CHAPTER 1: REVIEW OF LITERATURE

1. INTRODUCTION

1.0 The history of homocysteine

In the year 1932, du Vigneaud [1] first discovered homocysteine is a sulfur amino acid and as a product of methionine metabolism. Later in the year 1964, Mudd et.al., [2] and Carson et.al [3] in the year 1962, identified homocystinuria (excretion of homocysteine in urine) and hyperhomocysteinemia (increased levels of homocysteine in the blood) in mentally retarded children, as a result of deficiency of the enzyme cystathionine-β synthase. The link between homocysteine and vascular diseases was observed by Mc Cully in 1969 [4]. His studied identified vascular lesions in homocystinuria its associated with a defect in cobalamine metabolism. Later in the year 1976, Kanwar et.al [5] also observed that the vascular alterations were caused as a result of deficiency of the enzyme methylenetetrahydrofolate reductase in infants with homocystinemia.

The first link between homocysteine and ocular disease was made by Lowe, et. al [6] in the year 1952. He observed that disorder of an amino acid metabolism, mainly cystinosis in oculo-cerebral renal syndrome. Later, Carson et.al., in the year 1963 [7], observed that dislocation and crystalline deposit in the lens of the eye associated with moderate amount of homocysteine excreted in urine in two mentally retarded siblings. Ectopia lentis is the hallmark of homocystinuria due to the deficiency of the enzyme cystathionine-β-synthase (CBS) was observed by Spaeth in the year 1965 [8]. Spaeth proposed that homocystinuria, as a specific entity due to the malfunctioning of the enzyme CBS and pyridoxine deficiency [9]. In the same year, homocystinuria cases were observed in patients with cataract by Gaull [10], optic atrophy by Barber [11] and glaucoma by Kim and Spaeth [12] due to CBS and pyridoxine deficiency [11].

In the year 1996, Cruysberg showed that 76 % of patients having ectopia lentis had higher levels of homocystinuria due to the deficiency of CBS [13]. The importance of their study lies in the fact that deficiency of CBS, has been identified to be associated with other ocular complications, including cataract, strabismus, glaucoma, retinal detachment and unilateral blindness. An international survey on 629 patients with CBS deficiency by Mudd et.al., group in the year 1985 observed that these patients had a 50% risk of suffering a dislocation of lens and thromboembolic event before the age of 30 years [14] . The clinical implications of homocystinuria consist of dislocation of lenses, tendency to thromboembolic episodes, skeletal abnormalities includes spinal osteoporosis, arachnodactyly, thinning and elongation of bones, mental retardation, seizures and psychiatric disorders.

All the above studies provided the basis for further clinical and experimental studies on the relationship between increased levels of plasma homocysteine and vascular disease.

1.1 Measurement of plasma homocysteine

Total plasma Hcy consists of a free reduced compound, oxidized compound and protein-bound Hcy. In normal individuals and patients with mild to moderate amounts of Hcy is present as protein-bound. Blood sampling is a critical step in determining the total Hcy. The blood sample can be taken in EDTA tubes and separation of the plasma by centrifugation within 30 min will reduce the possibility of increase Hcy levels released by blood cells. Several methods have been developed for the routine measurement of total Hcy. They are given in following comparative table (table.1)

Table 1: Comparison of techniques

Techniques	**Detection**	Advantage	**Disadvantage**	**References**
TLC	Ninhydrin	Specific, sensitive, inexpensive equipment	Laborious sample preparation.	[15, 16]
Ion exchange	Ninhydrin	Reliable assay	Low sensitivity, low analysis time and low sample output.	[17]
Gas chromatography	Mass spectrometry	Specific, simultaneous determination of cysteine and methionine .	High specificity, not available in all the labs.	[18]
Radio isotopic techniques	Liquid scintillation counting	Specific, sensitive	Expensive more, scintillation counter necessary,sensitive to enzyme inactivity.	[16]
HPLC	Fluorescence	High specific, high sensitivity, measures other thiols	Careful maintenance of flow cell.	[19]
HPLC	Photometric	High sensitivity.	Laborious method	[20]
HPLC	Electrochemical	High sensitivity, specificity, No derivatization.	Detector maintenance. Needs two detectors. Flow cell maintenance.	[21]

1.2 Homocysteine metabolism

Homocysteine is a sulfur containing amino acid not available through diet. The known source of homocysteine in mammals is through the amino acid methionine. Homocysteine is produced through two pathways transmethylation and transsulphuration in methionine metabolism (Figure.1). In transmethylation, homocysteine acquires a methyl group from N-5-methyltetrahydrofolate (MTHF) from

methionine. The reaction with MTHF occurs in all tissues and its dependent on vitamin B_{12}. A proportion of methionine is then activated by adenosine triphosphate (ATP) to form S-adenosylmethionine (SAM). SAM serves as a methyl donor to a variety of acceptors, including nucleic acids, proteins, lipids, and hormones. S-adenosylhomocysteine (SAH), the byproduct of these methylation reactions, is subsequently hydrolyzed, thus regenerating homocysteine. It is a reversible reaction that favours the synthesis of SAH, and that elevated cellular concentrations of SAH are likely to be associated with all forms of hyperhomocysteinemia [22].

