CHAPTER 5: DISCUSSION

The initial aim was to develop a liquid chromatographic method to simultaneous determination of amino acids involved in Homocysteine pathway. The sulfur containing amino acids react with OPA as a thiol-containing component of OPA-β-mercaptoethanol reagent. The amino acids cysteine weakly reacts with OPA-β-mercaptoethanol derivative depend on presence of sulfhydryl groups on cysteine molecule. It was necessary to protect sulfhydryl groups of Homocysteine, cysteine and methionine before their reaction with OPA. Iodoacetic acid was added to standard mixture of amino acids after deproteinization of samples before addition of OPA reducing agent β -mercaptoethanol was used to sample to release Hcy and Cys. The data obtained demonstrate method of simultaneous measuring of Glut, Cys, Hcy, Gly, Met and Tau concentrations in blood plasma as OPA-β-ME derivatives in gradient elution mode. It took 30 min to separate OPA-β-ME adducts of above amino acids. We developed method to simultaneous detection of above amino acids using gradient elution with fluorescence detector in healthy donors and retinal vascular disease patients. It would provide a useful information about the level of amino acids and their roles involved in homocysteine pathway in development of vascular disease.

This method is useful for the simultaneous determination of amino acids involved in Homocysteine pathway, still now there is no method available. In the year 2013, Sochor et.al., [159] estimated the Cys and Hcy using electrochemical detector with capillary electrophoresis, it is a time-consuming for the analysis of Hcy in plasma. In the year 2012, pattengale et.al., [160] estimated the Hcy, Met, methylmalonic acid and methylcitric acid without the need of additional derivatization by LC/MS/MS. In the year 2003, Guan et.al., [161] estimated the simultaneous estimation of GSH, Hcy and Cys in biological samples using LC/MS, it is a selective and sensitive assay for the estimation of endogenous thiols. But in this method, cysteine was not able to estimated in biological samples due to the strong interferences of derivitizing agents [162]. In the year 2001, Tcherkas et.al., estimated the estimation of Hcy, Cys and Glutamic acid in plasma using isocratic reverse phase liquid chromatography with fluorescence detector [125] . All the above methods mentioned were able to estimate plasma Hcy only, some methods able to estimate Met, whereas some are able to estimate Glutamic acid, they were not able to estimate all the above mentioned amino acids involved in Homocysteine pathway.

But in the present study, developed a new simple method, fast, sensitive and simultaneous determination of above mentioned amino acids, involved in Homocysteine pathway.

The amino acids glutamic acid was significantly decreased whereas Hcy was significantly increased in ED and ARMD. Hcy estimation was done by this in-house developed HPLC method and compared with gold standard ELISA are comparable, thus validating the HPLC method is most reliable one [95, 116]. It shows the power of the assay, ELISA measure only Hcy, whereas HPLC being a separation method capable of measuring all the amino acids associated with Hcy in methionine metabolism. In the year 2006, Coral et.al., developed a method to estimate Hcy using OPA derivation with UV detector at 190 nm, in both the methods estimation of Hcy are correlated well [95]. The increased levels of Hcy in both ED and ARMD might be the role of Hcy as a risk factor in vascular eye diseases.

Glycine was significantly decreased in ED, while it was slightly increased in ARMD. Glycine has protective role against vascular damage. It decreases the expression of VEGF via reducing the advanced glycation end products (AGE) expression results in lowering nuclear factor kappa B (NFkappa B) in bovine retinal endothelial cells (BRECs) and thus glycine acts as anti-angiogenic molecule in endothelial cells by Barathi et.al., (in press) [163]. Glycine is an abundant aminoacid in extracellular matrix, as collagen is a major protein accounting for bulk of ECM, which contains glycine as 1/3 of its composition. Recently it is reported that MMP 9 activity is high in people with ARMD, justifying the increased levels of glycine in the plasma of people with ARMD [156, 164]. Similar to Glutamic acid, the amino acids cysteine decreased in both ED and ARMD, due to the decreased level of cysteine, glycine and glutamic acid, the plasma level of GSH was decreased in ED and ARMD. Surprisingly the levels of methionine were not changed in both the diseases.

