficiency of $N_5,N_10$-methylenetetrahydrofolate reductase, the enzyme involved in the vitamin $B_{12}$-dependent remethylation of homocysteine to methionine, may also lead to severe hyperhomocysteinemia (Mudd et al., 1972). Patients with this type of deficiency tend to have a worse prognosis than those with cystathionine $\beta$-synthase deficiency, in part because of the complete lack of effective therapy (Erbe et al., 1986; D'Angelo et al., 1997).
Pathological features of Homocysteine

![Diagram of the pathological features of Homocysteine]

**Fig. C.6.3 Impact of homocysteine**

Homocysteine is rapidly auto-oxidized when added to plasma, forming homocysteine, mixed disulfides, and homocysteine thiolactone (Velury et al., 1988; Stamler et al., 1993; Andersson et al., 1995). Potent reactive oxygen species, including superoxide and hydrogen peroxide, are produced during the auto-oxidation of homocysteine, and hydrogen peroxide (along with the hydroxyl radical), in particular, has been implicated in the vascular toxicity of hyperhomocysteinemia (Welch et al., 1997). There is extensive evidence that homocysteine-
induced endothelial-cell injury in vitro is largely due to the generation of hydrogen peroxide (Wall et al., 1980; de Groot et al., 1983; Starkebaum et al., 1986). Harker and colleagues have proposed that homocysteine-induced endothelial cell injury mediated by hydrogen peroxide exposes the underlying matrix and smooth-muscle cells, which in turn proliferate and promote the activation of platelets and leukocytes.

Auto-oxidation of homocysteine produces other cytotoxic reactive oxygen species, including the superoxide anion radical and hydroxyl radical (Misra et al., 1974; Heinecke et al., 1988). Superoxide-dependent formation of the hydroxyl radical has been shown to initiate lipid peroxidation (Rowley et al., 1982), an effect that occurs at the level of the endothelial plasma membrane and within lipoprotein particles (Heinecke et al., 1987; Heinecke et al., 1988). Homocysteine auto-oxidation has been shown to support the oxidation of low-density lipoprotein through the generation of the superoxide anion radical.

The production of endothelial-derived nitric oxide is also adversely affected by homocysteine. It has previously shown that normal endothelial cells detoxify homocysteine by releasing nitric oxide, which combines with homocysteine in the presence of oxygen to form S-nitroso-homocysteine. Nitrosation of the sulfhydryl group of homocysteine inhibits sulfhydryl-dependent generation of hydrogen peroxide (Stamler et al., 1993). S-nitroso-homocysteine is also a potent platelet inhibitor and vasodilator (Stamler et al., 1992).
effect of nitric oxide is eventually compromised as long-term exposure to hyperhomocysteinemia damages the endothelium sufficiently to limit nitric oxide production. Impaired endothelial production of nitric oxide leaves the endothelium vulnerable to unopposed homocysteine-mediated oxidative injury. Homocysteine may also decrease the bioavailability of nitric oxide by impairing its synthesis (Loscalzo et al., 1996; Welch et al., 1997). Homocysteine promotes lipid peroxidation, which may subsequently decrease the expression of endothelial nitric oxide synthase and directly degrade nitric oxide (Chin et al., 1992; Liao et al., 1995; Blom et al., 1995). It has been shown that homocysteine suppresses the expression of cellular glutathione peroxidase by endothelial cells, and this effect promotes lipid peroxidation by the reactive oxygen species elaborated during the oxidation of homocysteine (Upcharch et al., 1997).

It has also been demonstrated that homocysteine increases nitric oxide production in vascular smooth muscle cells by activating the transcription factor NF-κB (Welch et al., 1998). It appears that NF-κB is activated by a homocysteine-generated reactive oxygen species. Since NF-κB/rel activity is essential for the proliferation of vascular smooth-muscle cells (Bellas et al., 1995), these data suggest that homocysteine-mediated activation of NF-κB contributes to the mitogenic effect of homocysteine. Homocysteine also directly damages the vascular matrix by affecting the biochemical and biosynthetic functions of vascular cells. Homocysteine thiolactone, a highly reactive anhydrous byproduct of homocysteine oxidation, combines with low-
density lipoprotein to form aggregates that are taken up by intimal macrophages and incorporated into foam cells within nascent atheromatous plaques (Naruszewicz et al., 1994).

It has been observed that multiplicative increase in the risk of vascular disease in the presence of traditional risk factors and hyperhomocysteinemia (Nygard et al., 1995) may in part be related to the effect of homocysteine on lipid peroxidation. The vascular cytotoxicity of oxidized low-density lipoprotein has been linked to its content of lipid peroxidation products (Morel et al., 1983; Hughes et al., 1994). Homocysteine increases the formation of highly atherogenic oxycholesterols, increases lipid peroxidation, and increases the oxidation of low density lipoprotein in vitro (Parthasarthy et al., 1987; Heinecke et al., 1993). These observations suggest a potential role for antioxidant therapy in ameliorating homocysteine-dependent oxidative vascular injury.
C.7 Role of Garlic in CAD

Normalization of abnormal lipids and lipoproteins, hypertension, inhibition of platelet aggregation, and an increase in antioxidant status are believed to improve cardiovascular disease. Garlic (*Allium sativum*) is believed to have originated in Central Asia. It is used universally as a flavoring agent, traditional medicine, and a functional food to enhance physical and mental health. The beneficial effects of garlic consumption in treating a wide variety of human diseases and disorders have been known for centuries; thus, garlic has acquired a special position in the folklore of many cultures as a formidable prophylactic and therapeutic medicinal agent. It is even cited in the Egyptian *Codex Ebers*, a 3,500-y-old document, as useful in the treatment of heart disorders, tumors, worms, bites, and other ailments (Rahman et al., 2001). Garlic is also reported to inhibit the pathogenesis of cardiovascular disease and to prevent cancer and other chronic diseases associated with aging (Rahman et al., 2003). Over the last one-quarter century the role of garlic in treating cardiovascular disease has received much attention.

The majority of garlic (65%) is water, and the bulk of the dry weight is composed of fructose-containing carbohydrates, followed by sulfur compounds, protein, fiber, and free amino acids (Lawson et al., 1996). It also contains high levels of saponins, phosphorus, potassium, sulfur, zinc, moderate levels of selenium and Vitamins A and C, and low levels of calcium, magnesium, sodium, iron,
manganese, and B-complex vitamins; garlic also has a high phenolic content (Vinson et al., 2001). A majority of the compounds present in garlic are water-soluble (97%) with small amounts of oil-soluble compounds also present (0.15–0.7%). Over the years different garlic preparations have been investigated for their prevention and treatment of cardiovascular disease both in vitro and in vivo.

Several studies have indicated that garlic and its constituents inhibit key enzymes involved in cholesterol and fatty acid synthesis (Gebhardt et al., 1993; Yeh et al., 1994; Liu et al., 2001; Yeh et al., 2001). Direct measurements of enzyme activity have indicated that garlic and various constituents inhibit human squalene monooxygenase and HMG-CoA reductase, enzymes involved in cholesterol biosynthesis (Gebhardt et al., 1993; Gupta et al., 2001). This inhibition of HMG-CoA reductase by garlic has also been confirmed in one study (Augusti et al., 2005). It has also been shown that the more water-soluble compounds like S-allylcysteine (SAC) present in aged garlic extract are less cytotoxic and more efficient in inhibiting cholesterol biosynthesis than the lipid-soluble sulfur compounds such as diallyl sulfide (Yeh et al., 2001).

Garlic has also been shown to inhibit the LDL oxidation by scavenging superoxide (ROS) and inhibiting the formation of lipid peroxides (Dillon et al., 2003). It has also been shown that garlic consumption leads to the inhibition of platelet aggregation (Steiner et al., 2001; Banerjee et al., 2002; Rahman et al., 2003).
Thus, garlic has been shown to inhibit enzymes involved in lipid synthesis and consequently reduces cholesterol level, decrease platelet aggregation, prevent lipid peroxidation of oxidized erythrocytes and LDL, and increase antioxidant status.

**C.8 Association of Adipose Tissue with CAD**

The Greek physician Hippocrates observed that “Sudden death is more common in those who are naturally fat than in the lean,” in 400 BC. Obese hypertrophic adipocytes and stromal cells within adipose tissue directly augment systemic inflammation. This increase in systemic inflammation mediates multiple pathogenic mechanisms in the well-known but poorly understood associations between obesity, cardiovascular pathology, and the comorbidities such as dyslipidemia, type 2 diabetes mellitus, hypertension, and the metabolic syndrome. Discovery of the contribution of adipose tissue toward inflammation during acute infections prompted the question as to whether this physiological response to infection may also be dysregulated in obesity. It was discovered that tumor necrosis factor-α (TNF-α) expression was upregulated in adipose tissue of obese mice (Hotamisligil et al., 1993).