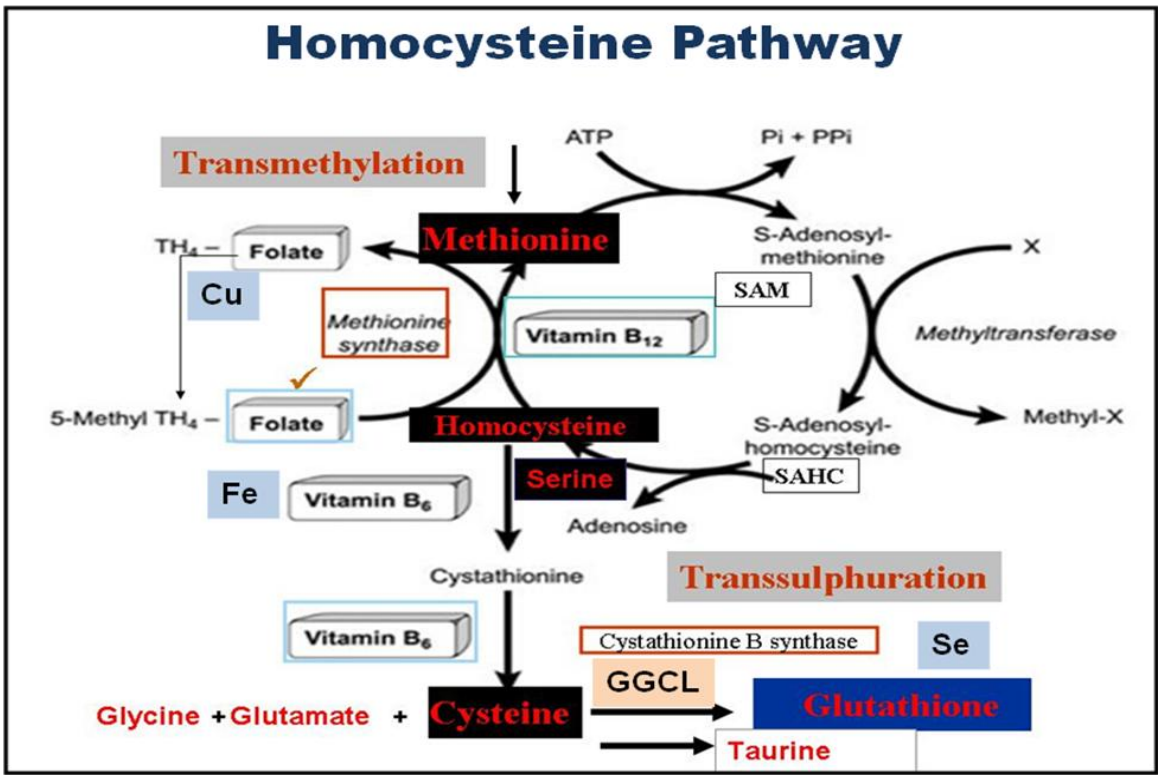

Figure 1 : Homocysteine Metabolisms

In the transsulphuration pathway, homocysteine condenses with serine to form cystathionine in an irreversible reaction catalyzed by the vitamin B_6 dependent enzyme, cystathionine β-synthase (CBS). Cystathionine is hydrolyzed by a second vitamin B_6 dependent enzyme, γ-cystathionase, to form cysteine. Excess cysteine is oxidized to taurine or is excreted in the urine [22, 23].

Since homocysteine is not a dietary constituent, methionine is the sole source of homocysteine. Due to the existence of a cellular homocysteine export mechanism, plasma normally contains a small amount of homocysteine averaging 5-15 μmole/L. Thus the catabolism of homocysteine through transsulphuration and transmethylation pathways help in the maintainance of low intracellular concentrations of this sulfur amino acid. In hyperhomocysteinemia, plasma homocysteine levels are elevated. The occurrence of hyperhomocysteinemia indicates that homocysteine metabolism has disrupted and excess homocysteine are liberates into the blood. The export mechanism limits intracellular toxicity, but leaves vascular tissue exposed to the possibly deleterious effects of excess homocysteine. Studies of the regulation of homocysteine metabolism have demonstrated that the utilization of homocysteine molecules by the transsulphuration and transmethylation pathways are also nutritionally regulated which is briefly discussed below [22, 23].

1.3 Homocysteinylation of proteins

Homocysteine-thiolactone is formed through a thio-ester of methionyl-tRNA synthetase (MetRS) [24]. It is due to an error editing reaction of protein biosynthesis by Hcy mistakenly replaced instead of methionine. Hyperhomocysteinemia is also occur through a homocysteine-thiolactone (HcyTL) apart from methionine diet [24]. In addition to the error editing, the accumulation of HcyTL becomes predominant also due to the inadequate supply of vitamins folate, B_{12} and B_6 [25, 26].

HcyTL which is capable of modifying proteins by binding amino acids lysine and cysteine proteins. These two protein-N-homocysteinylation and protein-S-homocysteinlation are reported in the literature [27] (Figure 2).

In addition to error editing formation of HcyTL, N-linked homocysteinylation and S-linked homocysteinylation are direct consequences of genetic effects of methylene tetrahydrofolate reducatase (MTHFR) and CBS [24]. Besides HcyTL also alters the function of proteins through a free thiol groups and inactivation of free amino acids affecting the redox potential of proteins and also triggering the activation of an inflammatory response [28].

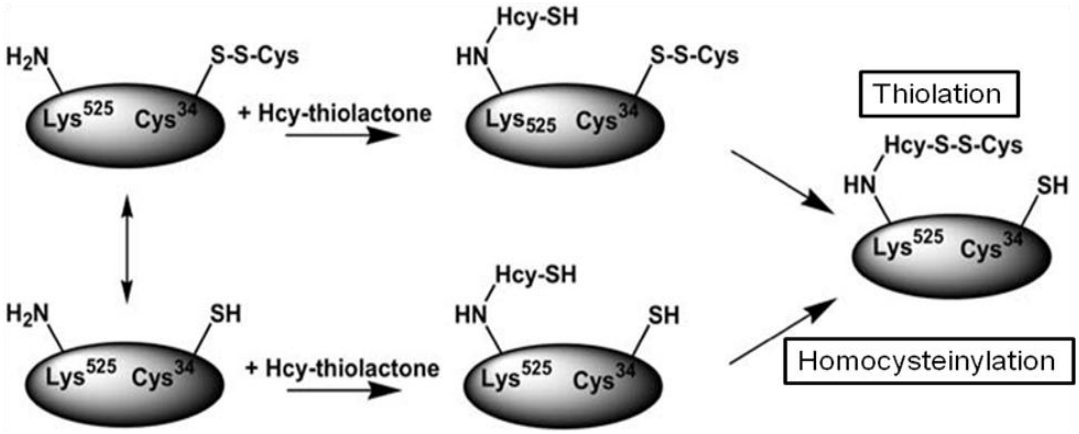

Figure 2: Homocysteinylation of proteins at lysine and cysteine residues

Adapted from Hieronim Jakubowski 2006.

