1.1 GENERAL OVERVIEW

Homocysteine, a non protein thiol amino acid, is formed intracellularly during methionine metabolism. It is a key branch point intermediate in the ubiquitous methionine cycle, the function of which is to generate one-carbon methyl groups for trans-methylation reactions. These trans-methylation reactions are essential to all life forms. Homocysteine [HSCH₂CH₂CH(NH₂)COOH] is the next higher homolog of cysteine and has molecular weight 135.18. Homocysteine was identified in 1932 by Butz and du Vigneaud at the University of Illinois as an amino acid of biological importance (Butz LW et. al., 1932). Mudd et. al. in 1964 for the first time identified the enzyme defects in cystathionine beta synthase causing homocysteinuria (Mudd SH et. al., 1964). Dr McCully for the first time speculated the role of high levels of homocysteine in atherosclerosis (McCully KS, 1969). On the basis of these observations, it was hypothesised that moderate elevations of homocysteine in blood may be a risk factor for atherosclerosis in the general population (McCully KS et. al., 1975). Wilcken and Wilcken in 1976 showed for the first time the increased levels of homocysteine-cysteine mixed disulfides after methionine load in CAD patients as compared to controls (Wilcken DEL et. al., 1976). After this landmark study, studies related to homocysteine have increased exponentially. In the last decade, research in the field of hyperhomocysteinemia received tremendous momentum as elevated levels of homocysteine has been found to be associated with several disorders like neural tube defect, Alzheimer’s disease, dementia, pregnancy complications, renal diseases, schizophrenia, osteoporosis etc (Mills JL et. al., 1995; Clarke R et. al., 1998; McCaddon A et. al., 1998; van Guldener C et. al., 2003; Applebaum J et. al., 2004; Villadsen MM et. al., 2005). Based on various retrospective, prospective and epidemiological studies from various parts of world, elevated levels of homocysteine has been accepted as an independent graded risk factor for cardiovascular diseases (Israelsson B et. al., 1988; Malinow MR et. al., 1990; Clarke R et. al., 1991; Graham I, 1994; Verhoeff P et. al., 1996). A 5 µM increment in plasma homocysteine level is associated with an increased risk of coronary heart disease (60% for men and 80% for women) (Boushey CJ et.
Prolonged lowering of homocysteine by 3–4 μmol/L has been shown to be associated with 30–40% reduction in the risk of getting CAD (Wald DS et al., 2002). Increased levels of homocysteine can confer its toxic effects to multiple organs/tissues through different mechanisms.

1.2 HOMOCYSTEINE METABOLISM

Homocysteine is a key branch point intermediate in the methionine cycle (Figure 1). Various enzymes and cofactors are involved in the metabolism of homocysteine. Homocysteine metabolism inside the cells is well regulated in healthy individuals and its concentration generally ranges from 5-12 μmol/L in the fasting state (Ueland PM et al., 1993). It is present at a much lower concentration inside the cell (1 μmol/L).

Methionine, obtained from dietary sources, gets converted into S-adenosyl methionine (SAM) in the presence of adenosine triphosphate (ATP) with the help of enzyme methionine adenosyltransferase 1, alpha (MAT1A). SAM primarily acts as a universal methyl donor and participates in many metabolic reactions. SAM is converted by various methyl-transferases to S-adenosyl homocysteine (SAH). During the conversion of SAM into SAH, methylation of macromolecules like DNA, RNA, proteins takes place. SAH is then hydrolysed by S-adenosyl homocysteine hydrolase (AHCY) to homocysteine and adenosine is released in the process. Conversion of SAH into homocysteine is a reversible reaction with the equilibrium favoring the synthesis of SAH. Hence the immediate removal of adenosine and homocysteine is required to check the resynthesis of SAH. Adenosine is removed with adenosine deaminase reactions. Homocysteine can be metabolized into two different metabolic pathways using different enzymes. It can be remethylated to methionine by enzyme methionine synthase (MS) through remethylation pathway and/or converted to cystathionine by cystathionine-βsynthase (CBS) through trans-sulfuration pathway. In the trans-methylation pathway, homocysteine is converted to methionine where the methyl group is transferred from 5-methyltetrahydrofolate and/or betaine. The vitamin B12 dependent reaction of homocysteine with 5-
methyltetrahydrofolate occurs ubiquitously in all cell types but the reaction of homocysteine with betaine is believed to take place only in liver and kidney.

**Figure 1:** Homocysteine metabolism

In the trans-sulfuration pathway, homocysteine condenses with serine to form cystathionine. This reaction is irreversible and takes place with the help of pyridoxal-5'-phosphate containing enzyme, cystathionine beta synthase (CBS). Cystathionine then gets converted into cysteine and α-ketobutyrate by hydrolysis by another enzyme gamma-cystathionase (CTH). Excess of cysteine is oxidized to taurine and other inorganic salts that are eventually excreted out by urine. Like many other organisms, conversion of cysteine to homocysteine is not reversible in humans and hence cysteine can’t act as a precursor for methionine. Cysteine formed in trans-sulfuration can be used in the protein synthesis. It can be utilized in the production of glutathione, taurine and many other metabolites.

Co-ordination between trans-sulfuration and trans-methylation pathways is tightly regulated. Dietary methionine decides the fate of two pathways. SAM regulates the methionine metabolism by various means depending on the amount of methionine received through diet. SAM is an inhibitor of MTHFR and activator of CBS (Prudova A et. al., 2006). Thus, as the concentration of methionine and consequently SAM increases, the rate of remethylation reaction decreases and that of trans-sulfuration increases thereby maintaining the intracellular concentration of methionine and SAM.
Defects in enzymes involved in homocysteine metabolism may elevate the level of homocysteine. Excess homocysteine is transported out of the cells presumably in the free reduced form. In circulation, homocysteine is believed to rapidly oxidise forming low molecular weight disulfides like homocystine (dimer of homocysteine) and homocysteine-cysteine mixed disulfides (20-30%). However, majority of homocysteine in circulation binds to proteins to form protein bound homocysteine (70-80%). In circulation, the concentration of homocysteine in healthy individuals as mentioned before is 5-12 µmol/L. However, in the mild hyperhomocysteinemic condition, homocysteine levels can be between 15-25 µmol/L while in intermediated hyperhomocysteinemic condition, homocysteine levels can be as high as 50 µmol/L. Under severe hyperhomocysteinemic condition, levels can reach up to 500 µmol/L.