A growing body of evidence demonstrates that increased adipose tissue mass contributes directly toward an increase in systemic inflammation. It has been reported a positive correlations between body mass and peripheral leukocyte counts (Nanji et al., 1985). Since then, a large number of studies have found that increased body mass
index (BMI) correlates with increases in systemic circulating levels of inflammatory proteins such as C-reactive protein (CRP), interleukin-6 (IL-6), platelet activator inhibitor-I (PAI-1), P-selectin, vascular cell adhesion molecule 1 (VCAM-1), fibrinogen, angiotensinogen, SAA3, and α1-acid glycoprotein. Considering that adipose and adipocytes produce all of these factors, it can be inferred that adipose itself is a large contributor to these systemic increases and coronary artery disease (Lin et al., 2000; Cottam et al., 2004).

Evidence for a connection between obesity and inflammation has also been found in the context of clinical weight loss studies. Whether the weight loss is attributable to decreased dietary intake, increased fuel use through exercise, liposuction, or bariatric surgery, loss of adipose tissue is associated with a decrease in markers of inflammation. Weight loss achieved through dietary intervention alone or diet and exercise resulted in decreased circulating IL-6, CRP, PAI-1, TNF-α, soluble TNF receptor, P-selectin, intercellular adhesion molecule-1 (ICAM-1), VCAM-1, and IL-18 in men and women of various age groups and BMIs (Heilbronn et al., 2001; Nicoletti et al., 2003).

In another form of dietary weight loss, gastric bypass surgery resulted in improved insulin sensitivity, amelioration of diabetes, and significant decreases in circulating IL-6 and CRP levels 14 months after the procedure (Kopp et al., 2003). Not only do these findings strengthen the evidence for the direct contribution of obese adipose tissue to systemic inflammation, they also imply that many of the
health benefits of weight loss are attributable to these decreases in inflammatory signals.

C.9 C-Reactive Protein (CRP) AND CAD

The first observations of the well-known association between CRP and cardiac risk was in 1954, when it was found that after myocardial infarction, there was a dramatic rise in circulating CRP levels and the amplitude of this rise correlated with poor prognosis (Kroop et al., 1954; Anzai et al., 1997). Subsequently, it was also found that preinfarct elevated CRP levels correlated with an increased risk of future cardiac events as well. A number of large, prospective epidemiologic studies have indicated that high sensitive CRP (hs-CRP) is a strong independent predictor of future cardiovascular events, including myocardial infarction, ischemic stroke, peripheral vascular disease, and sudden cardiac death among individuals without known cardiovascular disease (Ridker et al., 2001; Ridker et al., 2003). Research has focused on the use of hs-CRP, a marker of inflammation, in the detection of patients at increased risk for cardiovascular disease (CVD). Several prospective studies have demonstrated that hs-CRP is an independent predictor of future risk for cardiovascular events among healthy individuals, as well as among patients with acute coronary syndromes. In addition, because half of all cardiovascular events occur in persons with low to average levels of low-density lipoprotein cholesterol, hs-CRP may aid in identifying patients at high
risk for a first cardiovascular event who might otherwise be missed by lipid screening alone. Thus, hs-CRP is a potential adjunct for global risk assessment in the primary prevention of cardiovascular disease. The Centers for Disease Control and Prevention and the American Heart Association have therefore proposed joint guidelines for the use of hs-CRP in determining cardiovascular disease risk. C-reactive protein, an acute-phase reactant synthesized in the liver in response to the cytokine IL-6, is also a factor in the development of atherosclerotic plaque. Although CRP was initially believed to be only a marker of vascular inflammation, research indicates that it also plays an active role in atherogenesis (Blake et al., 2003; Ridker et al., 2003). It is detectable in the early stages of plaque development and is believed to be involved throughout the atherogenic process, facilitating everything from the initial recruitment of leukocytes to the arterial wall to the eventual rupture of the plaque.

Elevated CRP levels are unquestionably associated with increased risk of cardiovascular disease. Numerous additional studies have further strengthened the association of elevated CRP levels with nearly all the important cardiovascular risk factors, including insulin resistance and diabetes, metabolic syndrome, hypertension, smoking, and dyslipidemia (Saito et al., 2003). Numerous studies have demonstrated a linear relationship between circulating levels of CRP and CVD risk (Ridker et al., 2003). They revealed that elevated CRP levels in obese patients are not only prognostic for the development of
CVD but also predictive of the risk of progression to type 2 diabetes mellitus (Pradhan et al., 2001).

However, even before infarction, circulating CRP has atherogenic activities on vascular endothelium and smooth muscle. Elevated CRP levels have been associated with endothelial dysfunction in the form of inappropriate vascular constriction/relaxation. CRP has been shown to cause induction of endothelial adhesion proteins ICAM-1, VCAM-1, E-selectin, P-selectin, and angiotensin type 1 receptor (Pasceri et al., 2000; Verma et al., 2003). Additionally, CRP has been implicated in the activation of endothelial NF-κB, induction of endothelial IL-1β, PAI-1, IL-6, TNF-α, monocyte chemotactant protein-1 (MCP-1), endothelin-1, and tissue factor, and inhibition of endothelial NO synthase and NO signaling (Labarrere et al., 2004). Furthermore, many of these factors regulate CRP levels reciprocally; thus, even in the absence of tissue damage, CRP seems to be an amplifier of vascular inflammation. Increased expression of these adhesion proteins and cytokines results in increase in leukocyte adherence, chemotaxis, and extravasation into the inflamed subendothelial intima, with cellular inflammation and eventual foam cell accumulation. They also induce loss of endothelial and smooth muscle NO generation and proper vascular relaxation. Combined with increased vascular contractile signals by endothelin-1 and angiotensin II (AT-II), this results in inappropriate vascular contraction/relaxation and contributes to hypertension. Other effects include increased smooth muscle cell migration, proliferation, and vascular remodeling.
(Nickenig et al., 2002). The above mentioned effects on NO and induction of endothelin-1 and P-selectin have proaggregating effects on platelets (Ziccardi et al., 2002).

The earliest identifiable lesion is the fatty streak, an inflammatory lesion that consists of monocyte-derived macrophages (foam cells) and T lymphocytes. As a fatty streak progresses to an intermediate and advanced lesion, it forms a fibrous plaque—a process that involves a complex interaction between the endothelium, inflammatory cytokines, and numerous blood elements (Ross et al., 1999).

Calabro et al., (2003) have proposed that the smooth muscle cells of the human coronary arteries may also produce CRP as a local response to inflammatory cytokines. They further noted that this locally produced CRP may participate in the atherogenic process (Calabro et al., 2003). These multiple molecular mechanisms for vasculopathic effects by CRP may explain the central position of CRP within the context of cardiovascular risk factors.

**C-Reactive Protein Adds to Global Risk Scoring**

Using traditional risk factors, clinicians can predict approximately 50% to 60% of the variation in the absolute risk of a future coronary event in individual patients (Rader et al., 2000). The addition of hs-CRP to current strategies for global risk assessment, such as the Framingham Risk Score (FRS), may therefore have the
potential to increase the accuracy of cardiovascular risk prediction. Albert et al., (2003) demonstrated that hs-CRP levels are correlated with the calculated 10-year FRS in men, as well as in women not taking hormone replacement therapy. Data from the Augsburg cohort of the Monitoring Trends and Determinants in Cardiovascular Disease (MONICA) study also showed that hs-CRP enhances the assessment of global coronary risk as measured by the FRS, particularly in persons at intermediate risk for CHD (Koenig et al., 2004). In the Women's Health Study, both very low (<0.5 mg/L) and very high (>10 mg/L) levels of hs-CRP were useful for risk prediction across a full range of FRS (Ridker et al., 2004). Women with hs-CRP levels of less than 0.5 mg per liter had the lowest risk of future cardiovascular events. Women with hs-CRP levels of greater than 20 mg per liter had a risk almost 8 times higher (crude RR, 7.6; 95% CI, 4.7-12.1) than the women at lowest risk (Ridker et al., 2004).

**Guidelines for the use of hs-CRP in Risk Assessment**

In January 2003, joint guidelines from the Centers for Disease Control and Prevention (CDC) and the American Heart Association (AHA) named hs-CRP as the inflammatory marker of choice to assess cardiovascular risk (Pearson et al., 2004). The guidelines support the use of hs-CRP in primary prevention and set cutoff points according to relative risk categories: low risk (<1.0 mg/L), average risk (1.0-3.0 mg/L), and high risk (>3.0 mg/L). These cutoff points approximate the tertiles of hs-CRP observed in the adult population.
Ridker et al., (2003) suggested that the scope of hs-CRP values be extended from less than 0.5 mg per liter (very low) to greater than 10 mg per liter (very high). This extension of scope would provide clinicians with additional prognostic information on cardiovascular risk. The joint CDC-AHA guidelines state that the optimal use of hs-CRP is to help guide the evaluation and therapy for primary CHD prevention for patients at intermediate risk, as defined by NCEP ATP III (10%-20% CHD risk over 10 years) (NCEP Guidelines 2002). The joint guidelines also consider measurements of hs-CRP as a possible predictor of recurrent events in patients with stable coronary disease or acute coronary syndrome (ACS).