1.4 Homocysteine and Iron

The role of iron in homocysteine metabolism is important as a co-factor for the enzyme CBS and MTHFR. Iron occurs in the oxidation states Fe^{+2} and Fe^{+3}. The ferrous ions are soluble in biological fluids and generate in the presence of hydroxyl radicals. The ferrous ions are unstable in aqueous media and tend to react with molecular oxygen to form ferric ions and superoxide anion radical. Paradoxically, despite the fact that both forms of iron, ferrous and ferric are so inaccessible, iron is the key catalytic site of many of the enzymes and oxygen-transporting proteins in cells.

1.5 Cystathionine β- synthase and Iron

CBS from higher organisms is the only known pyridoxal-L-phosphate (PLP) dependent enzyme to contain a heme cofactor [29]. CBS contains heme in N- terminal, PLP in central and CBS in C - terminal as shown in figure 3. The minor change of hydrogen bonding in heme alters the active site of PLP and these alterations affect the synthesis of CBS. Heme is necessary for maximal activity of CBS in higher organisms. The reduction of heme iron decreases CBS activity, since cystathionine is a precursor of glutathione, an activation of CBS under oxidative conditions appears to be an auto corrective response to the adequate supply of glutathione [30]. The reaction of ferrous-CBS with oxygen leads directly to the formation of ferric-CBS with concomitant

formation of superoxide free radicals without detectable formation of an intermediate species, consistent with an outer sphere electron transfer to oxygen [30].

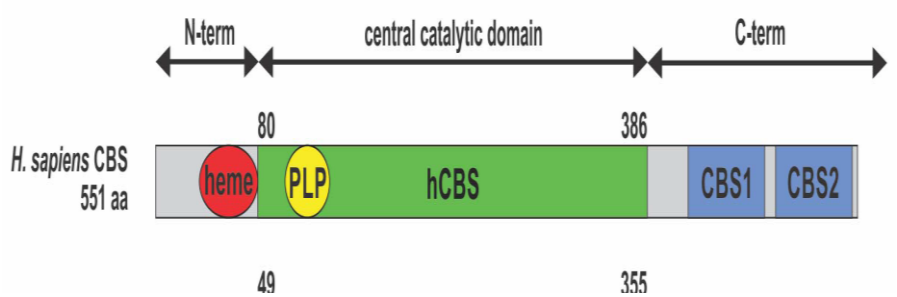

Figure 3: Structure of CBS enzymes

Adapted from Tomas Majtan 2014.

1.6 Homocysteine and Ferritin

Homocysteine-mediated cellular toxicity may be dependent in part on interactions with intracellular Fe^{2+} [31]. Nearly all iron in the body is tightly bound to specific carrier proteins (transferrin), storage proteins (ferritin), or other heme iron- containing and non-heme iron-containing proteins [31]. In non-erythrocyte tissues, a small intracellular labile pool of non heme and non ferritin-bound LMW-Fe^2 is maintained for incorporation into iron-containing proteins by a poorly characterized regulatory process that is sensitive to intracellular redox state and cellular iron stores [32]. This pool of intracellular LMW-Fe^2 is exchangeable with ferritin-bound iron, is readily chelatable, and is accessible as a catalyst for the production of hydroxyl radical in the Fenton reaction [33]. The hydroxyl radical is a highly reactive species that leads to lipid per oxidation, oxidative damage of DNA, cell dysfunction and death [34]. In cultured human dopaminergic neural cells, homocysteine-induced generation of reactive oxygen species is exacerbated by co-incubation with ferrous iron [35]. Inhibition of glutathione peroxidase (Gpx) by increased circulating homocysteine may promote hydroxyl radical production by maintaining metal ions in their reduced state [32].

A protein was purified from rat tissue that generated pABG (p-aminobenzoylglutamate) from 5-formyltetrahydrofolate [36]. The purified protein was identified as ferritin, the major iron-storage protein in the body [37]. Ferritin is

primarily localized in the cytoplasm and is comprised of heavy chain (HCF) and light-chain (LCF) subunits [37]. Both cell type and iron status determine the relative proportions of HCF and LCF [37]. Taher et.al., proposed that tetrahydrofolate derivative was catalyzed by iron [38]. HCF regulates intracellular folate concentrations, the mRNA and protein expression of ferritin was elevated in carcinomas cell [39]. In cancer, there is an increase in folate turnover and deficiency of folate due to increased ferritin levels especially HCF [39].

1.7 Clinical studies on the association of Homocysteine and Iron

The studies related to homocysteine and iron are limited until recently. Sullivan et.al., in the year 1981 [40] and Lapice et.al., in the year 2013 [41] proposed that excess iron and deficiency leads to cardiovascular diseases. Daher and Van Lente in the year 1995 [42] proposed that there is negative correlation between the serum iron and plasma homocysteine level while studies from Mattioli et.al [43] shows that there is a positive correlation between Hcy and iron in myocardial infarction. Pena- Duque et.al., shows that there is a significant positive correlation between Hcy and aortic-artery iron content in patients having coronary-artery surgery [44]. In the year 2011, Tamura et.al., observed that there is a significant positive correlation of serum ferritin and plasma homocysteine in cardiovascular diseases due to the deficiency of folate [45].

Above studies are showing that there is an association between the homocysteine and iron in vascular diseases. Further studies are needed to understanding the mechanisms of Hcy and iron in vascular diseases.

1.8 Glutathione synthesis

Glutathione (GSH) is synthesized from precursor amino acids glutamate, cysteine and glycine; it requires two ATP- dependent enzymes ϒ-glutamate-cysteine ligase (GCL) and glutathione synthetase by Meister 1983 [46, 47].

The first step of GSH biosynthesis is rate-limiting and catalyzed by γ-glutamate-cysteine ligase which exhibits an absolute requirement for Mg^{2+}. GCL is composed of a heavy (GCL-HS) and light (GCL-LS) [48]. The heavy subunit is active catalytically, it has a higher K_m value for glutamate and a lower K_i value for GSH compared with the holoenzyme [48]. Thus, the light subunit plays an important regulatory role for the

overall function of the enzyme and allows the holoenzyme to be catalytically more efficient and less subject to inhibition by GSH than the heavy subunit alone. The low affinity of the heavy subunit for glutamate and the high feedback inhibition exerted by GSH suggest that the heavy subunit alone is not likely to be active physiologically [49]. This remains to be proved.