1.3 FACTORS AFFECTING THE LEVELS OF HOMOCYSTEINE

Food contains traces of homocysteine and majority of it comes from metabolism of amino acid methionine. Several genetic and/or lifestyle factors (including diet) are known to influence homocysteine levels (Andersson A et. al., 1992; Nygard O et. al., 1995; Cravo ML et. al., 1996; Lussier-Caccan S et. al., 1996; Tonstad S et. al., 1997; Greenlund KJ et. al., 1998; Gudnason V et. al., 1998; Saw SM et. al., 2001). These factors singly or in combination might predispose an individual to hyperhomocysteinemia. Following are some of the factors known to be associated with homocysteine levels:

1.3.1 Life Habits
1.3.1.1 Physical Inactivity

Physical activity is inversely associated with homocysteine levels (Nygard O et. al., 1995), although few other studies failed to observe any such associations (Lussier-Caccan S et. al., 1996; Gudnason et. al., 1998; Saw SM et. al., 2001). Since physical activity is supposed to be associated with healthy lifestyle, it is expected that homocysteine levels would be low in that scenario.
1.3.1.2 Obesity

Childhood obesity is shown to be linked to hyperhomocysteinemia (Gallistl S et. al., 2001). Gallistl et. al. found out that correlation between changes in homocysteine and body weight was stronger as compared with the correlation between changes in homocysteine and folate (Gallistl S et. al., 2001). Another study by Lin et. al. investigated the pattern of obesity and plasma homocysteine levels in CAD patients (Lin YH et. al., 2008). They didn’t find any association of BMI with homocysteine levels. Rather, waist hip ratio was found to be strong predictor of homocysteine levels.

1.3.2 Non-Modifiable Factors

1.3.2.1 Age and Sex

Increases in age and male sex are associated with elevated homocysteine levels (Andersson A et. al., 1992a; Lussier-Caccan S et. al., 1996; de Bree A et. al., 2001). Age and sex are found to be among the few factors that shows strong and consistent association with homocysteine levels (Boers GH et. al., 1985; Ueland PM et. al., 1993). In a population based Hordaland Homocysteine study done on more than 16000 individuals of age group between 40-67 years, males were found with higher levels of homocysteine as compared with females (Nygard O et. al., 1995). Further, homocysteine levels increased with increasing age. Homocysteine levels are found to be higher in post-menopausal women as compared to pre-menopausal women suggesting the role of various sex hormones in regulating the levels of homocysteine (Andersson A et. al., 1992; Wouters MG et. al., 1995). Males have larger muscle mass as compared to females and formation of muscle is associated with formation of homocysteine (Norlund L et. al., 1998). Hence the difference between homocysteine levels between two sexes could be attributed to creatine/creatinine synthesis that takes place during muscle formation (Norlund L et. al., 1998).

1.3.2.2 Family History

Homocysteine levels have been shown to be influenced by family history (Tonstad S et. al., 1997; Greenlund KJ et. al., 1999). Family history is a predictor of increased plasma homocysteine levels and may be involved in
early-onset CAD (Greenlund KJ et. al., 1999). Tonstad et. al. also showed that in a stepwise multivariate regression analysis, history of parental CVD remained significantly associated with homocysteine level (Tonstad S et. al., 1997). Interestingly, a study by Nygård et. al. suggests that parents with CVD are more likely to have smoked cigarettes, and their offspring may have higher homocysteine levels because of passive smoking (Nygård O et. al., 1995). Thus, checking the levels of homocysteine is advisable for individuals who have a family history of premature CVD.

1.3.3 Modifiable Factors

1.3.3.1 Smoking

Various studies have checked the effect of smoking on homocysteine levels. Smoking is found to be positively associated with high levels of homocysteine in some of the studies (Nygard O et. al., 1995; Giles WH et. al., 1998; Rasmussen LB et. al., 2000; de Bree A et. al., 2001d) while others failed to find any association (Berg K et. al., 1992; Alftthan G et. al., 1994; Brattstrom L et. al., 1994; Bates CJ et. al., 1997). Cigarette smoke is known to disturb thiol redox status of plasma and hence might influence the homocysteine levels (Pryor WA et. al., 1993; Mansoor et. al., 1995; Bergmark C et. al., 1997). Effect of cigarette smoke was found to be more profound in females as compared to males (Nygard O et. al., 1995). Hordaland Homocysteine study is one large study performed on more than 16000 individuals that observed a strong association of cigarette smoking on homocysteine levels (Nygard O et. al., 1995). Cigarette smoke might inhibit the methionine synthase enzyme also thereby altering the homocysteine level.

1.3.3.2 Alcohol Consumption

Moderate alcohol consumption is known to be associated with low homocysteine levels whereas consumption of large amounts of alcohol leads to high homocysteine levels (Cravo ML et. al., 1996b; de Bree A et. al., 2001b; Koehler KM et. al., 2001). However the association between alcohol consumption and homocysteine levels is complex as there are studies which didn’t find any association between the two (Lussier-Cacan S et. al., 1996; Gudnason V et. al., 1998). Hordaland Homocysteine study found a complex
type of association between alcohol consumption and homocysteine levels (Nygard O et. al., 1995). They observed a weak association between alcohol intake and homocysteine levels.