The use of hs-CRP as an adjunct to lipid screening in primary prevention is intended to improve global risk prediction in patients not clearly identified as being at high risk by cholesterol levels alone (Ridker et al., 2003). This adjustment to the screening procedure is especially important for individuals with low LDL-C levels (<130 mg/dL) but high hs-CRP levels (>3 mg/L), an often-overlooked high-risk group. Preliminary data suggest that patients with low LDL-C and high hs-CRP levels may benefit from pharmacologic intervention, preferably with statin therapy. In the NCEP ATP III guidelines, hs-CRP was included among the emerging risk factors whose presence might affect clinician recommendations for therapeutic options.
Statins, C-Reactive Protein, and Coronary Risk

Clinical trials have shown that statins reduce patient levels of CRP by 15% to 28% as early as six weeks after treatment begins, independent of the magnitude of reduction in LDL-C levels (Jialal et al., 2001; Albert et al., 2001; Ridker et al., 2001). Data from a posthoc analysis of the secondary prevention Cholesterol and Recurrent Events (CARE) trial suggest that risk reduction of coronary events is greatest among patients with high baseline levels of hs-CRP.38 In this analysis, the risk reduction attributable to pravastatin therapy among patients with high levels of hs-CRP and another marker of inflammation, serum amyloid A (SAA), was substantially greater (54%) than in patients with lower levels of hs-CRP and SAA (25%). This finding held true even though total cholesterol, LDL-C, high-density lipoprotein cholesterol (HDL-C), and triglyceride levels were almost identical in the two groups (Ridker et al., 1998).

An important aspect of many of the recent hs-CRP studies in patients with ACS has been the observed correlation between more powerful LDL-C lowering and greater reductions in hs-CRP. This relationship was demonstrated again in the Reversing Atherosclerosis with Aggressive Lipid Lowering (REVERSAL) study, in which atorvastatin, 80 mg, lowered LDL-C by 46.3% (versus 25.2% with pravastatin, 40 mg) and hs-CRP by 36.4% (versus 5.2% with pravastatin, 40 mg; P<.001 for both) (Nissen et al., 2004). Similarly, in the 16-week ANDROMEDA study of 509 patients, rosuvastatin, which
was approved by the US Food and Drug Administration in August 2003, was shown to lower hs-CRP levels by 34% and 40% at doses of 10 mg and 20 mg, respectively. These results compared with observed reductions of 21% and 34% with atorvastatin, 10 mg and 20 mg, respectively (Betteridge et al., 2004).

In brief, High-sensitivity C-reactive protein, a marker of inflammation, is a strong predictor of future cardiovascular events in individuals both with and without overt CVD. Some studies have suggested that hs-CRP may assist in stratifying risk in patients presenting with coronary artery disease, particularly those with ACS. Further studies examining hs-CRP levels may help elucidate new therapeutic strategies for the secondary prevention of CVD. High-sensitivity C-reactive protein also adds prognostic information to the calculated FRS in individuals without overt coronary disease. When combined with lipid screening, hs-CRP improves global risk prediction in patients who would otherwise not be identified for primary prevention by lipid assessment alone. Although statin therapy has been shown to benefit individuals with elevated hs-CRP levels, it is not known whether aggressive statin therapy can reduce the risk of a first cardiovascular event in persons with low LDL-C but high hs-CRP.
C.10 Insulin Resistance and Hyperlipidemia

Resistance to normal action of insulin is related to an excessive postprandial release of free fatty acids (FFAs) from the fat cells. High FFA levels block glucose oxidation, causing insulin resistance (Randle et al., 1963). The high flux of FFA in the liver is the most likely driver of hepatic overproduction of TG and apoB, thereby contributing to an elevation in the concentration of VLDL (Lewis et al., 1995). Furthermore, insulin resistance contributes to decreased lipoprotein lipase activity, resulting in a reduced clearance of TG-rich lipoproteins (Taskinen et al., 1995). High TG-rich lipoprotein concentrations increase the presence of small dense LDL and decrease the high-density lipoprotein (HDL) concentration. These reactions are mediated by cholesteryl-ester transfer protein and hepatic lipase (Yasmashita et al., 1988; Zambon et al., 1998). So insulin resistance may coincide with alterations in lipid metabolism, such as hypertriglyceridemia, increased apoB levels, low HDL levels, and a predominance of small dense LDL particles (Reaven et al., 1993).

C.11 Vitamin E and CAD

In recent years, there has been a surge of interest regarding the use of vitamins to prevent or treat disease. Vitamin E, in particular, has been studied for various conditions, including hot flushes, hypercholesterolemia, fibrocystic disease, and hemolytic anemia. This vitamin has also been promoted to increase sexual potency, diminish wound scarring, augment resistance to infection, and decrease aging
(Whitney et al., 1990; Gaby et al., 1991). Lately, researchers have placed specific emphasis on the effects of vitamin E in coronary artery disease. In the last 40 years, much progress has occurred in the management of CAD and mortality has decreased. This progress is likely due to the impact of drug therapy and lifestyle modifications on people with hypertension and hyperlipidaemia, as well as the widespread use of thrombolytic drugs, angioplasty, and coronary artery bypass surgery (Farmer et al., 1997; Hunink et al., 1997). Traditional risk factors, such as hypertension, hypercholesterolemia, and smoking, explain some but not all CAD risk (Hopkins et al., 1981; Heller et al., 1984). Recent studies provide insight into the contribution of antioxidants, particularly vitamin E, in CAD prevention and treatment.

Vitamin E exists as at least 8 naturally occurring compounds, including α, β, γ, and δ-tocopherol and α, β, γ, and δ-tocotrienol. Of which, α-tocopherol is the most active component in vitamin E and naturally occurs as levo isomer. Dietary vitamin E is expressed in milligrams of α-tocopherol equivalents. The recommended dietary allowances (RDA) of vitamin E according to the National Research Council are 10 and 8 mg daily of α-tocopherol equivalents for men and women, respectively (approximately 13.5 and 10.8 IU/d). In general, 60% of dietary vitamin E is derived from vegetable and seed oils, such as margarine, salad dressings, and shortenings. Soyabean and wheat germ oils are particularly high in vitamin E, while corn, cottonseed,
and sunflower oils are of intermediate content. Animal fats, such as butter and milk, contain negligible vitamin E, although eggs and liver are substantial sources of this nutrient. Grains, fruits, and leafy green vegetables account for the remaining dietary intake (Whitney et al., 1990; Gaby et al., 1991).

Although small, short-term studies using vitamin E supplementation (100-800 IU/d) have shown no evidence of toxicity (Diplock et al., 1995; Takamatsu et al., 1995). A study involving α-tocopherol supplementation (50 IU/d) and its effects on the incidence of cancer found a higher mortality rate due to hemorrhagic stroke in patients receiving α-tocopherol supplementation than in patients receiving placebo (7.8 Vs 5.2 deaths per 10000 person-years). Additionally, high vitamin E intake is contraindicated in patients with coagulation defects caused by vitamin K deficiency, as it may promote hemorrhage (Diplock et al., 1995). Therefore, caution should be exercised in patients receiving warfarin or who have a malabsorption syndrome that may decrease vitamin K absorption. While vitamin E supplementation is often stated to be safe, this finding is frequently based on either small, short-term studies or long-term studies with inadequate end points to address the health consequences of vitamin E supplementation.

It has been reported that vitamin E decreased coronary artery lesion progression in patients consuming 100 IU/d or more of supplemental vitamin E compared with patients who had lower vitamin E intake (Hodis et al., 1995). However, it has also been
reported that vitamin E supplementation had no effect on the recurrence or progression of angina or on the incidence of major coronary events, in patients with a history of angina (Rapola et al., 1998).

So, far studies suggest that increased intake of vitamin E may attenuate the development of CAD; however the optimum dosage, duration, and method of consumption (dietary versus supplemental) plays a vital role in its effect. A low fat diet with high intake of fruit and vegetable sources containing vitamin E should be emphasized for all patients. Vitamin E supplementation may be considered in those at increased risk for CAD or with documented CAD, although this should be weighed against possible unknown long term adverse effects. If vitamin E supplementation is initiated, dosage titration appears necessary to derive cardiovascular benefit.

C.12 Platelets Activation and Atherosclerosis

Platelets are essential for primary hemostasis and repair of the endothelium, but they also play a key role in the development of acute coronary syndromes and contribute to cerebrovascular events. In addition, they participate in the process of forming and extending atherosclerotic plaques. Atherosclerosis is a chronic inflammatory process, and inflammation is an important component of acute coronary syndromes. The relation between chronic and acute vascular inflammation is unclear, but platelets are a source of inflammatory mediators, and the activation of platelets by inflammatory triggers may
be a critical component of atherothrombosis (Ruggeri et al., 2002; Wagner et al., 2003).