Over expression of GSH synthetase failed to increase GSH level whereas over expression of GCL increased the GSH level, consistent with the fact that GCL is the rate-limiting enzyme of GSH synthesis (Figure 4 and 5).

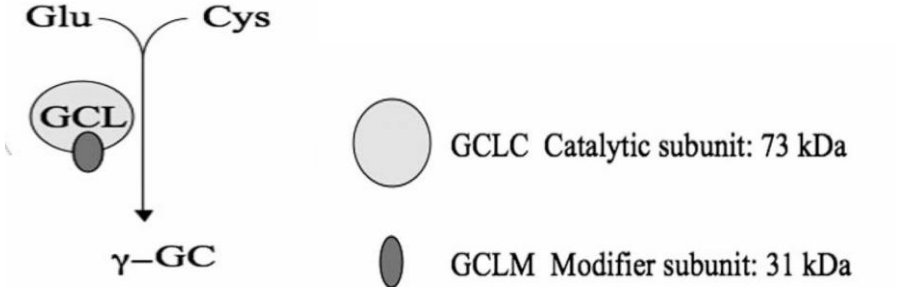

Figure 4: ϒ-glutamate-cysteine synthetase

Adapted from Christoper.C.Franklin, 2009.

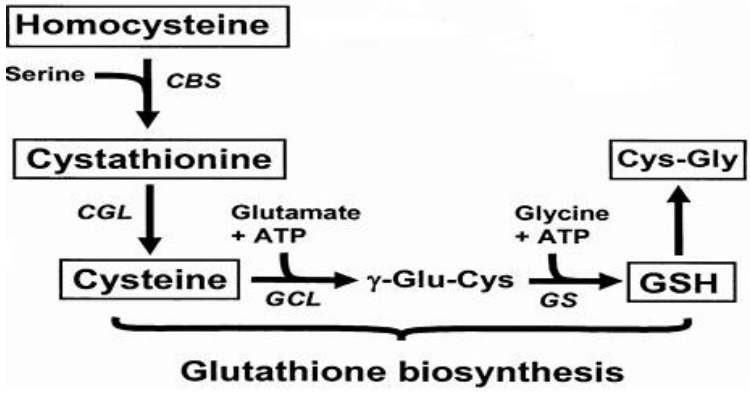

Figure 5: Glutathione synthesis

Adapted from Shelly, 2013

As a consequence of metabolism, all organisms are subject to a certain level of physiological oxidative stress. The intermediates that are formed, such as superoxide and hydrogen peroxide, can lead to the further production of toxic oxygen radicals that can cause lipid peroxidation and cell injury. The endogenously produced hydrogen

peroxide is reduced by GSH in the presence of selenium-dependent GSH peroxides. As a result, GSH is oxidized to GSSG, which in turn is reduced back to GSH by GSSG reductase at the expense of NADPH, forming a redox cycle. In the mitochondria, GSH is particularly important because there is no catalase enzyme present in mitochondria. GSH is critical in defending against both physiologically and pathologically generated oxygen free radicals. Severe oxidative stress may limit the ability of the cell to reduce GSSG to GSH, leading to accumulation of GSSG within the cytosol. To protect the cell from a shift in the redox equilibrium, GSSG can be actively exported out of the cell or react with a protein sulfhydryl group, leading to the formation of a mixed disulfide. Thus, severe oxidative stress depletes cellular GSH [49].

Why is GSH important for cellular function? To answer this question, The endogenous antioxidant protects cells from both oxidative and receptor-mediated glutamate toxicity without preventing depletion of GSH suggests its crucial role as a cellular antioxidant. GSH may be essential as an antioxidant to neutralize reactive oxygen species generated in mitochondria during normal respiration as well as by the metabolism of monoamines by mitochondrial monoamine oxidases, which have been implicated in glutamate toxicity. Understanding the cellular responses to GSH depletion may be helpful to the design of better methods for detecting oxidative stress in vivo as well as better treatments for diseases that involve oxidative stress.

1.9 Homocysteine and Growth Factors

CTGF (connective tissue growth factor) is a potent profibrotic factor implicated in fibroblast proliferation, angiogenesis and Extracellular Matrix (ECM) synthesis and its expression is regulated by several factors, such as transforming growth factor, high glucose, endothelin-1 and angiotensin II [50]. CTGF is a mediator in the ECM accumulation induced by homocysteine in human vascular smooth muscle cells (HUVSMCs). The molecular mechanisms linked to homocysteine induced ECM production in VSMCs involve several pathways such as homocysteine upregulates CTGF via activation of PKC in HUVSMCs by using two specific PKC inhibitors, H-7 and bisindolylmaleimide [50]. Another pathway implicated in the homocysteine action is the generation of intracellular ROS. It has been shown that hydrogen peroxide (H_2O_2) is a novel inducer of CTGF gene expression [51].

Transforming growth factor-β (TGF-β) has been identified as a potent inducer of CTGF expression and it is also a very important regulator of ECM synthesis in different cell types [52]. Thus TGF does not mediate the increase in CTGF expression stimulated by Homocysteine [53]. Intracellular ROS directly induces CTGF expression, through JAK activation, independently of TGF in human lens epithelial cells [51]. Chang et.al., show that a specific homocysteine induced down regulation of fibroblast growth factor disrupts endothelial integrity by both decreasing endothelial cell proliferation and inducing endothelial cell apoptosis [54].