1.3.3.3 Coffee Consumption

Coffee consumption is found to be associated with an increase in homocysteine levels in many observational studies (Nygard O et. al., 1997b; Oshaug A et. al., 1998; Stolzenberg Solomon RZ et. al., 1999; de Bree A et. al., 2001d; Jacques PF et. al., 2001; Koehler et. al., 2001). Hordaland Homocysteine study showed the strong relation between amount of coffee consumed and homocysteine levels (Nygard O et. al., 1995). Caffeine acts as an antagonist of vitamin B6 and hence inhibits the conversion of homocysteine to cysteine, thereby increasing the levels of homocysteine (Grubben MJ et. al., 2000). Presence of other polyphenols like chlorogenic acid in coffee may also be responsible for elevated homocysteine levels.

1.3.4 Diseases

1.3.4.1 Hypertension

Hypertension is one of the well-known risk factors for cardiovascular diseases (Kannel WB, 1996, Klag MJ et. al., 1996). Plasma homocysteine is found to be related to blood pressure (Glueck CJ et. al., 1995; Malinow MR et. al., 1995; van den Berg M et. al., 1996; Sutton-Tyrrell K et. al., 1997; Hoogeveen EK et. al., 1998; Folsom AR et. al., 1998; Gills WH et. al., 1998;). Cross-sectional studies have shown a positive association of systolic and diastolic blood pressure and hypertension with plasma homocysteine (Malinow MR et. al., 1995; Nygard O et. al., 1995; Hoogeveen EK et. al., 1998; Osganian SK et. al., 1999; Kahleova R et. al., 2002). Various mechanisms like homocysteine-induced arteriolar constriction, renal dysfunction and increased sodium reabsorption and increased arterial stiffness could explain the association of homocysteine and blood pressure (Stehouwer CD et. al., 2003). Framingham Heart study for the first time showed the association of hypertension incidence with homocysteine levels in a community-based setup (Sundström J et. al., 2003). Increased blood pressure is known to be associated with nephrosclerosis and decline in renal function that is linked
with CVD (Kasiske BL et. al., 1987; Perneger TV et. al., 1993; Klag MJ et. al., 1996; Tracy RE et. al., 1996; Fliser D et. al., 1998; Ruilope LM et. al., 1999). The Hordaland Homocysteine Study examined more than 16000 individuals and showed the association of plasma homocysteine with systolic and diastolic blood pressures that was stronger in younger individuals aged between 40 and 42 years (Nygard O et. al., 1995). NHANES III investigation also examined individuals with younger age with previous CVD and concluded that elevation of 5 µmol/L homocysteine was associated with a higher systolic blood pressure of 0.7 to 1.2 mm Hg and a higher diastolic blood pressure of 0.5 to 0.7 mm Hg in men and women, respectively (Lim U et. al., 2002).

1.3.4.2 Renal Failure

Majority of homocysteine is metabolized by liver and kidney in the body. Studies done on mice and humans have shown that nearly 70% of the plasma homocysteine is cleared by kidney from the body (Bostom A et. al., 1995; Guttormsen AB et. al., 1997). Hence it is expected that any problem in kidney function might lead to increased levels of homocysteine. Any insufficiency in the renal function leads to increased serum creatinine levels and almost all the studies done in patients with renal diseases have shown the positive correlation of serum creatinine with plasma homocysteine levels (Norlund L et. al., 1998; Bostom AG et. al., 1999a; Bostom AG et. al., 1999b; Wollesen F et. al., 1999). In the end stage renal disease, levels of both creatinine and homocysteine can be elevated up to 3-5 times higher than the normal levels (Wilcken DE et. al., 1980; Arnadottir M et. al., 1996; Bostom AG et. al., 1997). Studies done in the hemodialysis patients have shown that 85% of the patients were hyperhomocysteinemic and they had 3-4 times higher levels of homocysteine than the general population (Robinsosn K et. al., 1996).

1.3.4.3 Cancer

Cancer is found to be associated with increased levels of homocysteine (Refsum H et. al., 1990). Since in cancer, cells divide rapidly and DNA and protein synthesis takes place at a faster pace than the normal cells. These rapidly dividing cells need methyl groups for methylating DNA and proteins...
and hence demand for methyl group increases. This demand is fulfilled by methionine, which after donating the methyl group changes into homocysteine. Another reason could be the methyl group from folate cycle is preferentially used for DNA synthesis which hampers conversion of homocysteine into methionine that ultimately increases the levels of homocysteine.

1.3.5 Genetic Factors

1.3.5.1 Polymorphisms in \textit{MTHFR}

As the metabolism of homocysteine is controlled by a combination of various enzymes, any defect in these enzymes may lead to unusual elevation of its level in the cell. The gene, \textit{MTHFR} plays a key role in homocysteine metabolism. It changes methylenetetrahydrofolate to methyltetrahydrofolate that donates methyl group for the production of methionine from homocysteine. Frosst \textit{et. al.} in 1995 identified this polymorphism in exon 4 of \textit{MTHFR} gene at nucleotide position 677 that changes cytosine (C) to thymine (T) (Frosst \textit{P et. al.}, 1995). These polymorphisms present in exon 4 (C677T) and exon 7 (A1298C) in \textit{MTHFR} make the enzyme thermolabile leading to high homocysteine levels (Kang SS \textit{et. al.}, 1991). These polymorphisms have been shown to alter its activity (Weisberg I \textit{et. al.}, 1998). Enzyme activity reduces up to 35% in case of 1298CC while up to 70% in case of 677TT genotype (Frosst \textit{P et. al.}, 1995). Due to the thermolability, this enzyme becomes sensitive towards low concentrations of folate. \textit{MTHFR} C677T has been studied extensively in relation to the high levels of homocysteine. Prevalence of this polymorphism varies from 2% to 16% in different populations (Kumar J \textit{et. al.}, 2005). Various studies done in different parts of world have shown the association of 677TT genotype with elevated levels of homocysteine (Amouzou \textit{EK et. al.} 2004; Christensen B \textit{et. al.} 1997; Frosst \textit{P et. al.} 1995; Herrmann \textit{W et. al.} 2003; Mager A \textit{et. al.} 2005). Homocysteine levels were found to be even higher in the individuals having 677TT genotype along with folate deficiency (Nygard O \textit{et. al.}, 1999). Inbal \textit{et. al.} have shown the association of 677TT genotype with premature CAD (Inbal A \textit{et. al.}, 1999). This polymorphism is of great interest as it has also been shown to be associated with various other disorders like