![Diagram of Platelet Activation in CAD](image)

**Fig. C.12 (a) Role of Platelet activation in CAD**

Activated platelets release inflammatory and mitogenic substances into the microenvironment, primarily altering the chemotactic, adhesive, and proteolytic properties of the endothelium. Preformed platelet mediators, stored in granules, can be released immediately after platelet activation through a process of exocytosis triggered by increased intracellular calcium levels. Activated platelets are also capable of time-dependent synthesis of protein mediators, such as tissue factor and interleukin-1α. CD40 ligand is stored in the cytoplasm of resting platelets and rapidly presents on the surface after platelet activation. After cleavage, to generate a soluble, functional fragment (soluble CD40 ligand), the mediator is released into the extracellular environment, inducing inflammatory responses in the endothelium by binding CD40 on endothelial cells. P-selectin is released from platelet granules and binds to the P-selectin glycoprotein ligand 1 (PSGL-1) receptor on monocytes, enhancing the adhesion of the monocytes to vascular-cell adhesion molecule (VCAM) 1 and the other adhesins expressed on activated endothelial cells and inducing the production of tissue factor by monocytes. Activated platelets also release chemokines...
that trigger the recruitment of monocytes (e.g., regulated on activation normal T-cell expressed and secreted [RANTES]) or promote the differentiation of monocytes into macrophages (e.g., platelet factor 4), as well as matrix-degrading enzymes such as matrix metalloproteinase (MMP) 2 or 9. Interleukin-1α is a major mediator of platelet-induced activation of endothelial cells, causing enhanced chemokine release and up-regulation of endothelial adhesion molecules to promote the adhesion of neutrophils and monocytes to the endothelium. ICAM denotes intracellular adhesion molecule, mRNA messenger RNA, MCP-1 monocyte chemoattractant protein 1, and OH- hydroxyl radical.

**Platelets in Atherogenesis**

The evidence for a role of platelets in atherogenesis in humans is limited and largely indirect. Several platelet-derived chemokines and growth factors are detectable in atherosclerotic plaques (Pitsilos et al., 2003; Coppinger et al., 2004). Moreover, platelet activation is associated with increased wall thickness of the carotid artery and with progressive thickening of the artery in patients with type 2 diabetes mellitus (Koyama et al., 2003; Fateh-Moghadam et al., 2005). Persistent platelet activation, as reflected by enhanced excretion of thromboxane metabolites, has been reported in association with major cardiovascular risk factors that accelerate atherogenesis (Nowak et al., 1987; Davi et al., 1990; Davi et al., 1992; Di Minno et al., 1993; Minuz et al., 2002). These studies suggest that platelet activation links diverse metabolic and hemodynamic abnormalities to accelerated atherogenesis (Davi et al., 1997).
Isoprostane formation as a biochemical link between low-grade inflammation and platelet activation in human metabolic disorders.

A number of human metabolic disorders — hypercholesterolemia, diabetes mellitus, visceral obesity, and hyperhomocysteinemia — are associated with inflammatory signals generated from the metabolism of lipid, carbohydrate, and protein. Enhanced formation of reactive oxygen species leads to enhanced lipid peroxidation and free radical–catalyzed conversion of arachidonic acid into bioactive isoprostanes (e.g., 8-iso-prostaglandin F2 [8-iso-PGF2]) and other isoeicosanoids. Phospholipase-mediated release of these compounds from cell membranes and low-density lipoprotein particles triggers platelet adhesion and activation in the presence of low levels of other agonists. Several receptors for cytokines and oxidized lipids involved in the inflammatory response are also expressed on the platelet membrane. CD36, also known as glycoprotein IIIb, has been shown to interact with a variety of ligands. This receptor can take up oxidized LDL and thereby activate the platelet, causing the release of cytokines that amplify the inflammatory response and platelet activation. TP denotes thromboxane receptor, and TNF tumor necrosis factor.
C.13 Implications of Plasminogen Activator Inhibitor-I (PAI-1) in CAD

PAI-1 is a fast-acting inhibitor of plasminogen activation. It is produced by the vascular endothelium but is also present in platelets and is considered to be an important regulatory element in fibrinolysis and consequently in cardiovascular diseases (Kruithof et al., 1987).

PAI-1 is a linear glycoprotein that is composed of 379 amino acids and has a molecular weight of 48,000 (Kruithof et al., 1988). It binds rapidly to tissue-PA (t-PA) and to urinary-type plasminogen activator (u-PA), forming a stable complex with a ratio of 1:1 that is cleared from the circulation by hepatic cells (Lindahl et al., 1990; Owensby et al., 1991). The active form of PAI-1 is unstable, with a half-life of 30 minutes. Activated PAI-1 is the form synthesized in platelets as well as endothelial cells (Kooistra et al., 1986).

When platelets are stimulated by thrombin, PAI-1 is released on the platelet surface, protecting a blood clot from premature lysis. This mechanism causes a rapid local increase in the PAI-1 concentration in the circulation. Thrombin also stimulates the synthesis of PAI-1 in endothelial cells (Gelehrter et al., 1986).
The main coagulation reactions are divided into the intrinsic and extrinsic systems. Activation of factor XII on contact with a negatively charged surface initiates the intrinsic coagulation system. (The activated form of the factor is indicated by “a.”) The extrinsic coagulation system induces the formation of a complex composed of factor VII and tissue factor, which is released after tissue injury. Some of these reactions depend on calcium ions. Thrombin is formed by an enzyme complex called prothrombinase, composed of factor X, factor V, negatively charged phospholipids, and calcium ions. Intrinsic and extrinsic activation of the coagulation cascade leads to the generation of thrombin, the activation of fibrinogen, the release of fibrinopeptides, the formation of soluble fibrin, and finally, the formation of factor XIII-mediated, cross-linked, insoluble fibrin. The main fibrinolytic reactions involve the inhibition of fibrinolysis by plasminogen-activator inhibitor type 1 (PAI-1) and α-2 -antiplasmin. Fibrinolysis is initiated by tissue plasminogen activator (t-PA), urinary-type plasminogen activator (u-PA), and plasmin. Plasmin bound to the surface of fibrin initiates the lysis of insoluble, cross-linked fibrin, with the subsequent generation of fibrin-degradation products. Plasmin bound to the surface of fibrin is better protected from inhibition by α-2 -antiplasmin than is plasmin generated in the fluid phase.
PAI-1 in Coronary Artery Disease

The importance of the fibrinolytic system as a regulator of fibrin deposition in the vessel wall raises the question of the role of perturbations in this system in the development of vascular disease. In theory, at least, a decrease in fibrinolysis due to high plasma PAI-1 concentrations might be expected to result in an increase in the deposition of fibrin and subsequent formation of a thrombus. High plasma PAI-1 concentrations are indeed associated with various thrombotic disorders and are an independent risk factor for reinfarction in patients who have had a first myocardial infarction before the age of 45 years (Hamsten et al., 1985; Wiman et al., 1985; Auwerx et al., 1988; Margaglione et al., 1994; Thogersen et al., 1998). There is an association between the presence of coronary artery disease and low plasma fibrinolytic activity due to increased plasma PAI-1 concentrations (Francis et al., 1983).

Relation between PAI-1 and Cardiovascular Risk Factors

Fibrinolytic system in general, and PAI-1 in particular, have a role in the development of coronary artery disease is supported by the biologic characteristics of PAI-1 and the association between high plasma PAI-1 concentrations and other cardiovascular risk factors. Plasma concentrations of PAI-1 are lower during the day than at night, and it has been proposed that the higher incidence of myocardial infarction in the early morning hours could be due to higher plasma...
PAI-1 concentrations, and therefore lower fibrinolytic activity, at night (Andreotti et al., 1988; Angleton et al., 1989).

These data indicate the existence of an atherothrombotic syndrome associated with underlying insulin resistance and with familial segregation. Subjects with insulin resistance, whether they have normal glucose tolerance or diabetes, have high plasma PAI-1 concentrations (Juhan-Vague et al., 1989). Interventions that lower insulin resistance, such as weight loss, are invariably accompanied by a reduction in plasma PAI-1 concentrations (Svendsen et al., 1996). Clinical studies indicate that patients with coronary artery disease have both insulin resistance and high plasma PAI-1 concentrations (Bavenholm et al., 1995). These findings suggest that PAI-1 has a role in atherothrombotic disorders, principally through an association with other established risk factors in the presence of underlying insulin resistance.

![Diagram of Insulin resistance and abnormality in biochemical pathways](image)

**Fig. C.13 (b) Insulin resistance and abnormality in biochemical pathways**
C.14 Role of Apoptosis in CAD

Necrosis refers to a range of morphological changes resulting from the enzymatic digestion of the cell, the disruption of cellular membranes, and the denaturing of proteins that accompanies cell death. Apoptosis, in contrast, is a programmed, active, highly selective mechanism of cell death allowing for the removal of cells that are redundant or excessively damaged (Kerr et al., 1972). Apoptosis is initiated by a number of different stimuli, including DNA damage, intracellular damage, toxins, and extracellular signals (Wyllie et al., 1992; Sachs et al., 1996; Rowan et al., 1997). In multicellular organisms apoptosis is an essential component of development and cellular regulation (Risau et al., 1995; Haanen et al., 1996). Abnormal regulation of apoptosis can lead to disorders such as cancer, lymphocyte depletion in acquired immuno deficiency syndrome (AIDS), and atrophy or degeneration of tissues (Ameisen et al., 1994; Ellis et al., 1996). Apoptosis in both excessive and reduced amounts has pathological implications. Thus, control of the apoptotic mechanism may have significant therapeutic implications.