Vascular endothelial growth factor (VEGF) has been known to induce migration and proliferation of endothelial cells, enhance vascular permeability, stimulate angiogenesis and modulate thrombogenicity [55]. It has been demonstrated that VEGF activates monocytes and promotes their migration. Importantly, VEGF was remarkably expressed in activated macrophages, endothelial cells, and smooth muscle cells in human coronary atherosclerotic lesions, but not in normal artery [56]. Hcy induces VEGF expression through the activation of unfolded protein response in endothelial cells and retinal pigment epithelial cells, which leads to apoptosis [57, 58]. Hcy can specifically down regulate FGF2 (Fibroblast growth factor) expression in arterial ECs reveal a novel mechanism [54]. Hcy can therefore be exhibiting synergistic EC toxicity with ox-LDL by a shared pathway related to FGF2 [54]. On the basis of the significant Hcy concentrations on EC (endothelial cell) growth and survival before mechanistic studies investigating whether Hcy can alter the expression of FGF2 or VEGF through specific regulatory pathways. On the contrary, high-dose Hcy at proapoptotic levels decreased intracellular FGF2 as well as VEGF, consistent with the activation of degradative pathways and cell death. Tyagi et.al., observed that homocysteine inhibits the VEGF protein expression in the mouse micro vascular endothelial cells [59].

1.10 Homocysteine and vascular diseases

Wilcken and Wilcken showed that the concentration of homocysteine-cysteine mixed disulfide after a methionine load was slightly higher in coronary heart disease (CHD) patients than age and sex matched controls in the year 1976 [60, 61]. This pioneering work has led to important studies, that allowed, for the conclusion that elevated levels

of total homocysteine in men and women are independent risk factor for CHD, cerebrovascular or peripheral vascular disease. Studies on hyperhomocysteinemia and vascular disease as observed by Mc Cully in the year 1990 [62, 63]. Elevated plasma Hcy is associated with an increased risk of cardiovascular and occlusive artery disease observed by Ueland et.al., in the year 2001 [64].

1.11 Homocysteine and other diseases

Elevated homocysteine levels are typically caused by either genetic defects in the enzymes involved in homocysteine metabolism or by nutritional deficiencies in vitamin cofactors (folate, Vitamin B_{12} and Vitamin B_6). Other conditions affecting homocysteine metabolism include chronic kidney disease, hypothyroidism, psoriasis, certain cancers, and several drugs most commonly methotrexate, phenytoin, theophylline, niacin, and immunosuppressive agents.

Many drugs have an effect on the homocysteine metabolism. These drugs are decreases the synthesis of DNA and RNA nucleotides, which are necessary for cell function and reproduction. The drugs are listed in the table 2 as follows;

Table 2: Drugs induced the homocysteine metabolism and the mechanisms are listed

Drugs	Suggested mechanisms	References
Fibric acid derivatives	Reduction of glomerular function, There are no sources in the current document. Increased homocysteine, Increased creatinine, PPARα-activation.	[65]
Niacin	Increased homocysteine Vitamin B_6 metabolism Increases methylation demand	[66]
Diuretics	Increases of homocysteine, Decreased glomerular filtration rate	[65]

Statins	Increases homocysteine Decreased the MTHFR synthetase and ADMA synthesis.	[67]

During the past decade, several large prospective trials have established homocysteine as an independent risk factor for stroke, Coronary heart disease, venous thromboembolism, and death. The meta-analysis of Humphery et.al., in the year 2008 [68] reconfirms these findings and shows that homocysteine remains a significant predictor of new cardiovascular events in persons without known coronary heart disease, independent of other Framingham risk factors in the year 1971 [69].

In the *Heart outcomes Prevention Evaluation 2 trial* of patients with diabetes or vascular disease-vitamin therapy significantly reduced stroke (by 25%) but not myocardial infarction or death observed by Sleight et.al., [70]. Homocysteine concentrations were measured in only 19% of participants after 5 years, and the reduction in homocysteine concentration was not statistically significant. The clinical trials done are listed in the table.3.

Table: 3 Randomized Clinical Trials of Therapy to Decrease Total Homocysteine Level in Patients with Vascular Disease.

Study (reference)	Year of Study	Disease Study	Medication details	Sample Size	Results status
Vitamins to prevent stroke (VITATOPS)	2000-2002	Stroke or transient ischemic attack	folic acid 2 mg, vitamin B_6 25 mg, vitamin B12 500 μg.	8000	A vitamin lowers Hcy levels but still trial is going on. [71]
Vitamin Intervention For Stroke Prevention Trial	2002-2004	Stroke	40 mg of vitamin B_{12}, 25 mg B_6, 2.5 mg of Folic acid	3680	RR 1.0 (0.8 – 1.1). It was terminated within two years due to no difference in the vitamins of low and high doses of the patients. [72]
Randomized study by Wrone et al	2002-2004	Cardiovascular disease	12.5 mg of vitamin B_6, 6 g of vitamin B_{12}, 60 mg of vitamin C, 1.5 mg of vitamin B_1, 20 mg of vitamin B_3, 10 mg of vitamin B_5, and 0.3 mg of biotin.	510	No difference in treatment versus controls. [73]
Homocysteinemia in kidney and end-stage renal disease	2004	Chronic kidney disease	1 mg of folic acid, 10 mg vitamin B_6 and 6 μg of vitamin B_{12}	2006	Enrolling , study its still ongoing. [74]
Atherosclerosis and Folic Acid supplementation	2002-2006	Myocardial Infarction, Stroke and Cardiovascular disease	600 μg of folic acid.	315	RR 0.98 (0.66 - 1.47). Hcy-lowering response in the folic acid group were not significant. [75]
The Heart Outcomes Prevention Trial	2002-2006	Cardiovascular disease	2.5 mg of folic acid, 50 mg of vitamin B_6, and 1 mg of vitamin B_{12}.	5522	RR 0.95 (0.84 – 1.07). There was no difference in the treatment of vitamins. [76]