1.3.5.2 Polymorphisms in CBS

A whole range of mutations/polymorphisms are known in the CBS gene and many of them are found to be associated with hyperhomocysteinemia (Tsai MY et al., 1996; Tsai MY et al., 1997; Kluijtmans LA et al., 1998; Kraus JP et al., 1999; Blom HJ, 2000). Many missense mutations are known to decrease the CBS enzyme activity (Kraus JP et al., 1999). Deficiency of CBS has great impact on levels of homocysteine (Mudd SH et al., 1989). Because of defect in CBS, trans-sulfuration pathway is modulated and hence leads to elevated levels of homocysteine. CBS deficiency is the most common cause of inborn error of homocysteine metabolism (Mudd SH et al., 1989). Two of the most frequent polymorphisms (CBS I278T and G307S) present in the conserved domain of CBS are found to be associated with homocysteine levels (Kim CE et al., 1997; Gaustadnes M et al., 1999; Kluijtmans LA et al., 1999). A 68 base pairs insertion (844ins68) present in exon 8 of CBS gene has been found to be a common mutation with varying prevalence among different populations (Tsai MY et al., 1996; Sperandeo MP et al., 1996; Kluijtmans LAJ et al., 1997; Guisti B et al., 1997; Ramsbottom D et al., 1997). Association of this polymorphism with hyperhomocysteinemia has shown conflicting results (Orendac et al., 1992; Kluijtmans LAJ et al., 1997; Franco RF et al., 1997; Kluijtmans LAJ et al., 1998; Tsai MY et al., 1998; Orendac et al., 1998;). Several studies have confirmed the role of 844ins68 polymorphisms and MTHFR C677T together with hyperhomocysteinemia (de Franchis R et al., 2000; Guastadnes M et al., 2000).

1.3.5.3 Polymorphisms in other Genes

Polymorphisms in several other candidate genes that play a role in the lipid metabolism, folate metabolism, vitamin metabolism, homocysteine metabolism etc have been implicated in the elevation of homocysteine levels.
Multiple studies have already focused on the different polymorphisms in homocysteine metabolizing genes (Janosíková B et al., 2003; Janosíková B et al., 2005; Sharma et al. 2006; Fredriksen A et al., 2007). Fredriksen et al. have done a study in Norway in more than 10,000 individuals and seen the impact of 13 common polymorphisms present in genes involved in one carbon metabolism on different biochemical parameters (MTHFR C677CT, Ala222Val; A1298C, Glu429Ala, MTR A2756G, Asp919Gly; MTRR A66G, Ile22Met; MTHFD1 G1958A, Arg653Gln; BHMT G742A, Arg239Gln; CBS 844_845ins68 and C699T, Tyr233Tyr; TCN2 A67G, Ile23Val and C776G, Pro259Arg; SLC19A1 G80A, Arg27His; and PON1 T163A Leu55Met and A575G, Gln192Arg) (Fredriksen A et al., 2007). Some of these polymorphisms were found to be significantly associated with homocysteine, folate, betaine, cystathionine and methylmalonic acid. Janosikova et al. examined 11 genetic variants from methionine cycle in CAD patients and controls (Janosikova B et al., 2003). They observed that 844ins68 polymorphism in the CBS gene was associated with a significantly lowered risk of CAD and homocysteine levels after methionine load. Janosikova et al. had also studied about 42 coding SNPs present in genes in homocysteine metabolism in Czech population (Janosikova B et al., 2005). Functional polymorphisms in MTR A2756G and MTRR A66G had been found to be associated with significantly higher concentration of homocysteine (Harmon D et al., 1999; Gaughan DJ et al., 2001; Laraqui A et al., 2006). Recently in a genome wide scan, a new candidate gene called as the nicotinamide N-methyltransferase (NNMT) present at Chromosome 11q23 was found to be a major determinant of plasma homocysteine (Souto JC et al., 2005). One SNP (rs694539) present in intron 1 of this gene was found to be significantly associated with homocysteine concentrations.

1.3.6 Dietary Factors

1.3.6.1 Vitamin B

Along with all genetic factors, dietary factors like vitamin B12 and vitamin B6 play a role in influencing the levels of homocysteine (Ubbink JB et al., 1993; Franken DG et al., 1994; Pancharunity N et al., 1994; van der Berg N et al., 1994). Several studies have seen the association of homocysteine
levels with vitamin B intake (Kang SS et. al., 1991; Ubbink JB et. al., 1993; Pancharunity N et. al., 1994; Franken DG et. al., 1994; van der Berg N et. al., 1994; Dalery K et. al., 1995). Selhub et. al. in a cross sectional study have shown that about 67% cases of hyperhomocysteinemia were because of inadequate plasma concentration of vitamins (Selhub J et. al., 1993). Other studies also suggested the role of vitamin B12 on increased levels of homocysteine (Kang SS et. al., 1987; Stabler SP et. al., 1988). In the Framingham study, inverse correlation between homocysteine and vitamin B levels was observed (Selhub J et. al., 1993). Another report from UK National Diet and Nutrition Survey also reported the similar trend of vitamin B12 and B6 with homocysteine levels (Bates CJ et. al., 1997).

