CHAPTER 4 : RESULTS

4.1 HPLC analysis of amino acids involved in homocysteine pathway

In the laboratory practice of clinical biochemistry, a simple optimized method for the simultaneous determination of sulfur containing amino acids namely, homocysteine, methionine, cysteine is not available. It was necessary for this research on homocysteine metabolism, to have a protocol which has the power to determine these amino acids, and the other amino acids involved in homocysteine metabolism namely glutamic acid and glycine. A simple and convenient, HPLC method for the estimation of these related amino acids was developed. The details of this novel method, standard graphs for the individual amino acids are given under method section (p.no.35) of this thesis. The standards were eluted as follows, Glutamic acid, Cysteine, Homocysteine, Glycine, Taurine and Methionine with the retention time points of 10.8, 11.5, 12.1 , 12.9 , 13.3 and at 16.1 min respectively. The same protocol was used to determine these amino acids in samples derived from controls, ED and ARMD groups of subjects throughout the study. The sample preparation steps and the detailed extraction of amino acids, derivation protocols are given under methods. This attempt indeed, generated a fully validated, simple, precise and sensitive analytical method for the determination of all amino acids involved in homocysteine pathway and it's useful for clinical and routine diagnostic purposes. The raw data, area under curve (AUC) was used for calculating the concentration of amino acids and it was represented as µmole/L and given as mean and $\pm$ standard error mean. The levels of amino acids as shown in table 17 and 18. Student't' test was performed and p valve of < 0.05 is considered as a significant.

4.1.1 Levels of homocysteine and its related amino acids in plasma samples of ED

It is observed, that the mean Hcy level in patients with ED was 2.5 fold higher when compared to their control group. It is statistically significant with a p value of 0.001. This rise in Hcy indicates a plausible role for this amino acid in the disease mechanism. Further, all the three constituent amino acids for the endogenous production of glutathione, namely, glutamic acid, cysteine and glycine are lower to the tune of 0.6 to 0.8 fold in ED group compared to the controls. Interestingly, there is no

significant change in methionine while taurine yet another non protein sulfur containing amino acid similar to Hcy is also increased by 1.2 fold in ED cases, indicating errors in methionine metabolism. The results are given in table 17 and in figure 23, 24.

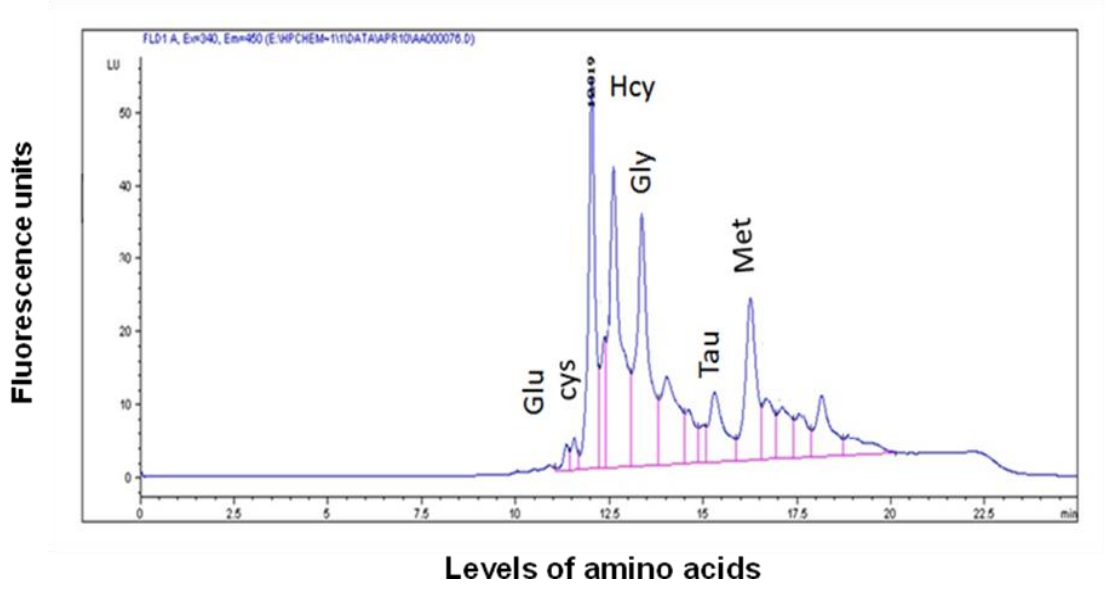

Figure 23: HPLC chromatogram of plasma samples of ED

Table 17: Comparative levels of plasma amino acids in controls versus ED

Sl. No	Amino acids	Controls (n = 20)	ED (n = 20)	P valve
1	Glutamic acid	30 ± 4	19 ± 1	0.018
2	Cysteine	228 ± 20	197 ± 12	0.193
3	Homocysteine	19 ± 2	49 ± 1	0.001
4	Glycine	108 ± 15	80 ± 6	0.08
5	Taurine	117 ± 9	149 ± 9	0.014
6	Methionine	158 ± 33	155 ± 8	0.9

All values are expressed as mean ± standard error mean.