Folic acid for lowering of tHcy in renal Transplantation	2006	Coronary, Cerebrovascular and Peripheral vascular disease.	5 mg folic acid,50 mg B_6, 1 mg B_{12}.	4000	Study it's still ongoing [77]
Norwegian Vitamin Trial	2006	Myocardial Infarction	0.4 mg of vitamin B_{12}, 40 mg B_6, 0.8 mg of Folic acid.	3749	RR 1.22 (1.00-1.50).No significant differences in vitamins treatment to lowering hcy. [78]
Western Norway Vitamin –B Intervention	2007	Cardiovascular death, Myocardial Infarction	0.8 mg folic acid, 0.4 mg B_{12} and 40 mg B_6.	3090	No significant differences in vitamins treatment to lowering hcy. [79]
Vitamins and Thrombosis (VITRO) study	2007	Thrombosis	5 mg folic acid,50 mg pyridoxine and 0.4 mg cyanocobalamine.	701	Vitamin B supplementation doesnot lowered homocysteine. [80]
The Heart Outcomes Prevention Trial 2	2009	Stroke	2.5 mg of folic acid, 50 mg of B_6 and 1 mg of B_{12}.	5522	Lowering of Hcy with folic acid,B_6 and B_{12} reduce the stroke but not in severity case. [81]

1.12 Other Diseases

There is continuing uncertainty whether Hcy has a causal role in the development of atherosclerosis or is simply as a marker for increased vascular risk. Evidence to support a direct role for Hcy in the pathogenesis of vascular disease has emerged from studies showing a dynamic and inverse relationship between plasma Hcy and vascular endothelial function.

Hcy metabolite generates reactive oxygen species that can directly injure the endothelium. Hcy has been shown to inhibit nitric oxide synthase activity, leading to endothelial dysfunction [82]. These observations are consistent with reports of dose and time dependent effects of Hcy on endothelial cellular function in-vitro [82]. These

findings suggest that even diet-related increments in plasma Hcy contribute to the development and progression of atherosclerosis.

The reduced Hcy directly alters vascular cell function. Because reduced Hcy undergoes oxidation *invivo*, one could argue that the Hcy oxidation products such as hydrogen peroxide, superoxide anion radical, and other reactive oxygen species are the injurious agents. Many of the *in vitro* studies using cultured endothelial and smooth muscle cells from human and animal vessels during the past decades used exceedingly high concentrations of reduced Hcy, so far exceeding physiological and pathophysiological concentrations of reduced Hcy. Another problem is that many of these studies failed to demonstrate specificity for Hcy and in many cases found a general thiol effect [83, 84].

Recent *invivo* work by Lentz and colleagues has determined that elevated plasma Hcy concentrations impair the normal vasodilatory response of the endothelium leading to increased arterial thrombosis. In mice and other animals, HHcy leads to endothelial dysfunction, vascular hypertrophy, accelerated thrombosis, and predisposition to atherosclerosis [85-88]. Similar vascular phenotypes are observed when HHcy is induced by a variety of different genetic or dietary approaches. These findings in animal models suggest, but do not prove, that elevated Hcy itself is a causative factor in vascular dysfunction.

The kidney plays a major role in plasma amino acid clearance and metabolism [89]. There is considerable controversy surrounding the extent of homocysteine metabolism and its mechanisms in kidney. One potential mechanism for endothelial dysfunction in renal disease is the accumulation of asymmetric dimethylarginine an endogenous inhibitor of nitric oxide synthase [90]. The dialysis patients advised to increase their nutrient intake to gain weight and to increase their serum cholesterol and Hcy concentrations. It will be necessary to confirm the relationships between Hcy, renal function, and vascular outcomes in larger data sets and other trials before concluding that mild hyperhomocysteinemia is simply a marker of decreased renal function rather than an independent vascular risk factor.

1.13 Homocysteine and Eye

In the year 1993, a case control study, reporting that elevated plasma Hcy level as an independent risk factor for retinal vascular occlusive disease. In this study, the estimation of Hcys was done by high performance liquid chromatography [15]. There is a debate on the role of Homocysteine in ocular diseases. Hyperhomocysteinemia may cause vascular endothelial injury, hypercoagulability, occlusion and ischemia changes disturb the ocular development observed by Maestro de las Casas in the year 2003 [91] and Lattanzio et.al., in the year 2006 [92]. However, De Luis in the year 2005 observed that there is no association between homocysteinemia and diabetic retinopathy [93]. In the year 2000, Sulochana et.al., observed the association of homocystinuria and also decreased levels of GSH in 29 persons among 29 patients , 5 had subluxation of lens, 4 had developmental cataract and 20 had congenital cataract children [94]. In a meta analysis of seven independent studies on a total of 465 people with central retinal vein occlusion having a higher plasma Hcy levels in cases when compare to controls in the blood. Similarly, the plasma Hcy levels were higher in patients suffering retinal artery occlusion compared to controls. In the year 2006, Coral et.al., observed that hyperhomocysteinemia with decreased thiol content play an important role in exudative ARMD leads to vascular dysfunction [95]. Bharathi et.al., observed that, hyperhomocysteinemia with decreased arylesterase activity as a risk factor for CRVO in the year 2007 [96]. In the year 2008, Coral et.al., observed that there is an association between intravitreal homocysteine and proliferative diabetic retinopathy [97]. Nguyen in the year 2009 confirmed that there is an association between homocysteinemia and diabetic retinopathy, surprisingly but they are not found the same association after normalizing for other causative factors [98]. Ganapathy et.al., in the year 2009 observed that elevated homocysteine due to the deficiency of cystathionine-β-synthase enzyme leads to retinopathy in some cases of diabetic patients not in all the cases [99]. Besides, they have also showed that loss of retinal ganglion cells are due to the pathologic effect of diabetes itself, but not due to the elevated Homocysteine [100]. A case controlled study by Bhanuprakash Reddy et.al., in the year 2011, observed that vitamin B_{12} deficiency could be a risk factor for diabetic retinopathy, because deficiency of vitamin B_{12} leads to hyperhomocysteinemia [101]. Retinal and optic nerve alterations are mainly due to the deficiency of cobalamine and cystathionine-β-synthase. Elevated plasma Hcy levels were observed

in secondary open angle glaucoma in the presence of a pseudo exfoliation syndrome (PEX), due to a mutation of the CT genotype of an enzyme MTHFR [102]. Hyperhomocysteinemia is the risk factor leads to vascular diseases of the patients with exfoliation syndrome [103]. Homocysteine was not elevated in aqueous humour of exfoliation syndrome observed by Tuomo puusti et.al., in the year 2004 [104] however, it is contributing in the abnormal ECM formation and associated with the PXF associated systemic and ocular vasculopathy [105]. However, the biochemical link between Hcy and pseudoexfoliation syndrome is still unclear. Hyperhomocysteinemia is one of the causative agents for endothelial damage in Behcet's disease and it may be an additional risk factor for retinal vascular diseases was observed by H Er et.al., in the year 2002 [106]. However the etiology between the homocysteine and ocular diseases is still unclear.