Taurine is another sulfur aminoacid, which is not a constituent of proteins, but produced endogenously in Methionine metabolism. There was a mild elevation in the level of Taurine in ED, while it was slightly lowered in ARMD. The levels were not statistically significant in both cases. Taurine is getting importance as a nutritional supplement as this aminoacid is reported to antioxidant and anti-atherogenic functions in human [165]. These interesting results warrant further research in order to get the insights into their roles in endothelium.

Hcy has been shown to undergo autoxidation to generate reactive oxygen intermediates (ROI), such as superoxide anion (O_2^-) and hydroxyl radical (OH^-) thus initiating oxidation

of LDL, and this has been shown to induce vascular dysfunction [150]. HcyTL is a product of an error-editing reaction in protein biosynthesis which forms when Hcy is mistakenly selected by methionyl-tRNA synthetase [150]. HcyTL having the ability to form isopeptide bonds with protein lysine and cysteine residues, which alter the protein's function [150]. The present study shows elevated protein-Cys bound Hcy; protein-Lys bound Hcy, HcyTL and diminished GSH in patients with ED and ARMD, implying altered protein function playing an important role in disease.

An important factor affecting proteins and thereby producing vascular changes is through angiotoxic and atherogenic effects of HcyTL [25]. The amount of HcyTL formed is decided by concentrations of Hcy; the higher the Hcy higher the HcyTL [126, 152]. There are reports that some other factors could cause increased formation of HcyTL. Moreover, biochemical half-life of serum or plasma HcyTL is also quite low, i.e., 0.5 h and 1.5 h, respectively [126]. However, in its short survival period and in increased concentration, the HcyTL reacts with proteins and causes homocysteinylation. Homocysteinylation results in severe damage to protein structure resulting in changes in their function, leading to vascular pathology [152]. Hcy, as well as its metabolite, thioester HcyTL, play an important role in atherothrombosis in following ways. HcyTL modifies LDL [166, 167] and inhibits lysyl oxidase as well as insulin signalling [167]. Of special significance there is a degeneration of multiple retinal cells and photoreceptors by HcyTL in HHcy, which was observed in the present study. Hence, it is suggested that the retinal proteins get homocysteinylated to a significant extent in ED and ARMD, and this could contribute to ocular pathology.

The aetiopathogenesis of ED is not clearly known. Eales' disease is an idiopathic. However, recently literature associated ED with tuberculosis, but still now the active organism was not cultivated. The disease still remains idiopathic, with no diagnostic test available in the lab. Direct evidence of infection is not made. Similar to homocysteine, Iron also act as an independent risk factor of cardiovascular diseases. Roest et.al [168] observed increased iron stores leads to cardiovascular diseases similar to that of hyperhomocysteine. Sullivan et.al [169] observed that increased iron stores leads to endothelial dysfunction and that elevated homocysteine is dependent on iron overload. In this study, it was observed increased serum iron binding capacity but no change in serum iron, thus increased iron binding capacity is an indicative of early stages of development of iron overload. This excess iron generates free hydroxyl radical and causes oxidative stress through Fenton reaction. Schiepers et.al., [170]

observed that changes in both homocysteine and ferritin levels in cardiovascular disease. The serum ferritin is frequently used as a measure of iron stores. The present findings indicate iron storage since; there is an elevated level of ferritin and lowered transferrin in ED.

It is known that, red cell breakdown can occur outside or inside the vascular compartment in ED. Increased serum Hb in ED indicates the vascular damage due to intra vascular and extra vascular hemolysis. Extravascular hemolysis are occur due to phagocytosis whereas intravascular hemolysis as a result of mechanical injury and toxic factors. Heme is the prosthetic group of heme proteins such as haemoglobin, is an essential molecule plays a crucial role in cell differentiation and other functions. Free heme namely unbound heme can be toxic to cells, because it results in production of reactive oxygen species and causes cell damage. The absolute levels of Heme are regulated by its biosynthesis and catabolism. Heme biosynthesis is regulated by ALAS and its degradation to Fe, bilirubin and CO is catalyzed by HO, in this study, it is observed that both the enzymes are elevated in ED.

