Homocysteine in Occlusive Vascular Disease

3

3.1 Pathophysiology of Homocysteinemia-Induced Occlusive Vascular Disease

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

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

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

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

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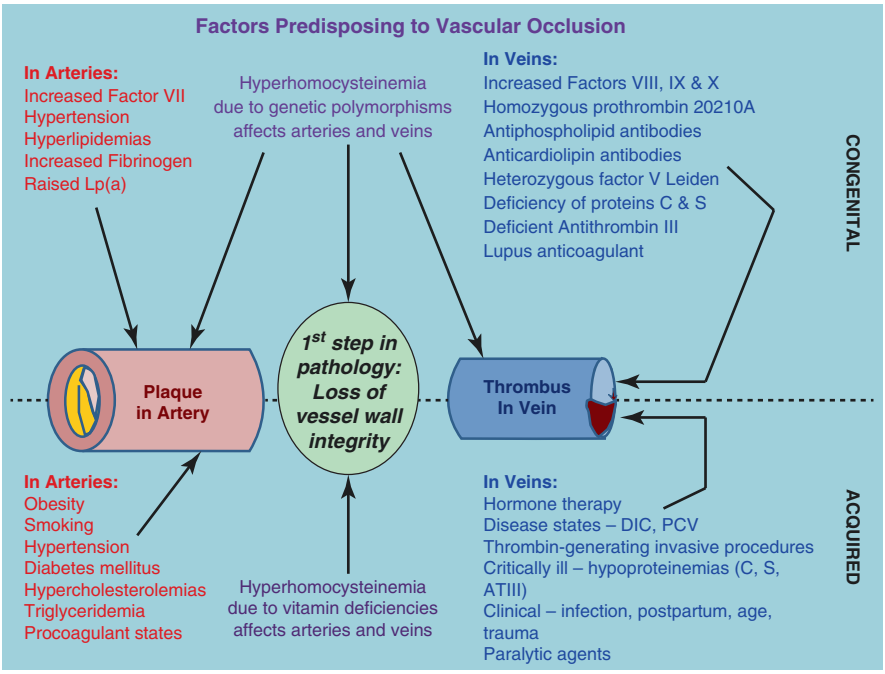

Fig. 3.1 Causes of occlusive disease of arteries and veins

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3.2 Homocysteine and Occlusive Vascular Disease

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

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

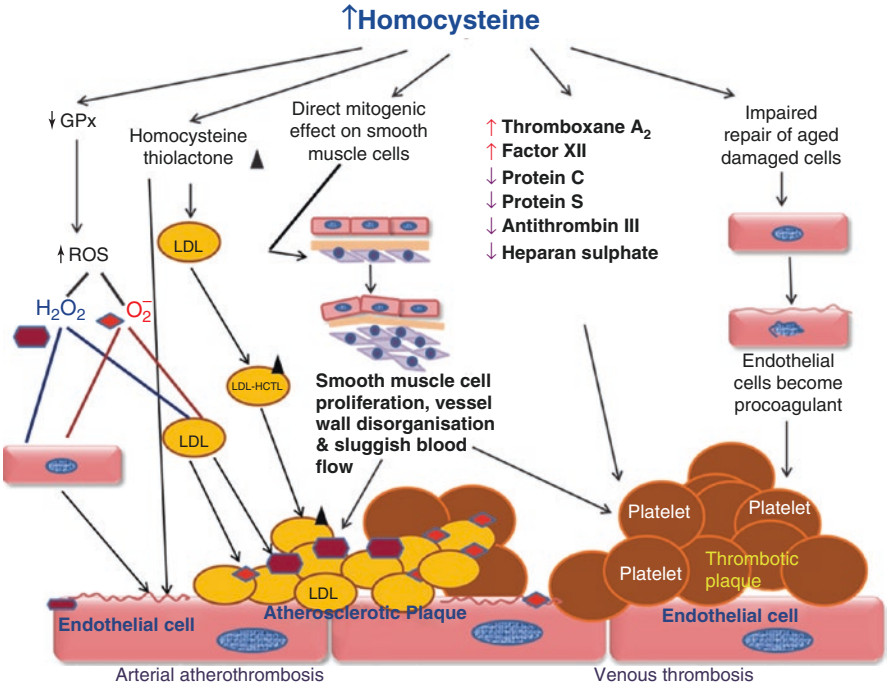

Fig. 3.2 Several mechanisms by which homocysteinemia causes atherothrombosis. *GPx* Glutathione peroxidase, *ROS* reactive oxygen species, H_2O_2 hydrogen peroxide, O_2^- nascent oxygen, *LDL* low-density lipoprotein cholesterol, *LDL-HCTL* homocysteinylated LDL, ↑ increased, ↓ decreased