Majority of Indian population follows vegetarianism that predisposes them to vitamin B12 deficiency. Because of the vitamin B12 deficiency, prevalence of hyperhomocysteinemia in Indian population is high (Kumar J et. al., 2005). Vitamin supplementation has been known to normalize the elevated levels of homocysteine (Kang SS et. al., 1991; Ubbink JB et. al., 1993; van der Berg N et. al., 1994; Franken DG et. al., 1994; Pancharunity N et. al., 1994; Dalery K et. al., 1995;).

1.3.6.2 Folate

Folate is an important factor in one carbon metabolism and plays a key role in methionine metabolism. Because of folate deficiency, one carbon metabolism gets affected, leading to accumulation of various intermediate metabolites like homocysteine that may have negative consequences (Kang SS et. al., 1987). Independent of other dietary factors, high folate intake is shown to be associated with decreased levels of homocysteine (Rasmussen LB et. al., 2000; de Bree A et. al., 2001c; Jacques PF et. al., 2001). Various observational studies focused on intake of folate and their effect on homocysteine levels also agreed with these findings (Selhub J et. al., 1993; Bates CJ et. al., 1997; Shimakawa T et. al., 1997; Ubbink JB et. al., 1998; Koehler KM et. al., 2001; Saw SM et. al., 2001). Folic acid deficiency has been shown to be associated with the predisposition to atherosclerotic cardiovascular disorders (Boushey CJ et. al., 1995). Folate fortification of cereals and grains in US in 1998 has greatly reduced the frequency of deficiency of folate in that population thereby
reducing the frequency of elevated levels of homocysteine (Jacques PF et. al., 1999). Many studies have shown the damage done by increased levels of homocysteine can be reversed by high doses of folate (Bellamy MF et. al., 1999; Verhaar MC et. al., 1999).

1.3.6.3 Lipids

Association between homocysteine and total cholesterol has been observed in many studies (Ueland PM et. al., 1989; Refsum H et. al., 1990; Nygard O et. al., 1995; Giles WH et. al., 1998; Schneede J et. al., 2000; Carmel R et. al., 2001; Ganji V et. al., 2003; de Bree A et. al., 2005;). Most of the common lipid disorders can be treated by dietary management. Mikael et. al. has reported recently that homocysteine inhibits the synthesis of apoA-1 (main HDL apolipoprotein) (Mikael LG et. al., 2006). Another study supported similar finding where they observed the reduction of HDL cholesterol concentration by homocysteine (Schwahn BC et. al., 2007). Various factors including hyperhomocysteinemia induce ER stress that has been shown to affect lipid metabolism through sterol regulatory element-binding protein (SREBP) (Shank KJ et. al., 2001; Werstuck GH et. al., 2001; Lee JN et. al., 2004). Expression of enzymes required for cholesterol and fatty acid biosynthesis and uptake are regulated by SREBPs (Shimano H et. al., 1996).

1.3.6.4 Other Factors

Various drugs were found to be having an impact on homocysteine levels (Refsum H et. al., 1990; Ermens AA et. al., 1991; Tonstad S, 1997; Schneede J et. al., 2000). Antiepileptic drugs interfere with folate metabolism and hence influence the homocysteine concentration (Refsum H et. al., 1990). Methotrexates are folate antagonists and act by depleting cells of folate leading to high homocysteine levels (Ueland PM et. al., 1986). These drugs that inhibit cell reproduction are given for treatment of cancer and rheumatoid arthritis. Nitrous oxide that is used as anesthetic inactivates methionine synthase enzyme and hence causes the elevation of homocysteine levels (Ermens AA et. al., 1991). Drugs used for lipid lowering also have been shown to elevate the levels of homocysteine (Tonstad S, 1997).
1.4 MECHANISM OF ACTION OF HOMOCYSTEINE

Hyperhomocysteinemia has been shown to be associated with various complex disorders like neural tube defect, pregnancy complications, end stage renal disease, Alzheimer’s diseases, stroke, diabetes and is known as an independent risk factor for cardiovascular disorders. Pathological basis by which elevated levels of homocysteine mediates these diseases are still actively pursued. Past research in the field of homocysteine has identified key mechanisms which can be causal for the numerous physiological changes associated with hyperhomocysteinemia. The following hypotheses provide a causality clue to hyperhomocysteinemia.

1) Oxidative Stress hypothesis.
2) Molecular Target hypothesis.
3) Endoplasmic stress hypothesis.
4) Epigenetic modulations.

1.4.1 Oxidative Stress Hypothesis

This was the earliest of hypothesis put forward to understand hyperhomocysteinemia (Starkebaum G et. al., 1986). Most of the thiols auto-oxidize in the presence of transition metal catalysts and molecular oxygen thereby creating oxidative stress by generating hydrogen peroxide and superoxide radicals (Starkebaum G et. al., 1986). Homocysteine, being one of the thiol compounds, also undergoes auto-oxidation generating reactive oxygen species (ROS) (Loscalzo J, 1996). Because of the presence of a reactive sulfhydryl group in homocysteine, it can undergo auto-oxidation in the presence of molecular oxygen (O₂) at physiological pH thereby producing ROS.

\[ 2\text{RSH} + \text{O}_2 \xrightarrow{\text{M}^+} \text{RS-SR} + \text{H}_2\text{O}_2 \]

Thus the reactive oxygen species that is generated from this process can damage various cells or cell organelles like platelets, leukocytes, Golgi apparatus etc and hence can damage the cellular functions in a number of ways. Various studies showed the impairment of endothelial cell functions due
to increased levels of homocysteine (Tawakol A et. al., 1997; Woo KS et. al., 1997; Chambers JC et. al., 1998; Bellamy MF et. al., 1998; Lambert J et. al., 1999; Chambers JC et. al., 1999). Elevated levels of homocysteine can also decrease the ability of cells to respond to toxic effects of $\text{H}_2\text{O}_2$ by impairing the activities of antioxidant enzymes (Upchurch GR et. al., 1997). Upchurch et. al. in a study have shown that oxidative stress due to hyperhomocysteinemia has been linked with decreased expression of potent anti-oxidant enzymes like glutathione peroxidase leading to decrease in bioactive nitric oxide (NO) and increase in the formation of peroxides. Recently, Kumar et. al. have also shown that the growth defects in yeast exposed to hyperhomocysteinemic conditions had no link to oxidative stress as a causal factor (Kumar A et. al., 2006).