Heme oxygenase (HO) plays an important role in regulating the heme level by catalyzing the initial and rate limiting step of heme degradation and resulting in the formation of carbon monoxide, iron and bilirubin. Heme oxygenase exists as three isoforms; HO-1, HO-2 and HO-3. HO-1, the inducible 32-kDa isoform, HO-2, the constitutive 36-kDa isoform, and HO-3, has no activity and is not expressed in humans. The HO-1 is a member of the heat shock proteins, and its expression is influenced by hypoxia [171], heavy metals, ROS such as H_2O_2 [172], reactive nitrogen oxides [173], TNF α, interleukin β and interferon ϒ [174]. The biological functions of HO-1 are associated against oxidative and cellular stress. HO-1 represents a crucial mediator of antioxidants and possesses anti-inflammatory and anti-apoptotic properties[175] . L'Abbate et.al., [176] have shown that induction of HO-1 was associated with a parallel increase in the serum levels of adiponectin, which has a well documented anti-inflammatory property. The peroxisome proliferator-activated receptor (PPAR-ϒ) regulates the expression of HO (HO-1) in human vascular cells [177].

Over expression of HO-1 contributes in the revascularization of damaged tissue. In terms of neovascularisation, HO-1 having a proangiogenic, anti-inflammatory and antiapoptotic enzyme in regulation of wound healing [178]. Product of HO activity the bilirubin is a powerful antioxidant thereby, protecting the retinal cells. CO, a yet another product of heme

catabolism though not an antioxidant, it causes the induction of antioxidant genes [178]. It decreases superoxide anion and increases glutathione in blood vessels. Recent focus on understanding, how CO work as vasodilator resulted in the following facts, CO can increase cGMP similar to NO by increasing activity of soluble guanyl cyclase, thereby protect blood vessels against cellular ischemia [179].

However, during haemorrhage, the iron and bilirubin excessively produced and are neurotoxic, have deleterious consequences. HO-1 is an inducible enzyme whose activity increases in response to iron as well as heme, light, oxidative stress, and inflammation. The main condition for the initiation of neovascularisation is hypoxia [180]. Hypoxia up regulates the expression of angiogenic factors directly or through the hypoxia-inducible factor (HIF-1) [180] . HIF-1 activates several genes related to iron metabolism such as HO-1, endothelin-1, transferrin, transferrin receptor and ceruloplasmin [180].

HO, cleavage of the heme ring will release intracellular iron, which in turn increases the stimulation of ferritin. Dulak et.al observed that HO plays an important role in angiogenesis during hypoxia, similarly nitric oxide synthase, by VEGF production [181]. Alternately VEGF can stimulate HO-1 to promote angiogenesis and inhibiting leukocyte adhesion and transmigration [136]. In the present study, it's observed that both HO and VEGF increased due to inflammation and non-inflammatory responses.

Hepcidin, a circulating peptide hormone is mainly synthesized by the liver hepatocytes, and also in eye plays a major role in regulating iron homeostasis in the body [182]. The mature form is 25 amino acids with four inter subunit disulfide bonds. The massive iron overload found in hepcidin knock-out mice suggests that hepcidin is an iron stores regulator involved in communication of body iron status to the intestine and also in the retinal pigment epithelial cells [182]. The mechanism of hepcidin activity depends on hepcidin interactions with ferroportin. Ferroportin is the only known mammalian cellular iron exporter. Hepcidin regulates posttranslational ferroportin expression [182]. Hepcidin binds to ferroportin and causes its internalization and degradation in turn blocks the iron transport via ferroportin [182]. Hepcidin is reported to be elevated in chronic inflammation condition, anemia, more specifically wherein iron accumulates inside the cells [183].

Hepcidin synthesized can be induced by inflammatory cytokine IL-6, inflammation, infection [184]. IL-6 acts via its receptor and causes phosphorylation of signal transducer and activator of transcription 3 (STAT 3), STAT3 activation requires the presence of SMAD 4 to affect the HAMP gene expression [183]. Under normal conditions HAMP gene expression is regulated by BMP/SMAD and STAT3 pathways. Another hepatocyte iron sensors activating hepcidin synthesis are hemochromatosis protein (HFE) and transferrin receptor 2 (TfR2) [183].