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3.3 Homocysteine and Occlusive Vascular Disease in Indians

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

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

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

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

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

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

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

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

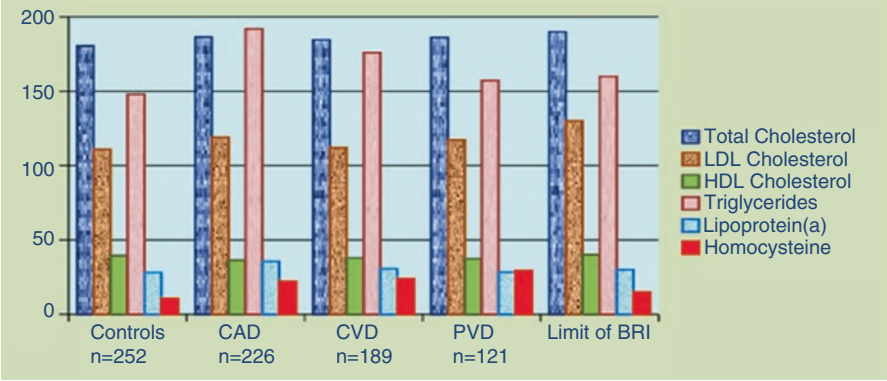

Fig. 3.3 The mean blood concentrations of cholesterols (mg/dL), triglycerides (mg/dL), lipoprotein(a) (mg/dL) and homocysteine (μmol/L) in Indian controls and patients of vascular disease. *CAD* Coronary artery disease, *CVD* cerebrovascular disease, *PVD* peripheral vascular disease, *BRI* biological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