Oxidative stress theory has its own inherent drawbacks. Cysteine, being another thiol compound, can undergo auto-oxidation in a similar way as homocysteine and has been shown to undergo oxidation faster than cysteine (Sengupta S et. al., 2001). Based on these findings, Jacobsen and colleagues argued that cysteine should be the major causal agent for oxidative stress. But clinical findings implying cysteine in CAD have not been reported. Homocysteine may result in oxidative stress indirectly by decreasing the catalytic activity of anti-oxidant enzymes like glutathione peroxidase and superoxide dismutase. Studies have shown the altered activities of these enzymes in aortic endothelial cells and vascular smooth muscle cells when exposed to homocysteine (Upchurch Jr GR et. al., 1997; Lang D et. al., 2000).

Thus a more direct evidence for homocysteine causation was put forth in the molecular target hypothesis.

1.4.2 The Molecular Target Hypothesis

An alternative hypothesis to the oxidative stress hypothesis is molecular target hypothesis which tells that homocysteine attacks certain molecular targets resulting in their structure and function modulation. Various studies using homocysteine have shown that it can modulate the structure and function of proteins.
Till date about 20 different proteins have been identified through in vitro, in-vivo and clinical studies as molecular targets for homocysteine (Sengupta S et. al., 2001; Undas A et. al., 2001; Majors AK et. al., 2002; Lim A et. al., 2003). Inhibition or modulation of endothelial cell proliferation, neuronal NMDA-like receptor activity, tissue plasminogen activator binding to homocysteinylated annexin II, activation of matrix metalloproteinases such as latent MMP-2 and the induction of expression and secretion of monocyte chemoattractant protein-1 and interleukin-8 in human aortic endothelial cells have given the evidences in support of the hypothesis (Wang H et. al., 1997; Lipton SA et. al., 1997; Hajjar KA et. al., 1998; Bescond A et. al., 1999; Sengupta S et. al., 2001; Poddar R et. al., 2001). These studies provide evidence that homocysteine may directly target and alter protein function. In a recent study based on certain criteria that include cysteine content, solvent accessibility of cysteine residue, pKa of cysteine and dihedral strain energy, Sundaramoorthy et. al. have identified different potential protein targets of homocysteine (Sundaramoorthy E et. al., 2008). Most of these potential targets, when analyzed in a pathway context fell within the disease processes associated with hyperhomocysteinemia such as coagulation, LDL metabolism and notch signaling. In another study, Notch and fibrillin were identified as key targets for homocysteinylation (Hubmacher D et. al., 2005; Hutchinson S et. al., 2005). Since fibrillin is a key component of the extracellular matrix, especially with relevance to vasculature, molecular targeting of fibrillin has been proposed as a key pathogenic mechanism.

Homocysteine thiolactone is a cyclic thioester synthesized by aminoacyl tRNA synthetases. Thiolactone is a reactive moiety that binds to side chain amines of lysine residues resulting in structural and functional perturbation. Pioneering work of Jacubowski et. al. (Jacubowski H, 1991; Jacubowski H, 1997; Jacubowski H, 1999) led the investigation in this field. They were able to show the homocysteinylation in the presence of homocysteine thiolactone leads to protein damage.

1.4.3 Endoplasmic Reticulum Stress

Endoplasmic Reticulum (ER) is the destination for secretory and extracellular proteins. ER is the organelle in the cell which is responsible for
proper protein folding prior to export to their destination. However, accumulation of misfolded and unfolded proteins results in ER stress. Cells respond to this stress by generating unfolded protein response (UPR) (Kokame K et. al., 1996). Excess ER stress however leads to apoptosis. Elevated levels of homocysteine have been shown to alter the cellular redox state resulting in ER stress (Outinen PA et. al., 1998; Outinen PA et. al., 1999). The first direct link between hyperhomocysteinemia induced ER stress causing dysregulation of cholesterol metabolism was shown by Werstuck et. al. (Werstuck GH et. al., 2001). This was the first direct evidence linking hyperhomocysteinemia with atherosclerosis. Kumar et. al. have shown in yeast, an organism which has been extensively utilized for understanding ER stress, that homocysteine induced growth retardation in yeast is probably mediated due to ER stress (Kumar A et. al., 2006). In a recent review by Sharma et. al., it has been mentioned that elevated levels of homocysteine cause the up regulation of ER stress proteins resulting in apoptosis (Sharma P et. al., 2006). Furthermore, ER stress also interferes the lipid metabolism which may lead to cardiovascular disorders. Thus, ER stress induced by homocysteine emerges as the common pathway that relates to apoptosis and atherosclerosis.