In the cardiovascular system, apoptosis has been recently found in association with ischemic and idiopathic dilated cardiomyopathies, myocardial cell death after infarction, arrhythmogenic right ventricular dysplasia, long-QT syndrome, and other conduction system disorders (Yao et al., 1996; Cheng et al., 1996; Mallat et al., 1996; James et al., 1996). Apoptosis has also been implicated as a prominent feature in coronary artery disease associated with advanced atherosclerosis and
transplant arteriopathy. These finding are supported by evidence of the increased expression of molecular markers of apoptosis in atherosclerotic tissue (Geng et al., 1995; Bochaton-Piallat et al., 1995; Dong et al., 1996; Mallat et al., 1997).

Vasoactive mediators that are altered in atherosclerosis, such as nitric oxide, endothelin, and angiotensin II, regulate vascular smooth muscle and endothelial cell apoptosis (Fukuo et al., 1996; Pollman et al., 1996; Shichiri et al., 1997; Dimmeler et al., 1997). Furthermore, inhibition of endothelin-1 by endothelin receptor antagonists increases apoptosis (Sharifi et al., 1997). The exact role of apoptosis in the pathophysiology of coronary disease is as yet unknown, but the association of the cardiovascular risk factors, hypertension and hypercholesterolemia, with increased apoptosis suggests that apoptosis may play a role in the pathophysiology of atherosclerosis. Additionally, apoptosis has been implicated in the pathophysiology of syndromes that develop from coronary atherosclerosis, including myocardial infarctions and heart failure.

In coronary atherosclerosis, cholesterol may be important in the induction of apoptosis. Although cholesterol itself has no direct angiototoxicity, it forms cholesterol oxides that have multiple toxic effects on the vasculature (Imai et al., 1980). Cholesterol oxides, including 7b-hydroxycholesterol, 7-ketocholesterol, 19-hydroxycholesterol, cholesterol 5a,- 6a-epoxide, and 25-hydroxycholesterol all promote the loss of cell adhesion and increase the rate of apoptosis in cultured endothelial cells (Lizard et al., 1996).
Cholesterol oxides promote disruption of actin microfilaments, most notably with the disappearance of stress fibers within the cell body (Palladini et al., 1996).

Additionally, oxidized LDL increases apoptosis in vitro in a dose-dependent fashion and is associated with increased caspase-3, 1 of the ICE-like proteases also known as CPP32 (Dimmeler et al., 1997). Caspase-3 cleaves actin in cell-free extracts, which may be a mechanism by which cholesterol oxides can cause apoptosis (Song et al., 1997). However, the role of apoptosis in hypercholesterolemia and early atherosclerosis in vivo remains to be proven. Vasoactive substances that are often altered in atherosclerosis are regulators of apoptosis. Nitric oxide, an important mediator of vasodilatation, platelet inhibition, and suppression of smooth muscle proliferation, upregulates Fas and induces apoptosis in vascular smooth muscle cells (Nishio et al., 1996). Other vasodilators such as atrial natriuretic peptide and C-type natriuretic peptide also induce apoptosis in vascular smooth muscle cells (Trindade et al., 1995). Additionally, angiotensin II, a mitogen and vasoconstricting peptide, antagonizes nitric oxide–induced apoptosis (Pollman et al., 1996). Another important vasoconstricting peptide, endothelin-1, also counterbalances apoptotic promoters, and endothelin receptor antagonism is associated with increased apoptosis (Shichiri et al., 1997; Sharifi et al., 1997). It may be speculated that the critical balance between vasodilators that are growth inhibitors and vasoconstrictors that are growth promoters may involve the apoptotic
process. Abnormalities in this balance associated with atherosclerosis may be a mechanism of atherosclerosis progression. Thus, it has been demonstrated that apoptosis is present in atherosclerotic and postangioplasty lesions and confirmed by the additional findings of increases in other known markers of apoptosis. Apoptosis may be enhanced through multiple mechanisms including those associated with hypertension and hypercholesterolemia. The essential regulatory system of cell proliferation and apoptosis is fundamentally important to mediate responses to injury and the subsequent pathological processes in the blood vessels. In summary, dysregulation of cell proliferation and apoptosis are clearly seen in multiple forms of vascular disease, including hypertension, transplant arteriopathy, and atherosclerosis. Apoptosis may play a significant role in the pathogenesis of coronary atherosclerosis and may be initiated by atherosclerotic risk factors. Previous studies support the hypothesis that a central balance between vasodilators with antimitogenic properties and vasoconstrictors with growth-promoting abilities is a major determinant of the response to injury and the effects of remodeling on the vessel wall. It may be speculated that one of the mechanisms by which this balance contributes to progression or repair in atherosclerosis is through regulation of cell apoptosis. A better understanding of the mediation of these events and a better knowledge of the role of proliferation and apoptosis in the pathophysiology of these disorders will further our understanding of
cardiovascular disease and will help us to tailor therapeutic options to some of these important regulatory mechanisms.

C.15 Lipoprotein Lipase (LPL) in Atherosclerosis

LPL that is bound to the components of extracellular matrix of the arterial intima can lead to retention of LDL in these structures by acting as a molecular bridge. Retention increases the residence time of LDL in the intimal matrix, thus allowing the particles to be modified more extensively than they would otherwise have been. During such modification, biologically active lipids, such as oxidized phospholipids, oxysterols, free fatty acids, and ceramide, are formed in the LDL particles. Free fatty acids and lysophosphatidylcholine are released from the modified particles to some extent and, when bound to albumin, may be transported to the intimal cells or, when the modified LDL particles are ingested by the cells, may be taken up by them with other modified lipids. The biologically active lipids may have several effects on the intimal endothelial cells, macrophages, and smooth muscle cells: they are able to trigger inflammatory reactions, and at high concentrations, they may even be cytotoxic. Finally, LPL also bridges native and modified lipoproteins to cell surface heparan sulfate proteoglycans and to various lipoprotein receptors on the cell surfaces and thus facilitates the uptake of lipoproteins by the intimal cells. According to one finding, LPL appears to cause selective uptake of cholesterol from LDL, a process that requires cell surface proteoglycans but is independent of lipoprotein receptors and LPL.
activity (Seo et al., 2000). During lipolysis of chylomicrons and VLDL by LPL, the formed free fatty acids have been shown to release the particles with LPL from the endothelial surface (Saxena et al., 1989), allowing the remnant particles to be transported to the liver or to extrahepatic tissues. In addition to LDL, IDL particles, small VLDL particles, and chylomicron remnants have also been shown to enter the arterial wall, where they have even been suggested to be preferentially retained (Nordestgaard et al., 1995; Proctor et al., 1998). In an elegant study, Rutledge et al showed that LPL increased the retention of VLDL in perfused arteries and that when both the surface and core of VLDL were followed; LPL appeared to generate surface remnants of VLDL that accumulated in arteries as "lakes." Interestingly, particles that closely resemble remnants of triglyceride-rich lipoproteins hydrolyzed by LPL in vitro have been isolated from the human arterial intima (Chung et al., 1994). This important finding provides strong supportive evidence for the view that LPL plays a role in the retention of remnants of triglyceride-rich lipoproteins in the arterial intima. Hydrolysis of VLDL by LPL generates free fatty acids, which are able to increase the permeability of the arteries to LDL (Rutledge et al., 1997), so tending to further promote the entry and retention of LDL. Finally, fatty acids resulting from lipolysis of VLDL by LPL have been shown to fuel phagocytosis by macrophages in the presence of low concentrations of glucose (Yin et al., 1997), to enhance the production of tumor necrosis factor-α by monocyte/macrophages (Mamputu et al., 1999), to cause proatherogenic changes in the
production of proteoglycans by smooth muscle cells (Olsson et al., 1999), and to induce synthesis of LPL by monocytes (Michaud et al., 2001). Finally, LPL may have some actions potentially relevant to the development of atherosclerosis that are more or less independent of direct interaction with lipoproteins. Thus, LPL can (1) enhance the adhesion of monocytes to the endothelium, presumably as a result of the ability of the dimeric LPL to bind heparan sulfate proteoglycans on endothelial and monocyte surfaces (Obunike et al., 1997; Mamputu et al., 1997), (2) enhance the production of proteoglycans by macrophages (Obunike et al., 2000), and (3) have a proliferative effect on vascular smooth muscle cells that is independent of the presence of lipoproteins (Mamputu et al., 2000).

C.16 Insulin like Growth Factor and Atherosclerosis

Insulin-like growth factors I and II (IGF-I and -II) are regular constituents of human blood plasma. Over the past decade, the functions of circulating IGFs have become clearer, but the actions of locally produced IGFs are still ill defined. Accumulating evidence now indicates that IGFs and their regulatory proteins, secreted by cells of the cardiovascular system, are growth promoters for arterial cells and mediators of cardiovascular diseases (Cercek et al., 1991; Delafontaine 1995; Grant et al., 1996; Wilson et al., 1996). Dysregulated actions of these factors contribute to coronary atherosclerosis and restenosis.

Systemic IGF-I and IGF-II levels are determined mainly by production in the liver. However, many cells of the body synthesize
these growth factors (Daughaday et al., 1989). The IGFs have a broad range of physiological actions starting with early embryonic development and extending throughout life. Metabolic functions, particularly glucose metabolism, constitute an important aspect of IGF-I and -II activities (Jacob et al., 1989). The IGFs also induce differentiated functions of cells stimulating amino acid uptake and protein synthesis (Boulware et al., 1992), and promoting migration (Bornfeldt et al., 1993). Another prominent aspect of IGF-I is regulation of cell cycle progression and mitogenesis (Stiles et al., 1979; Baserga et al., 1993). IGFs may also function as survival factors by decreasing apoptosis in various cells (Parizaas et al., 1997).