Due to an intracellular storage of iron, there is an increased production of hepcidin. In the present study, there is an increased storage of ferritin levels and upregulation of hepcidin. Inflammation leads to cellular iron sequestering through IL-6 upregulation of hepcidin. Iron can be export from the cell with the help of iron exporter ferroportin. In the present study, we also observed that hepcidin expression was increased and ferroportin was decreased in ED compared to controls. It indicates that hepcidin binds to ferroportin, triggering its internalization and degradation. Interestingly cellular iron accumulation caused diminished ferroportin after hepcidin binding. There is an increased expression of hepcidin may be due to increased iron, inflammation and infection. Importantly, a cellular iron act as a cofactor of HIF factor, in the present study, it shows that there is a significantly increased expression of HIF1 α and also an increased expression of HIF2α. Previous reports says that hypoxia is a negative regulator of hepcidin expression, but in the present study, shows that HIF 1α and HIF 2α expression was increased in ED may be due to inflammation and also by IL-6. The interaction between hepcidin and ferroportin binding is a key step to control an iron homeostasis.

From the present study, observed that increased serum iron binding capacity, serum and intracellular ferritin, heme and HO, hepcidin play an important role in the vasculitis. The present study establishes the role for iron in disease pathogen. The increased levels of these proteins may be due to an infection and inflammation conditions. The real significance of these findings needs to be understood in an animal model or an in-vitro cell culture experiments. Thus dysregulation of iron homeostasis in ED as shown in figure 63.