1.4.4 Epigenetic Modulation

Methylation of cellular macromolecules is intimately linked to one carbon metabolism. Fate of trans-methylation reactions is dictated by homocysteine levels thereby altering the methylation status (Ulrey et. al., 2005). Wang et. al. in a study checking the effects of homocysteine on the growth of vascular endothelial cells observed the increased production of S-adenosylhomocysteine (a potent inhibitor of methyltransferases) causing hypomethylation. Hypomethylation led to decrease in vascular endothelial cell growth which is a hallmark of atherosclerosis (Wang H et. al., 1997). It has been shown that in patients with chronic alcoholism, homocysteine levels are positively correlated with global DNA methylation (Bonsch et. al., 2004). Hypomethylation of DNA in vascular patients has been shown because of hyperhomocysteinemia (Castro et. al., 2003). Another study revealed that the pattern of global DNA methylation changes in the initial stages of
atherosclerosis (Lund et. al., 2004). Recently, in a study by Yi-Deng et. al., hypomethylation of markers like Alu and LINE1 elements was observed in vascular smooth muscles cells grown in culture that were exposed to high concentrations of homocysteine (Yi-deng et. al., 2007). Interestingly, hypomethylation was observed inspite of increased activity of DNMT3a and DNMT3b. Another study by this group have shown that when cultured monocytes were treated with 100µM homocysteine, total cholesterol levels increases while ApoE mRNA decreases through hypermethylation of promoter sequences (Yi-deng et. al., 2007). Recently, Sharma et. al. have shown that hyperhomocysteinemia can alter the methylation profile of lymphocyte DNA in CAD cases compared to control (Sharma P et. al., 2008). Also this modulation had a graded effect with regard to the concentration of plasma homocysteine. Thus it remains to be seen whether this global methylation profile change is causal in CAD. To understand this, gene specific methylation changes occurring in CAD cases are currently under investigation by our group.

1.5 HOMOCYSTEINE IN CARDIOVASCULAR DISEASES

World Health Organization reports indicate that CVD are number one cause for deaths worldwide. Among CVD, coronary heart disease is one of the most prevalent diseases (American Heart Association Update, 2003). Coronary artery supplies blood to heart and any obstruction in this leads to improper functioning of heart. Obstruction in coronary artery often starts with atherosclerosis that is characterized by deposition of cholesterol, calcium and other cellular waste products in the inner layer of arterial walls. This deposition, known as plaque, can increase in size and block the artery completely thereby affecting the functioning of heart.

Many classical risk factors like male sex, age, family history, high blood pressure, alcohol, physical inactivity, obesity, high blood glucose etc are known that causes CAD (Enas EA et. al., 1995; Sheth T et. al., 1999; Anand SS et. al., 2000). But a significant proportion of CAD cases cannot be explained by these risk factors and hence search for other risk factors is required (Enas EA et. al., 1995). One such emerging risk factor found to be associated with
CAD is elevated levels of homocysteine. Many case-control studies have shown the positive association of homocysteine with coronary atherosclerosis (Wilcken DEL, et. al., 1976; Kang SS, et. al., 1986; Genest JJ Jr, et. al., 1990; Ubbink JB, et. al., 1991; Pancharunits N, et. al., 1994; Von Eckardstein A, et. al., 1994; Dalery K, et. al., 1995; Robinson K et. al., 1995;). Follow up studies have shown that mortality rate in CAD patients with high homocysteine levels were much higher than the CAD patients with normal homocysteine levels (Nygard O et. al., 1997). It has also been demonstrated that disease mortality among those who are in their highest quartile of homocysteine are two-fold higher when compared with those who are in the lowest homocysteine quartile (Bostom A et. al., 1999). Population based studies have shown that about 10% events of CAD are due to increased levels of homocysteine (Boushey CJ et. al., 1995). Because of its potential role in the pathogenesis of atherosclerosis, factors causing the alteration in homocysteine metabolism are getting increased attention. It is still not known whether increased levels of homocysteine caused cardiovascular problems or it is a surrogate marker.

McCully based on his clinical observations hypothesized that elevation in homocysteine levels may be a risk factor for atherosclerosis. Homocysteine was administered parenterally and also through alimentary canal to rabbits and baboons and its atherogenic effects were observed subsequently (McCully KS et. al., 1970; Harker LA et. al., 1974; McCully KS et. al., 1975). Studies done in cultured endothelial cells also demonstrated that homocysteine has adverse effect on these cells (Tsai JC et. al., 1994). Vascular damage done by homocysteine has been related to oxidative stress, production of hydrogen peroxide and superoxide, inactivation of nitric oxide and inhibition of glutathione peroxide activity and synthesis (Welch GN et. al., 1997). Effect of hyperhomocysteinemia on multiple coagulation factors like platelets, factors V, VII and XII leads to increased propensity to thrombosis (D’Angelo A et. al., 1997). Homocysteine modifies low density lipoprotein by formation of homocysteine thiolactone and changes them to small, dense LDL particles. These particles are taken up by macrophages to form foam cells that leads to oxidative modification of LDL, deposition of cholesterol and lipids, thrombogenesis, intimal damage and alteration of connective tissue of
developing arteriosclerotic plaques (Naruszewicz M et al., 1994; McCully KS, 1996). Today, numerous clinical studies have been done in humans showing the role of homocysteine in cardiovascular, cerebrovascular and peripheral vascular diseases (Malinow MR et al., 1994; Boushey CJ et al., 1995).

Various clinical and epidemiological studies done across various parts of the world have now concluded that moderate hyperhomocysteinemia is an independent risk factor for CVD. Hyperhomocysteinemia is comparable to other risk factors for CVD like age, sex, smoking and hypercholesterolemia (Clarke R et al., 1991; Graham IM et al., 1997). In the Framingham heart study done on elder population, deficiency of folate, vitamin B12 and vitamin B6 were found to be associated with elevation in plasma homocysteine levels (Selhub J et al., 1993). Several of the studies done on animal models, cell culture studies and various epidemiological and clinical studies formed the basis of homocysteine theory of arteriosclerosis (McCully KS et al., 1975; McCully KS et al., 1983; McCully KS, 1996; McCully KS et al., 1997). According to this theory, hyperhomocysteinemia is caused by various factors like age, sex, smoking, alcohol consumption, genetic defects in enzymes participating in homocysteine metabolism, nutritional deficiencies of various factors like folate vitamin B12 and vitamin B6, diabetes etc and atherogenesis is the secondary consequence of hyperhomocysteinemia. In the Nurse Health study, significant decrease in the morbidity and mortality due to CVD was observed during a period of 14 years after dietary intake of folate and vitamin B6 (Rimm EB et al., 1998). In the Framingham heart study also, it was shown that women who had lowest intake of folate and vitamin B6 were at highest risk of mortality and myocardial infarction (Selhub J et al., 1993). Norwegian study looking into the prospective cardiovascular mortality risk due to hyperhomocysteinemia in CAD patients also observed the same findings (McCully KS, 1983). Study done by Bellamy et al. in 18 healthy adults also showed that a reduction of homocysteine from 14.9 ± 7.4 to 8.7 ± 2.5 μmol/L after 6 wk of oral folate supplementation (5 mg/d) enhanced endothelium-dependant brachial artery dilatation (Bellamy MF et al., 1999). These findings were supported by other studies (Bellamy MF et al., 1999; Kanani PM et al., 1999). The largest population based study, The Hordaland Study, checked the
relation between plasma homocysteine and risk factors for CVD (Refsum H et al., 2006).