The ultimate cell response to IGFs depends on the context of IGF binding proteins (IGFBPs). Six different IGFBPs have been identified. The IGFBP that carries most circulating IGF (90% in adult serum) is IGFBP-3 (Baxter et al., 1994). This complex restricts the extravascular transit and is a circulating store for IGFs. At the cellular level, IGFBPs form a binary complex with IGFs and critically modulate local IGF actions (Kelley et al., 1996).

Among the various growth factors involved in atherosclerotic plaque development, IGFs play a relevant role. The different cell types of atherosclerosis secrete IGFs, and type I IGF receptors are present on smooth muscle cells (Pfeifle et al., 1983), inflammatory cells (Hochberg et al., 1992), and arterial endothelial cells within the atherosclerotic lesion (Bar et al., 1984).
Activation of Vascular Smooth Muscle Cells (VSMCs)

VSMC dysregulation at atherosclerotic sites is associated with a shift from the so-called contractile-to-synthetic phenotype and displays many features of growth factor activation. Several in vitro studies of both animal (Clemmons et al., 1985; Delafontaine et al., 1991) and human VSMCs show that IGF-I induces cell cycle changes resulting in VSMC proliferation and migration. IGF-I and platelet-derived growth factor (PDGF) act synergistically to stimulate VSMC proliferation (Banskota et al., 1989). IGF growth-promoting effects are also interactive with the effects of angiotensin II and basic fibroblast growth factor (Clemmons et al., 1984; Delafontaine et al., 1993).
A study of severely atherosclerotic patients showed higher IGF-I mRNA expression in regions containing densely packed VSMCs within the active plaque, compared with lower levels found in stable plaques (Wilson et al., 1996). Some statin effects also are mediated by IGF-I, because lovastatin efficiently blocks intracellular signaling pathways activated by IGF-I and limit VSMC proliferation (Martinez-Gonzalez et al., 1997).

Migration of VSMCs from the media is a major pathologic vascular response leading to the development and progression of the lesions of atherosclerosis. IGFs are potent stimuli of VSMC migration.
and the effect appears to be mediated through type I IGF receptor (Gockerman et al., 1995). VSMC apoptosis occurs in the evolutionary process of atherosclerotic plaques (Bennet et al., 1995). This programmed cell death can be suppressed by high IGF-I and PDGF concentrations, a finding consistent with recent studies of IGF-I and PDGF as potent survival factors for rat VSMCs (Bennet et al., 1994). Likewise, constitutive overexpression of IGF-I prevents cell death of viable myocardium after infarction, limiting ventricular dilation, myocardial loading, and cardiac hypertrophy (Li et al., 1997).

Fig. C.16 (b) Pathological feature of IGF in atherogenesis

IGF axis elements are synthesized by the different cells of the atherosclerotic plaque acting in an autocrine/paracrine manner. IGFs stimulate VSMC proliferation, migration, and extracellular matrix synthesis. In macrophages, IGFs promote excess...
LDL cholesterol uptake, release of proinflammatory cytokines, and chemotaxis. This inflammatory environment digests the fibrous cap that overlies the lipid-rich core, thus leaving the plaque prone to rupture. IGFs also stimulate endothelial cell migration and organization, forming vascular conduits that may become newly formed capillary networks under additional angiogenic stimuli. SMC indicates smooth muscle cells; ACE-I, angiotensin-converting enzyme inhibitors; HMGCoA reductase-I, 3-hydroxy-3-methylglutharyl- coenzyme-A reductase inhibitors; and TNF-a, tumor necrosis factor-a.

**Macrophage Activation**

Macrophage accumulation is an early event in atherosclerosis. Macrophages are crucial in inflammatory processes associated with tissue injury through the ability to induce phagocytosis and to release proteases and cytokines (Noble et al., 1993). High-affinity type I IGF receptors on the macrophage surface allow IGFs to modulate macrophage concentrations at injury sites (Hochberg et al., 1992). Human macrophages also synthesize and secrete IGF-I (Nagaoka et al., 1990) and some of the binding proteins (Li et al., 1996). IGF-I secreted within the atherosclerotic lesion is important for monocyte chemotaxis, activation, and cytokine release (i.e., tumor necrosis factor-a) (Renier et al., 1996). It is likely that macrophage-derived IGF enhances cellular LDL uptake and degradation and also the macrophage cholesterol esterification rate (Hochberg et al., 1992).

**Angiogenesis**

Normal coronary arteries have no vessels within the inner media or intima. Angiogenesis occurs as part of the normal wound-healing process and also in atherosclerosis (Kumamoto et al., 1995; Kwoon et al., 1998). Many growth factors regulate angiogenesis, stimulating
migration, proliferation, proteolytic activity, and organizational behavior of endothelial cells (Folkman et al., 1992; Thompson et al., 1996). These angiogenic factors include basic fibroblast growth factor, vascular endothelial growth factor, transforming growth factor-β, and IGF-I (Grant et al., 1993; Nicosia et al., 1994). Endothelial cells from both capillaries and arteries possess specific receptors for IGF-I25 and secrete IGF-I and IGFBPs (specifically IGFBP-2, -3, and -4) (Delafontaine et al., 1995). Locally synthesized IGF-I at sites of lesion formation stimulates vascular injury repair promoting endothelial cell migration. IGF-I has a chemotactic action on vascular endothelial cells and induces endothelial tube-forming activity in vitro (Grant et al., 1987; Nicosia et al., 1994). These endothelial conduits receive the influence of other factors in the process of maturation to become fully organized capillaries.

Inflammatory angiogenesis occurs in atherogenesis and involves both endothelial cells and macrophages. Animal studies of inflammation-linked angiogenesis produced after microembolization of a coronary artery showed alterations in gene expression of IGF-I and the binding proteins 3, 5, and 6 in macrophages (Kluge et al., 1997).

In conclusion, alterations in the balance of the components of the IGF axis in the vessel wall influence the cell growth, survival, migration, and extracellular matrix synthesis that modulate atherosclerotic plaque progression and neointimal formation of restenosis. A better understanding of IGF axis dynamics could identify new targets to limit or prevent these vascular pathologies.
C.17 Proteases and CAD

Atherosclerosis and Restenosis

Vascular leukocyte and medial smooth muscle cell invasion, migration, and proliferation into the subendothelial space are major pathobiologic vascular hallmarks leading to initiation of atheromatous plaque. Similarly, intimal smooth muscle cell accumulation caused by invasion, proliferation, and migration from the media is the key to neointimal formation after balloon or stent angioplasty. Extracellular proteases (ECPs) are fundamental in these cellular actions as demonstrated by plasminogen activator and matrix metallo proteinase (MMPs) such as gelatinases, which increase markedly after artery injury during vascular smooth muscle cell proliferation and migration from the media to the intima (Bendeck et al., 1994; Reidy et al., 1996). Vascular lumen cells such as monocytes and lymphocytes use ECP to erode the basement membrane. Similarly, medial smooth muscle cells use these molecules to break the internal elastic lamina. Proteases are especially important in the latter because the internal elastic lamina is rich in elastin, one of the most resistant molecules to proteolysis. ECPs also induce intracellular signals for vascular cell migration and proliferation both by direct cell stimulation (such as urinary Plasminogen Activator (uPA and uPAr) or indirectly by modifying cell-cell or cell-matrix interactions such as laminin-5 cleavage by MMP-2 or liberation of E-cadherin by MMP-3 and MMP-7 (Odekon et al., 1992; Okada et al., 1996; Giannelli et al., 1997; Noe et al., 2001).
Protease implications at molecular, cellular, and clinical atherosclerotic disease.

Protease contribution to invasion, migration, and proliferation is supported by many experimental studies. Protease over-expression as with uPA (Plekhanova et al., 2001) and MMP-9 (Mason et al., 1999) after balloon angioplasty increase cell migration, proliferation, and neointimal formation. Conversely, protease deficiency such uPA and MMP-9 knockout mice show less neointimal formation after vascular injury (Carmeliet et al., 1997; Galis et al., 2002).

Apoptosis

Apoptosis contributes to plaque progression, rupture, and thrombus formation in atherosclerosis. Many circumstances involving ECP may trigger programmed cell death. Although the best apoptotic molecules known are the intracellular caspase family of cysteine proteases, extracellular MMPs can also mediate the initiation of apoptotic signaling intervening in Fas-mediated cell apoptosis (Kayagaki et al., 1995). ECPs also promote apoptosis by cleaving cell-matrix interactions, a curious phenomenon known as “anoikis,” as demonstrated for the plasminogen activation system in vascular smooth muscle cells (Meilhac et al., 2003). Smooth muscle cell apoptosis in plaque shoulder regions may contribute to acute coronary syndrome (ACS) by favoring plaque weakness because smooth muscle cells compete with proteases ECM degradation by secreting ECM and therefore increase fibrous capsule strength. Extracellular proteases, by
releasing apoptotic cytokines such tumor necrosis factor-α (TNF-α), also contribute to cell apoptosis.