1.14 The effect of vitamin supplementation in hyperhomocysteinemia

Folic acid promote an endothelial function and support cell growth through mechanisms that are independent of lowered homocysteine levels [107]. This effect may explain the increased rate of in-stent restenosis in patients treated with B vitamins after percutaneous interventions [108]. Moreover, folic acid and vitamin B_{12} may alter the methylation potential of vascular cells, resulting in a change in the cell phenotype that promotes the development of plaque [107]. Vitamin B_6 is involved in numerous enzymatic reactions and biological functions, including cell growth, immunocompetence, and cholesterol metabolism, high levels of B_6 may inhibit angiogenesis.

Until now, there have been no controlled data for vitamins B_2, B_6 and B_{12} on the effects of homocysteine lowering treatment of vascular function or clinical end points. The precise mechanisms by which homocysteine mediates its adverse effects are unknown, but they may relate to impaired smooth muscle cell function.

In the case of homocystinuria, the results of most prospective studies, and the relation between hyperhomocysteinemia and atherosclerosis suggest that elevated Hcy is a causal risk factor for CVD, including venous thrombosis. Hyperhomocysteinemia as an isolated phenomenon probably confers minor risk, but it further increases the risk

when it occurs in combination with other factors that provoke vascular lesions. Thus, hyperhomocysteinemia seems to be a particularly strong risk factor in subjects with an underlying disease and predicts the short-term outcome in such individuals. The impairment of the nitric oxide – dependent flow-mediated vasodilatations during transient hyperhomocysteinemia provides one plausible mechanism accounting for the effect [107]. Further studies are needed to conclude whether homocysteine is a causal risk factor for the cardiovascular disease or arteriosclerosis.

Homocysteine, however, remains an important field of study as an unconventional risk factor. The relevance of much of the experimental data to patients with hyperhomocysteinemia remains unclear and further work must be performed for homocysteine to examine the specific mechanisms of atherogenesis and thrombosis. An understanding of these mechanisms by which homocysteine can modulate angiogenesis may suggest novel therapeutic strategies for cardiovascular disease and cancer.

Further studies are now being conducted in order to estimate whether total homocysteine is a risk factor of ocular diseases. Furthermore, the studies will estimate whether homocysteine is induced by iron or vice versa will accomplish a reduction of the rate limiting enzyme ϒ-glutamate-cysteine (EC 6.3.2.2) and leads to decrease the synthesis of glutathione.

1.15 Factors influencing plasma homocysteine levels

1.15.1 Age and gender

Age and gender among male are associated with a higher total Hcy concentration [109]. Since the total Hcy concentration was found higher in post-menopausal women compared with premenopausal women [109, 110]. Hcy concentration was higher in male compared to women and increased progressively with age. In our hospital based study (tertiary eye care hospitals – Sankara Nethralaya) of homocysteine estimated by ELISA for four years, we have observed that the elevated plasma levels of homocysteine in the age group of 20 - 40 years are more significant in males when compared to the females follow by the 0-20 years and 40-60 years people, but in the age group of 60 – 80 years the values are not significant, it suggested that the elevated

levels of plasma homocysteine is may be due to the nutritional, genetic and environmental factors. The normal value of homocysteine is 0-15 μM. The values (unpublished SNSC laboratory reference data) are listed in the table 4 as follows.

Table 4: Demographic details of the patients based on the age and sex matched groups.

Age / Years	Estimation of Homocysteine by ELISA		P Value
	Male	Female	
0 – 20	17.2 ± 10.9	12.8 ± 6.5	0.02
20 – 40	16.5 ± 9.7	11.8 ± 5.5	0.001
40 – 60	16.4 ± 10.0	12.8 ± 4.6	0.04
60 – 80	17.4 ± 12.2	14.8 ± 6.7	0.2

1.15.2 Dietary factors

Several intervention studies have provided evidence for the importance of B vitamins in homocysteine metabolism. Supplements with folic acid and combinations of folic acid, vitamin B_2, B_6, and B_{12} effectively reduced the Hcy concentration [111, 112]. Randomized clinical trials showed that folic acid supplementation reduced Hcy concentrations by 25% with similar effects in a daily dosage of 0.5 to 5.0 mg [113]. Conversely, vitamins B_2, B_6, and B_{12} are not utilized when homocysteine is metabolized; they function as cofactors of enzymes involved in homocysteine metabolism [114]. Most of the homocysteine lowering trials with folic acid are currently underway in vascular patients, which are expected to clarify whether or not folate therapy is protective and relevant to vascular diseases [114].

1.16. Retinal vascular diseases such as Eales' Disease and Age related macular degeneration

1.16.1 Eales' Disease

Eales' disease (ED) is an idiopathic retinal periphlebitis characterized by capillary non-perfusion and neovascularization. The fundus image was shown in figure 6. It was first described by Henry Eales in 1880. The disease is more prevalent in the Indian subcontinent than anywhere else in the world [115]. It occurs primarily in otherwise healthy adult males between 15 and 40, initially presenting as retinal periphlebitis and later as retinal ischemia that may lead to neovascularization. Oxidative stress at the level of systemic circulation and local environment (i.e. Vitreous humour and epiretinal membrane) has been found to be related to ED pathology [116, 117]. Accumulation of AGE and lipid oxidation end products in epiretinal membranes, observed in patients with ED [118]. The pathophysiological mechanism of Hcy toxicity, however, remains unclear to date. The mechanism of Homocysteine and Homocysteine thiolactone are need to study, we taken up the ED as a model for retinal vascular diseases.