Elevated levels of homocysteine were found to be associated with multiple clinical conditions, whereas a low homocysteine levels with better physical and mental health. Individuals having high levels of homocysteine were found with increased risk of cardiovascular morbidity, cardiovascular and non cardiovascular mortality. Framingham study done in elderly population demonstrated that when individuals in lowest quartiles of homocysteine were compared with those in highest quartile, there was a two fold increase in all-cause cardiovascular disease mortality (Bostom AG et al., 1999). Elevated levels of homocysteine have shown the graded effect on risk of getting CAD and the extent and severity of the disease (Chao et al., 1999).

Inconsistent results have been reported from various observational studies. Some of the studies have shown weak or no association of homocysteine with CVD (Stampfer MJ et al., 1992; Alfthan G et al., 1994; Arnesen E et al., 1995; Chasan-Taber L et al., 1996; Taber L et al., 1996; Chasan- Evans RW et al., 1997; Taber L et al., 1996; Verhoef P et al., 1997; Folsom AR et al., 1998; Fallon UB et al., 2001). In a nested case-control study from South Wales, UK, observed no association of elevated levels of homocysteine with CAD risk (Fallon UB et al., 2001). Another prospective case-control ARIC study failed to observe the association of homocysteine with coronary heart diseases in males (Folsom AR et al., 1998). In a prospective case-control study done on more than 14000 male physicians, high homocysteine levels did not find to predict the events of getting myocardial infarction.

1.6 CAD AND HOMOCYSTEINE RESEARCH IN INDIA

CAD is probably the largest cause of mortality and morbidity word wide and is reaching epidemic proportions in developing countries (Reddy KS et al., 1998). In India, mortality due to CAD increased from 1.17 million to 1.59 million from 1990 to 2000 and is expected to rise to 2.03 million by 2010 (Ahmad N et al., 2004). It has been predicted that deaths due to CAD are likely to be more than any other disease in India (Balarajan R, 1991). Cross-sectional studies done in India also have shown that CAD prevalence is many
folds higher as compared to the developed countries (Mathur KS et al., 1960; Miller GJ et al., 1982; Beckles GL et al., 1986; McKeigue PM et al., 1988; McKeigue PM et al., 1989; Balarajan R et al., 1991; Enas EA et al., 1996; Anand SS et al., 2000; Bahl VK et al., 2001). Studies done on Indians living in different parts of the world also have shown the increased risk of getting cardiovascular diseases as compared to the native populations (Mathur KS et al., 1960; Miller GJ et al., 1982; Beckles GL et al., 1986; McKeigue PM et al., 1988; McKeigue PM et al., 1989; Balarajan R et al., 1991; McKeigue PM et al., 1991; Enas EA et al., 1996; Anand SS et al., 2000). Further, in Indians, CAD tends to occur much earlier than any other ethnic groups (Bahl VK et al., 2001).

Prevalence of CAD in rural and urban populations of India has been investigated by many investigators and it is estimated that urban populations have more prevalence (8-13%) as compared to the rural population (3-7%) (Bahl VK et al., 2001). The difference could be attributed to the different risk factors present in two different setups. The proportion of the urban dwellers rose from 23.3% in 1981 to 32.1% in 2001 and a further increase to 42.8% is expected by 2021 (Reddy KS et al., 1993). People living in urban areas have increased access to higher fat and energy rich diet and are more likely to be living sedentary life style as compared to the people living in rural areas (Drewnowski A et al., 1997; Reddy KS et al., 1998, Yusuf S et al., 2001). Increased use of tobacco is also associated with urbanization in certain parts of the world (Muna FTW et al., 1993). Studies done in North India have shown prevalence of about 10% (Chadha SL et al., 1990) while it was found to be about 11% in Southern part of India (Mohan V et al., 2001).

Many studies have been done to look into the role of homocysteine in CVD in India and among Indians living in other countries (Karthikeyan G et al., 2002). Studies related to homocysteine levels done on Indians have given conflicting results (Chacko KA, 1998; Gheyse S et al., 1999; Chambers JC et al., 2000; Deepa R et al., 2001; Sastry BKS et al., 2001; Puri A et al., 2003). SHARE study involving about thousand individuals from three different ethnic groups (Europeans, Chinese and South Indians) confirmed that South Asians had high concentration of homocysteine as compared to other two
ethnic groups (Anand SS et. al., 2000). Although high levels of homocysteine were not found to be associated with CAD, Chambers et. al. conducted two parallel studies on Europeans and Indians to check the effect of post methionine load homocysteine on CAD (Anand SS et. al., 2000; Chambers JC et. al., 2000). High levels of homocysteine were found to be independently associated with CAD in both Europeans and Indians. Many other studies confirmed high levels of homocysteine in Indians but failed to show any association with CAD (Chacko KA, 1998; Deepa R et. al., 2001). In a recent study, Jain et. al. reported the significant high levels of plasma total homocysteine in hypertensive patients than controls (Jain S et. al., 2003).