**Extracellular Proteases in the Diagnosis and Prognosis of Arterial Disease**

Besides protease function and vascular effects, protease detection and quantitation in peripheral blood may help detect atheromatous disease stages and aid in clinical decision-making. Elevated tissue Plasminogen Activator (tPA) levels predict coronary artery disease events and stroke in healthy subjects, independent of established risk factors, as well as recurrent coronary events and cardiovascular death in patients with established coronary artery disease (Thompson et al., 1995; Thogersen et al., 1998).

Serum MMP-2 and MMP-9 are elevated in ACS, but not in stable angina (Kai et al., 1998). PAPP-A plasma levels deserve special consideration for detecting ACS. PAPP-A levels identify high risk patients since they are found elevated in ACS patients with normal troponins and indeterminate C-reactive levels (Bayes-Genis et al., 2001). Such patients might otherwise remain undiagnosed. Plasma PAPP-A has recently been demonstrated as a strong independent predictor of ischemic cardiac events and the need for revascularization in ACS patients with low troponin levels (Lund et al., 2003). Protease shedding may be fundamental for detecting atherosclerotic process molecules and may have value for diagnostic or prognostic information in apparently healthy and asymptomatic populations. Potential candidates are soluble intercellular adhesion molecule-1 or soluble P-
selectin, which when detected in peripheral blood shows some clinical usefulness (Ikeda et al., 1995; Ogawa et al., 1999). ECP detection and measurement in peripheral blood is finding use in other cardiovascular diseases, in which ECP such as MMP-2 and MMP-9 are found in chronic heart failure or patients affected by abdominal aortic aneurysms.

In brief, They participate not only in disease through abnormal tissue destruction as in extracellular matrix degrading molecules but also by direct effects on cells such as by modulating cell behavior and leading to cell proliferation, migration and invasion, apoptosis, and morphogenesis. In addition, ECP by acting on other proteins (modulating the inflammatory response, growth factors availability, or trans-membrane proteins) can indirectly modulate cell behavior. ECP modes of action are autocrine, paracrine, and endocrine. In addition to these complex and extended actions, antagonist effects as seen in inflammation, apoptosis, angiogenesis, or though shedding complicate the pathobiologic complexity of these molecules even more.

C.18 Estrogen and CAD

The incidence of cardiovascular disease differs significantly between men and women, in part because of differences in risk factors and hormones. The incidence of atherosclerotic diseases is low in premenopausal women, rises in postmenopausal women, and is reduced to premenopausal levels in postmenopausal women who receive estrogen therapy (Grady et al., 1992; Barrett-Connor et al.,
Until recently, the atheroprotective effects of estrogen were attributed principally to the hormone's effects on serum lipid concentrations. However, estrogen-induced alterations in serum lipids account for only approximately one third of the observed clinical benefits of estrogen (Bush et al., 1987; Mendelsohn et al., 1994). Reviews of the data suggest that the direct actions of estrogen on blood vessels contribute substantially to the cardiovascular protective effects of estrogen (Farhat et al., 1996).

Estrogen increases vasodilatation and inhibits the response of blood vessels to injury and the development of atherosclerosis. Estrogen-induced vasodilatation occurs 5 to 20 minutes after estrogen has been administered and is not dependent on changes in gene expression; this action of estrogen is sometimes referred to as "nongenomic." The estrogen-induced inhibition of the response to vascular injury and the preventive effect of estrogen against atherosclerosis occur over a period of hours or days after estrogen treatment and are dependent on changes in gene expression in the vascular tissues; these actions are sometimes referred to as "genomic."

Blood vessels are complex structures, with walls containing smooth-muscle cells and an endothelial cell lining. Vascular endothelial and smooth muscle cells bind estrogen with high affinity, and estrogen receptor α has been identified in both types of vascular cells in women and men, as well as in myocardial cells (Karas et al., 1994; Losordo et al., 1994; Venkov et al., 1996; Kim-Schulze et al., 1996; Caulin-Glaser et al., 1996; Grohe et al., 1997).
Vascular endothelial and smooth-muscle cells express the two known estrogen receptors. Estrogen has both rapid vasodilatory effects and longer-term actions that inhibit the response to vascular injury and prevent atherosclerosis. These effects are mediated by direct actions on vascular endothelial cells (red) and smooth-muscle cells (purple). The rapid effects of estrogen on the blood-vessel wall are believed to occur without any changes in gene expression (nongenomic effects), whereas the longer term effects involve changes in gene expression (genomic effects) mediated by the estrogen receptors, which are ligand-activated transcription factors.

Fig. C.18  Direct effects of Estrogen on Blood Vessels

Eestrogen

Endothelial cells

Smooth-muscle cells

Rapid effects
(nongenomic)

\[ \uparrow \text{Dilatation} \]

\[ \uparrow \text{Nitric oxide} \]

Longer-term effects
(genomic)

\[ \downarrow \text{Atherosclerosis} \]

\[ \downarrow \text{Vascular injury} \]

\[ \uparrow \text{Endothelial-cell growth} \]

\[ \downarrow \text{Smooth-muscle-cell growth} \]
C.18.1 Effects on Serum Lipoproteins

The effects of estrogen therapy on serum lipid concentrations result largely from estrogen-receptor–mediated effects on the hepatic expression of apoprotein genes. Many studies, including one large, randomized, controlled trial have documented that estrogen therapy in postmenopausal women decreases serum total cholesterol and LDL-C concentrations, increases serum HDL-C and triglyceride concentrations, and decreases serum lipoprotein (a) (Lp(a)) lipoprotein concentrations (Writing group). The route of administration of estrogen influences its effects on serum lipids. Transdermally administered estrogen has less of an effect on serum lipid concentrations than does orally administered estrogen. Coadministration of a progestin can blunt the changes in serum lipids due to estrogen; the magnitude of this effect depends on the specific progestin. Raloxifene has effects on serum lipid concentrations that are similar to but less pronounced than those of estrogen, but raloxifene may lower serum Lp(a) lipoprotein concentrations more. Whether raloxifene has cardiovascular protective effects remains uncertain and requires further study (Bjarnason et al., 1997; Clarkson et al., 1998; Walsh et al., 1998).

C.18.2 Effects on the Response to Vascular Injury and on Atherosclerosis

Estrogen accelerates endothelial cell growth in vitro and in vivo (Morales et al., 1995; Krasinski et al., 1997). The rapid re-endothelialization induced by estrogen after vascular injury may be
due in part to increased local expression of vascular endothelial growth factor (Krasinski et al., 1997). Estrogen also inhibits apoptosis of cultured human endothelial cells in an estrogen-receptor-dependent manner (Spyridopoulos et al., 1997). Early restoration of endothelial integrity by estrogen may contribute to the attenuation of the response to injury by increasing the availability of nitric oxide, which can directly inhibit the proliferation of smooth-muscle cells (Cornwell et al., 1994). Estrogen directly inhibits the migration and proliferation of smooth-muscle cells in vitro and, in some but not all studies, the expression of adhesion molecules by vascular cells (Cid et al., 1994; Kolodgie et al., 1996; Bhalla et al., 1997). Estrogen contributes to long-term vascular protection by inhibiting the proliferation of vascular smooth-muscle cells and accelerating the growth of endothelial cells.

Abundant evidence from both prospective and retrospective observational studies demonstrates that estrogen therapy reduces the primary risk of cardiovascular disease in previously healthy postmenopausal women by 35 to 50 percent (Grodstein et al., 1996; Grodstein et al., 1997). Less is known about the effects of combined therapy with estrogen and progestin in postmenopausal women, but one large study suggests that it too is beneficial. These observational data are extensive and consistent, but definitive evaluation of the efficacy of estrogen therapy for the primary prevention of coronary heart disease in women must await the results of ongoing randomized clinical trials such as the Women's Health Initiative. The few studies of
estrogen therapy as secondary prevention also support a cardiovascular benefit (O'Brien et al., 1996; Sullivan et al., 1997; O'Keefe et al., 1997). However, in the Heart and Estrogen/Progestin Replacement Study, the first randomized trial to examine the effect of therapy with conjugated equine estrogens plus medroxyprogesterone in postmenopausal women with coronary disease, treatment for an average of 4.1 years did not reduce the overall rate of events associated with coronary heart disease but increased the rates of thromboembolic events and gallbladder disease (Hulley et al., 1998). There was an increase in coronary events in the first year in the treated women and a decrease in coronary events in years 4 and 5. These findings may be due to the effects of the conjugated equine estrogens, medroxyprogesterone, or both on nonvascular tissues, such as hepatic effects.