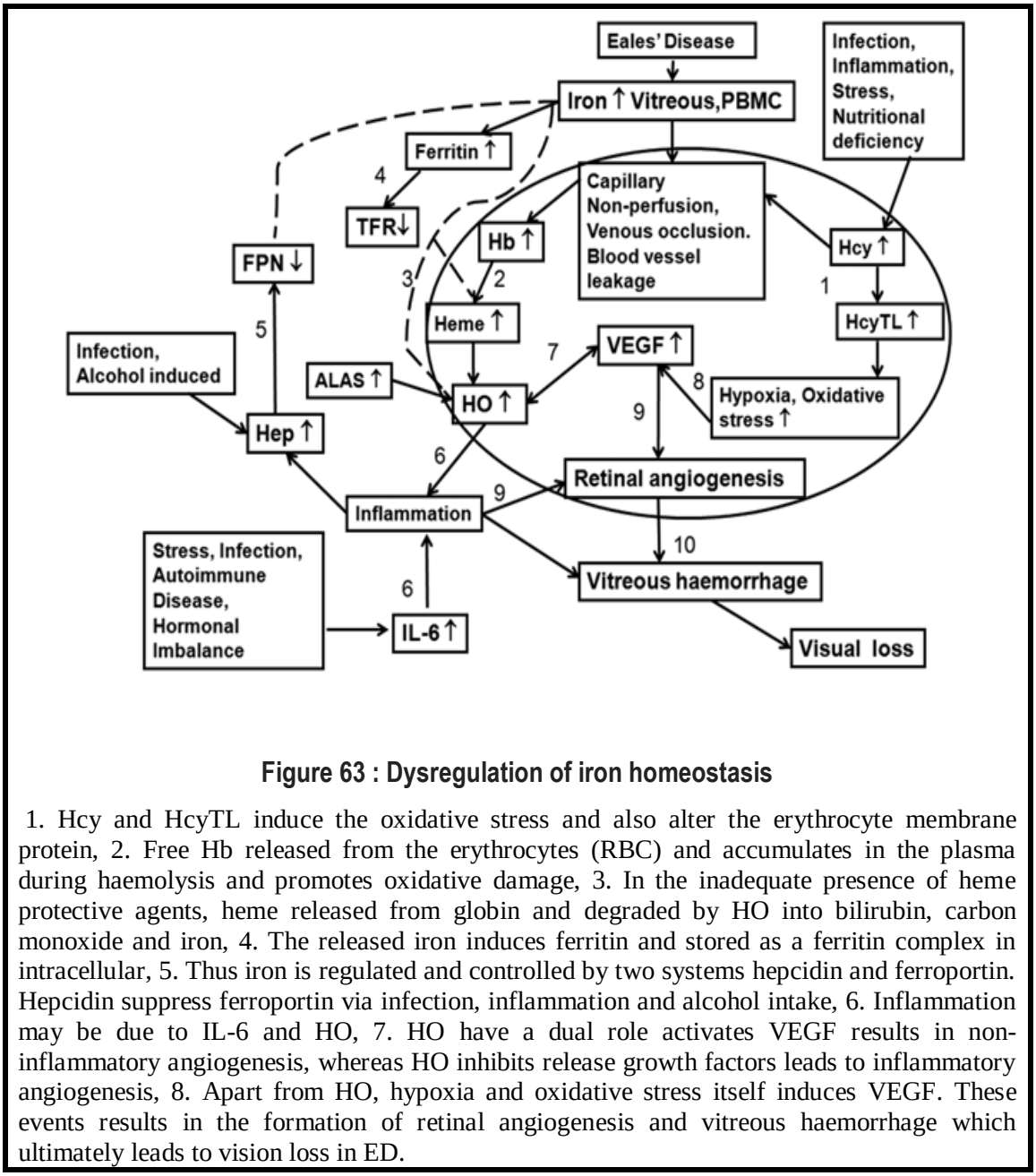

Figure 63 : Dysregulation of iron homeostasis

1. Hcy and HcyTL induce the oxidative stress and also alter the erythrocyte membrane protein, 2. Free Hb released from the erythrocytes (RBC) and accumulates in the plasma during haemolysis and promotes oxidative damage, 3. In the inadequate presence of heme protective agents, heme released from globin and degraded by HO into bilirubin, carbon monoxide and iron, 4. The released iron induces ferritin and stored as a ferritin complex in intracellular, 5. Thus iron is regulated and controlled by two systems hepcidin and ferroportin. Hepcidin suppress ferroportin via infection, inflammation and alcohol intake, 6. Inflammation may be due to IL-6 and HO, 7. HO have a dual role activates VEGF results in non-inflammatory angiogenesis, whereas HO inhibits release growth factors leads to inflammatory angiogenesis, 8. Apart from HO, hypoxia and oxidative stress itself induces VEGF. These events results in the formation of retinal angiogenesis and vitreous haemorrhage which ultimately leads to vision loss in ED.

GSH is the most important endogenous antioxidant in humans. Maintaining the intracellular thiols, such as GSH, in their reduced form, may allow for the maintenance of Hcy and other intracellular thiols in redox states [185]. It is often accompanied by other endogenous thiols, such as cysteine, cysteinylglycine and even Hcy. These thiols scavenge reactive oxygen species (ROS) and are involved in preserving the pro-oxidant–antioxidant balance in human tissues [186]. It has been reported that GSH and thiol content may decrease in certain pathological states and on account of the aging process. This study confirms the findings of the earlier study with respect to the diminished plasma levels of GSH in ED and ARMD patients [95].

GCL is a rate limiting enzyme and play a role in the GSH homeostasis and also in oxidative stress [187]. GCL is a heterodimer protein contains two subunits namely catalytic (GCLC) and modifier (GCLM) subunits [187]. The GCLC subunit having the catalytic activities of the enzyme, however GCLM subunit increases GCL activity (Vmax and Kcat), substrate affinity for glutamate and ATP, and the Ki for GSH feedback inhibition [187].

Thus the present study shows the decreased level of GSH. It also shows the decreased level of reduced and oxidized GSH in the plasma of ED. This is the first study, shows the level of GCL activity is decreased in serum and PBMC of ED. Thus the study shows that the decreased level of GSH synthesis is due to the decreased level of a rate limiting enzyme GCL activity through the decreased levels of amino acids cysteine, glycine and glutamic acid in ED. Both the GCLC and GCLM mRNA expression was decreased in PBMC of ED due to the decreased mRNA expression of nuclear related factor (Nrf2).

This was the first study proved the modelling and MD studies revealed that GCLC enzyme can bind to the native Cys and Cso (639) forms in presence of Glu (638) and cofactors namely, ADP (640) and $3Mg^{+}$ ions (641,642 and 643). The protein backbone RMSD of both the complexes (Cys-GCLC and Cso-GCLC) exhibits a stable backbone conformation which suggests that Cys639 and Cso639 are not playing any major role in GCLC protein backbone stability. Therefore, role of cofactors were investigated through binding pose analysis. The result suggests that in case of Cys-GCLC complex, due to the active role of Mg642 and Mg643 all the cofactors interacts with each other which results Glu638 and Cys639 closure enough to take part in the ligation reaction for Glutathione synthesis. Whereas, in case of Cso and GCLC complex Mg643 interacts with Cys and Mg642 interacts with ADP and Glu. No direct interactions were observed in between the Glu638 and Cso639. It suggests that Cys enhances the Glutathione synthesis by taking part in the ligation reaction while Cso brings hindrance in the Glutathione synthesis.