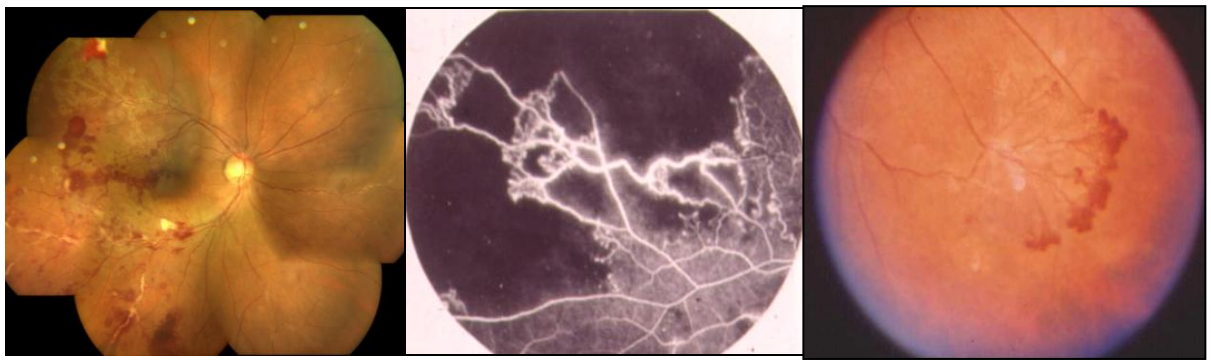

Figure 6: Fundus image of Eales' Disease

1.16.2 Age related macular degeneration

Age-related macular degeneration (ARMD) is a leading cause for legal blindness in the western world in persons older than 60 years of age [119]. It is a complex multifactorial disease that affects the central region of the retina. It has two main presentations, atrophic and exudative. Exudative ARMD is characterized by the

presence of choroidal neovascularization with disciform scar [120]. The fundus image was shown in figure 7. Although the cause of ARMD remains unknown a number of associated risk factors with ARMD have been identified. These factors include age, heredity, high blood pressure, high levels of serum cholesterol and obesity [121, 122]. In addition exposure to cigarette smoke is strongly associated with the disease [123]. Increased oxidative stress and decreased nitric oxide levels have been implicated in choroidal perfusion and drusen formation in ARMD patients [124]. Increased plasma levels of vascular endothelial growth factors, von willebrand factors, and endothelial cell damage were also reported in ARMD. These findings together, suggest an association of markers of angiogenesis, hemostasis, oxidative stress and endothelial dysfunction with ARMD. The mechanism of Homocysteine and Homocysteine-thiolactone are need to study, we taken up the ARMD as a model for retinal vascular diseases.

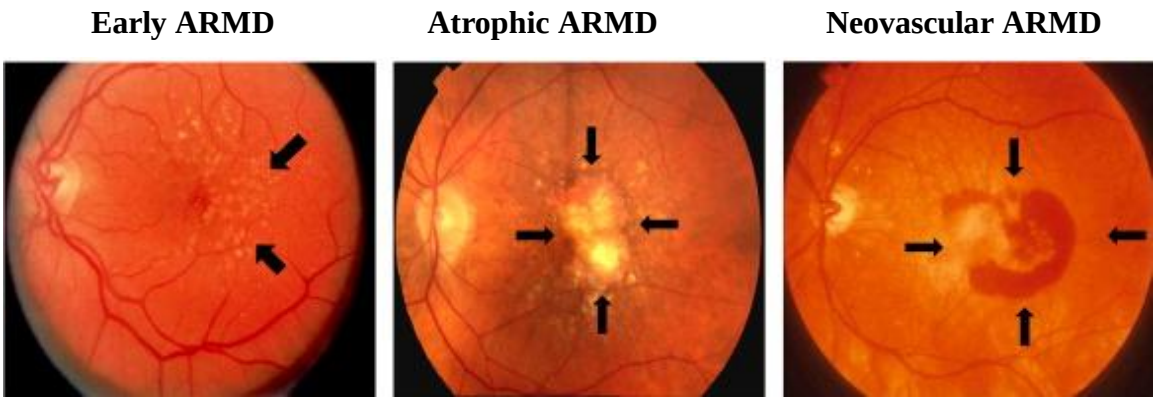

Figure 7: Fundus image of ARMD

Gaps in Existing Research

From the above literature review, it's clearly understandable that there is no effective medical treatment for hyperhomocysteinemia available presently which can combat these complications. Medical treatment would only be possible if the exact biochemical mechanisms leading to the pathogenesis of hyperhomocysteinemia are known. Considerable progress has been made in understanding the mechanisms of this complication, however, many gaps need to be filled in this area.

There is a little doubt about the hyperhomocysteinemia is associated with vascular diseases. Over the decades, some of the studies showing that hyperhomocysteinemia is an independent risk factor of cardiovascular diseases, stroke, myocardial infarction and

coronary heart diseases, whereas some studies shows that homocysteine is a casual risk factor for vascular diseases. In vitro experiments, homocysteine induces the expression of ϒ-glutamate-cysteine (GGC) through the transcription factor such as nuclear erythroid factor (Nrf2) in the hepatoma cell lines, whereas in other HUVSMCs lines it inhibited the expression GGC. Recent studies show that there is a link between homocysteine and iron involvement in vascular diseases. Still, there is a controversy.

1. Whether homocysteine is a really friend or foe to vascular diseases.
2. Is homocysteine really affects the GSH synthesis?
3. Is homocysteine affects the GCL activity?
4. Is there any relation between homocysteine and iron?
5. Is homocysteine induced iron or viceversa leads to vascular diseases.
6. Whether any infection or inflammation leads to increased the homocysteine level.

There is also no report about the levels and the methods of amino acids Methionine, Glycine, Cysteine, Glutamic acid, Taurine, Homocysteine and its role in Homocysteine metabolisms. There has been no substantial report on the association of homocysteine which in turn modify the proteins by Protein-Cys bound Hcy and Protein-Lys bound Hcy, antioxidant glutathione (GSH), ϒ-glutamate-cysteine in ED, ARMD and there transcription factors related to homocysteine. To answer the above questions, we proposed the study objective is to find out the mechanisms of iron and GSH related to homocysteine in peripheral blood mononuclear cells, retinal pigment epithelial cells and human umbilical vein endothelial cells. This would be a pioneering effort as till now no such study has been reported either at the national or international level.