C.19  Myopathy associated with Hypolipidemic agents

C.19.1  Statins associated Myopathy

As a class, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) have transformed the treatment of patients with lipid disorders and substantially altered the approach to primary and secondary prevention of coronary atherosclerotic events. Emerging data now suggest that statins offer significant benefits to an even broader range of patients at high risk for coronary heart disease (LaRosa et al., 1999; Serruys et al., 2002). In fact, high risk patients appear to benefit from statin therapy, regardless of their LDL-C levels.
Statin therapy has been proven both safe and well tolerated in millions of patients over nearly 15 years of clinical use. Thus, the issue of statin-related myopathy must be viewed in the context of the considerable potential benefits that long-term statin therapy offers to patients. While rare, myopathy and rhabdomyolysis have been reported for all statins (Omar et al., 2002), and fatal rhabdomyolysis has been reported for all statins except for fluvastatin (Staffa et al., 2002). Cerivastatin was voluntarily withdrawn from the global market by its manufacturer in 2001, raising concerns regarding the safety of the entire class (SoRelle et al., 2001). Relative to other statins, cerivastatin had a higher reporting rate for rhabdomyolysis, including fatalities, particularly at the highest recommended dosage (0.8 mg/d) or when it was taken in combination with gemfibrozil (Furberg et al., 2001; Staffa et al., 2002). In about 50% of all cases of statin related rhabdomyolysis, a drug-drug interaction was suspected (Omar et al., 2002). Changing concepts of who will benefit from statin therapy along with more aggressive treatment goals for lowering LDL-C will significantly enlarge the population that will receive statin therapy in the future (Wood et al., 1998). At the same time, the pool of patients who will benefit most from statin therapy are the same patients who may be at the greatest risk for myopathy. Patients at highest risk for CHD—regardless of their lipid profiles—include older individuals, patients after transplantation, and patients with hypertension, diabetes, or multivessel atherosclerotic disease. These individuals are also the most likely to need multiple medications and thus are at
greatest risk for drug-drug interactions while receiving statin therapy. Another group of patients, those with mixed hyperlipidemia, may benefit greatly from combination lipid lowering therapy. Yet combinations of statins and fibrates are known to increase the risk of myopathy. It has been reported in randomized controlled trials (RCTs) that there is no relationship between lowering cholesterol levels with statin therapy and increased mortality from any cause (Farmer et al., 2000; Serruys et al., 2002).

The reported rates of serious adverse events (SAEs) among statins as a class have been very low (<1%) and include a slight risk for elevation of liver enzymes and myopathy (Bottorff et al., 2000). The risk for reversible elevation of liver transaminases (defined by alanine aminotransferase and/or aspartate aminotransferase levels >3 times the upper limit of normal [ULN]) is approximately 1% for all statins (Maron et al., 2000).

At the outset of a discussion regarding drug-related myotoxicity, it is important to define 4 conditions: myalgia, elevated creatine kinase (CK) levels with or without symptoms, myopathy, and rhabdomyolysis. Myalgia is a patient-reported symptom of muscle soreness or pain that has been associated with the use of all statins and is also common among placebo treated patients. Although the physician must investigate any patient report of myalgia appropriately, most are not associated with any increase in CK values. Thus, statistical reports of myalgia found in clinical trial reports or product information are not helpful in determining risk for myopathy. Elevated CK levels are
biochemical markers of the muscle damage associated with myopathy from any cause. Reference values for total CK in adults vary by analytical method and reference population. In the clinical setting, asymptomatic elevations of CK level of less than 5×ULN may be considered benign, whereas elevations of 5 to 10×ULN require evaluation. Myopathy has traditionally been defined as CK level greater than 10×ULN with symptoms (e.g., generalized myalgia, fatigue, or weakness). However, the definition of myopathy varies among studies and reports of statin-related myopathy are not based on a consistent definition. Likewise, reports of rhabdomyolysis have been confounded by its varied definitions.

Rhabdomyolysis is a clinical syndrome that results from severe and widespread injury to skeletal muscle and the subsequent accumulation of toxic muscle products in the blood and urine (Omar et al., 2001). Although initially defined by the US Food and Drug Administration (FDA) as a CK level greater than 10000 U/L, more recently rhabdomyolysis has been defined by the FDA as an appropriate diagnosis only when organ damage (typically renal insufficiency) occurs in association with elevated CK levels (Pierce et al., 1990). Severe myopathy and rhabdomyolysis are often characterized by marked elevations of CK levels (often >100×ULN). Rhabdomyolysis is accompanied by findings such as myoglobinuria, myoglobinemia, and evidence of target-organ damage, such as decreased renal function or acute renal failure. If untreated, rhabdomyolysis may be fatal. Rhabdomyolysis may result from a wide
range of diseases and disorders. It's most frequent causes are alcohol abuse, excessive exercise, acute viral infections, major trauma, surgery, hypothyroidism, and numerous medications. Notably, the progression from myopathy to rhabdomyolysis can almost always be reversed by early diagnosis and treatment of symptomatic elevations of CK levels with adequate hydration and cessation of potentially offending drugs (Pierce et al., 1990; Omar et al., 2001).

The mechanism by which statins cause myopathy is not completely understood. However, the association appears to be dose dependent, and the risk is known to increase when statins are prescribed in combination with agents that are also myotoxic when used as monotherapy or increase the serum concentration of the statin. The risk is also enhanced in patients who have preexisting risks for myopathy, such as those mentioned previously, as well as in women and the elderly (Shek et al., 2001).

However, it has recently become evident that the magnitude of the problem was markedly greater for cerivastatin than for other statins. Cases of rhabdomyolysis involving the concomitant use of gemfibrozil and cerivastatin led to a change in cerivastatin prescribing information in 1999 to include an unequivocal warning against prescribing these 2 agents concurrently. In May 2001, a "Dear Doctor" letter was widely distributed, prohibiting the concomitant administration of the 2 agents (SoRelle et al., 2001). Nonetheless, additional cases of rhabdomyolysis with this combination continued to
be reported through 2001 (Alexandridis et al., 2000; Bruno-Joyce et al., 2001; Lau et al., 2001; Garcia et al., 2001).

Drug-drug interactions with statins are significantly more likely to be associated with myopathy compared with statin monotherapy. For example, one source reported that the incidence of myopathy for lovastatin monotherapy was 0.15%, but increased to 2%, 5%, and 28% in patients receiving concomitant niacin, niacin plus cyclosporine, or cyclosporine plus gemfibrozil, respectively (Tobert et al., 1988).

Most of the clinically important drug-drug interactions that occur with statins are attributable to the concurrent use of statins that are recognized by CYP3A4 and other agents that are potent inhibitors or substrates of this enzyme—in particular, the azole antifungals, some macrolide antibiotics, and cyclosporine. Other CYP3A4 substrate agents (e.g., the calcium channel antagonists) may compete for the enzyme, thereby also potentially increasing the serum concentration of the statin. An interaction also occurs between statins and coumarin anticoagulants (Corsini et al., 1999); the coadministration of statins to patients receiving warfarin causes a small increase in the anticoagulant effect of warfarin that requires monitoring of the international normalized ratio and potentially a reduction in warfarin dosage. The mechanism of the interaction between statins and warfarin is uncertain; given that both CYP3A4 and CYP2C9 isoenzymes are involved in the metabolism of warfarin, competition with statins at this level may be a contributing factor in the potentiation of warfarin effects (Corsini et al., 1999). Cases of
rhabdomyolysis have been reported with the combination of warfarin and any statin, but it is not clear whether these cases were due to a warfarin-statin interaction (Omar et al., 2002). However, it should be noted that the risk for myopathy also appears to increase when statins are combined with drugs that may not be metabolized via the CYP3A4 pathway, such as fibrates and niacin. Drug interactions at the excretion level might potentially occur as a consequence of competition for carrier-mediated transport across the bile canalicular membrane.

In addition, changes in the absorption and excretion of drugs independent of CYP metabolism can alter drug disposition and may contribute to the interaction potential of statins. A newly recognized class of active drug transporters, P-glycoproteins, are known to affect the disposition and bioavailability of many drugs including 3A4 substrates. P-glycoproteins are transmembrane proteins that function as drug efflux pumps that actively transport drugs from intestinal, renal, brain, and hepatic cells (Yu et al., 1999). Lovastatin and simvastatin are very potent and effective inhibitors of P-glycoprotein transport, whereas atorvastatin and pravastatin have less inhibitory activity (Christians et al., 1998; Wang et al., 2001). In contrast, fluvastatin is not a substrate of P-glycoprotein (Fischer et al., 1999).
C.19.2 Fibrates associated Myopathy

Monotherapy with fibrates appears to pose an independent risk for myopathy that is greater than the risk posed by statin monotherapy. Although rare, myopathy occurred more frequently in patients using either statins or fibrates than in the general population; however, current fibrate users were 5.5 times more likely to develop myopathy than were current statin users. Myopathy related to the statin-fibrate combination appears likely to occur by more than a single mechanism and does not always involve CYP3A4 pathways. For example, gemfibrozil was shown to increase plasma concentrations of lovastatin without inhibiting CYP3A4, whereas benzafibrate demonstrated no significant effect on the pharmacokinetics of lovastatin. Although all fibrates have been associated with cases of CK elevations and myopathy in combination with statins, the risk for the development of myopathy may be greater for gemfibrozil compared with benzafibrate or fenofibrate use (Kyrklund et al., 2001). Fibrate monotherapy may impair liver function independently; therefore, patients with impaired liver function should not receive combination statin-fibrate therapy. Furthermore, fibrates, which are excreted primarily through the kidneys, may increase the risk for myopathy in patients with even mild renal impairment.