Retinal pigment epithelial cells are taken as a model for retinal vascular diseases. The study observed that increased concentration of Hcy decreased the levels of amino acids involved in the Hcy pathway. The level of intracellular GSH content was decreased while increasing the concentration of Hcy whereas the intracellular GSH content was increased while increased the concentration of cysteine. It could be noticed along with co-incubation of Hcy and Cys to

the cells, the intracellular GSH level was retrieved by 60%. It was proved by insilico modelling and MD studies of binding pose analysis of GCL with Hcy and Cys.

The mRNA expression of GCLC, GCLM and Nrf2 was not altered after exposing of Hcy in to the cells. GCL gene expression was induced by other factors including oxidative stress [188] and chemotherapeutic agents [189]. Mani et.al., observed that increased the mRNA expression and protein expression of GCLC and Nrf2 were increased with increased concentrations of Hcy in hepatoma cell lines [157].

Human umbilical endothelial cells are taken as a model for vascular disease. The level of intracellular GSH content was decreased while increasing the concentration of Hcy. The mRNA expression of GCLC, GCLM and Nrf2 was decreased while increasing the concentration of Hcy in to the cells. This is the first study proved that the GSH synthesis could be inhibited by Hcy through the inhibition of GCL and could be retrieved by adding Cys by both the *invivo* and *invitro* cell culture experiments. The alteration of GSH synthesis in ED as shown in figure 64.

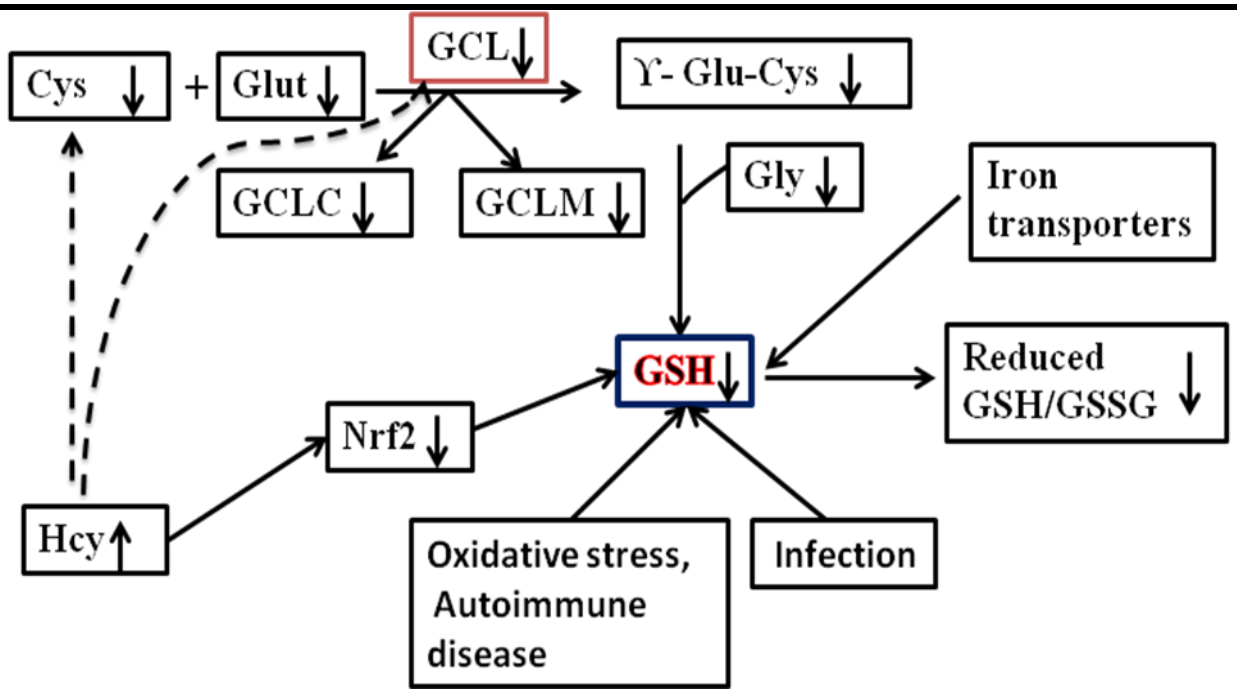

Figure 64 : Altered GSH synthesis in ED.