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

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

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

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

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

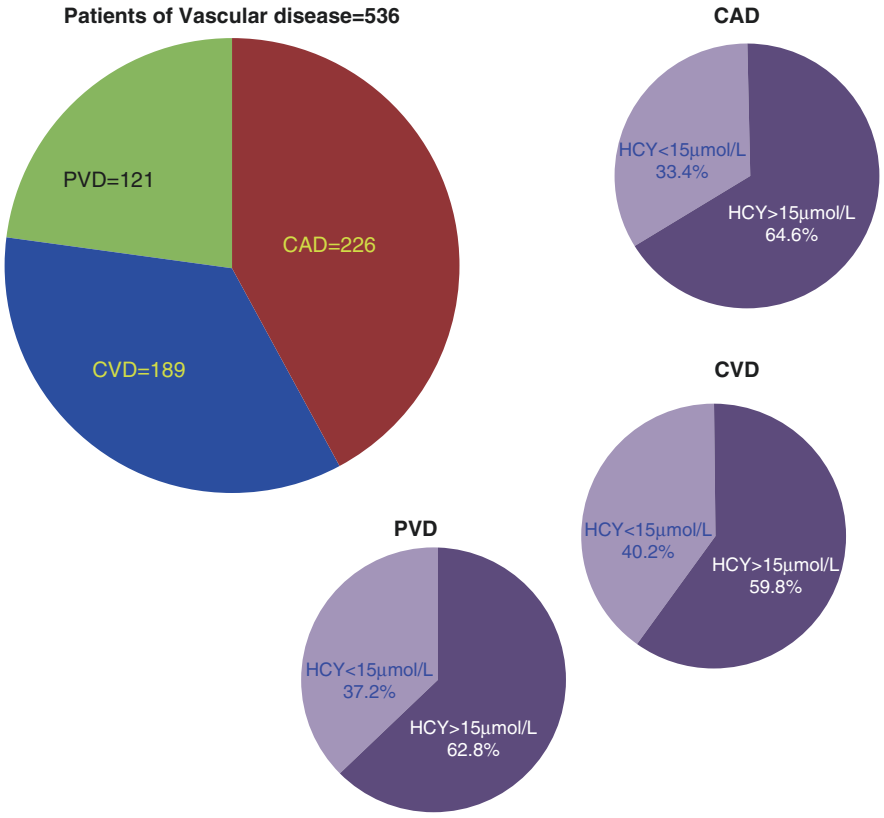

Fig. 3.4 Distribution of homocysteinemia in Indian patients of vascular disease, coronary artery disease, cerebrovascular disease and peripheral vascular disease. Prevalence of homocysteinemia in Indian patients of CAD, CVD and PVD was 64.6%, 59.8% and 62.8%, respectively (Bhargava et al. Current Medicine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

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

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

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

Table 3.2 Prevalence of T allele of the MTHFR C677T polymorphism

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

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

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

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

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

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

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

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

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

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

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

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

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

3.4 Homocysteine, Lipid Peroxidation and Antioxidant Status

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

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

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

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

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

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

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

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

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

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

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

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

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

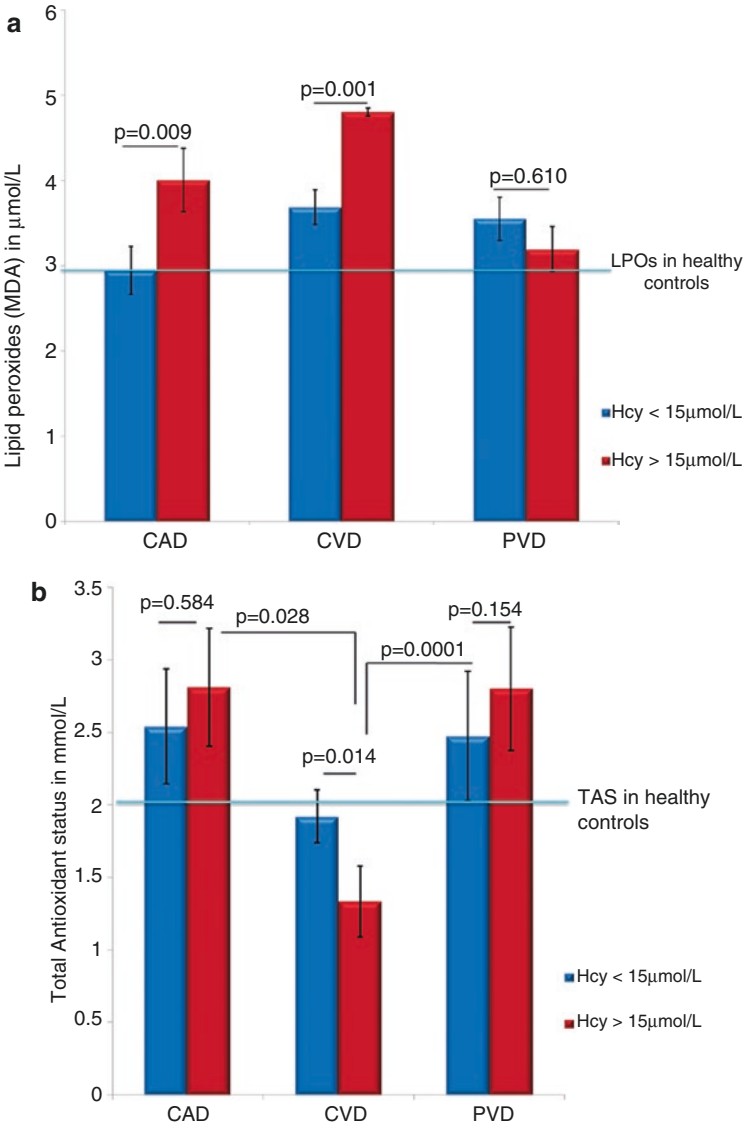

Fig. 3.5 Serum LPOs in Indian patients of occlusive vascular disease with hcy more than and less than 15 µmol/L. (**a**) Lipid peroxide levels were significantly higher in CAD and CVD patients with hcy >15 µmol/L than in those with hcy <15 µmol/L. In PVD patients, there was no significant difference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

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

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

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

Lacunae in Knowledge

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

Clinical Message

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

3.5 Homocysteinemia and Skeletal Muscles

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

Clinical Message

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