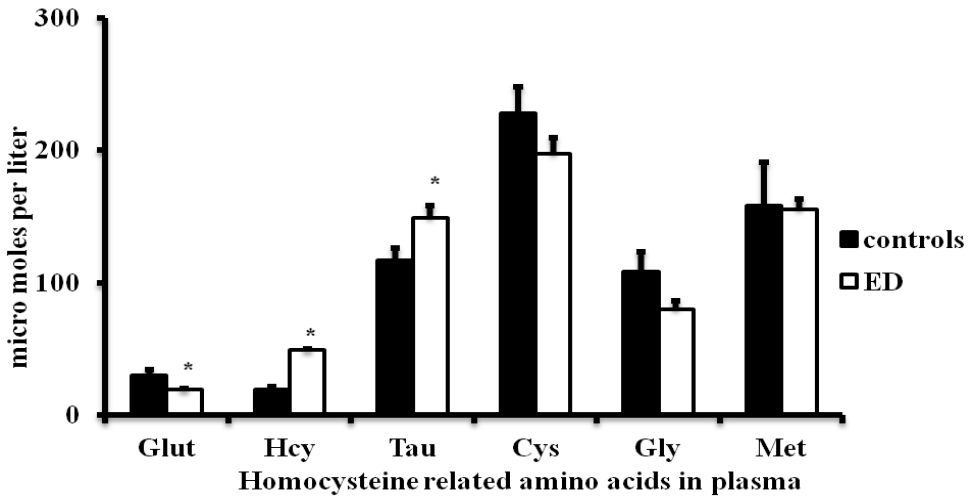

Figure 24 : The Bar diagram of plasma amino acids in controls versus ED

4.1.2. HPLC analysis of Homocysteine and its related amino acids in plasma samples of ARMD

Homocysteine level was 2.5 fold increased in ARMD compared to controls. Mean levels of, glycine was 1.0 fold increased, while glutamic acid and cysteine were decreased by 0.6 fold in ARMD group compared to controls. Both methionine and taurine were lowered in ARMD group when compared to control. The profiles of these amino acids collectively indicate the disease mechanism, even though HHcy is indicatively in both diseases ED and ARMD the pathophysiology of them seems to be different. The results are given in table.18 and figure.25, 26.

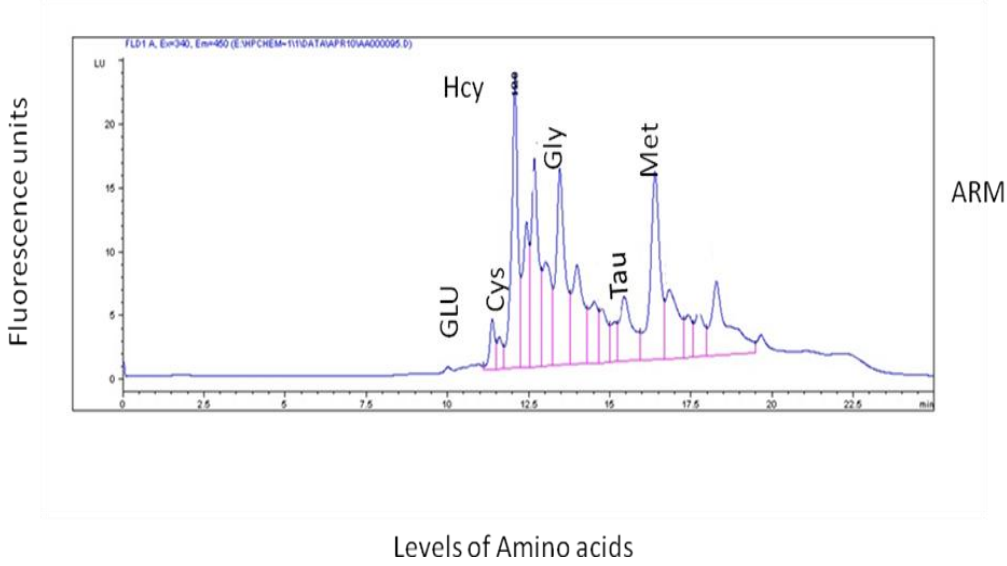

Figure 25: HPLC chromatogram of plasma samples of ARMD

Table 18: Comparative levels of plasma amino acids in controls versus ARMD

S.NO	Amino acids	Controls (n = 16)	ARMD (n = 16)	P valve
1	Glutamic acid	47 ± 5	30 ± 2	0.006
2	Cysteine	386 ± 68	227 ± 53	0.070
3	Homocysteine	11 ± 1	27 ± 2	0.01
4	Glycine	114 ± 14	122 ± 14	0.70
5	Taurine	53 ± 8	49 ± 8	0.47
6	Methionine	119 ± 38	84 ± 28	0.70

All values are expressed as mean ± standard error mean.

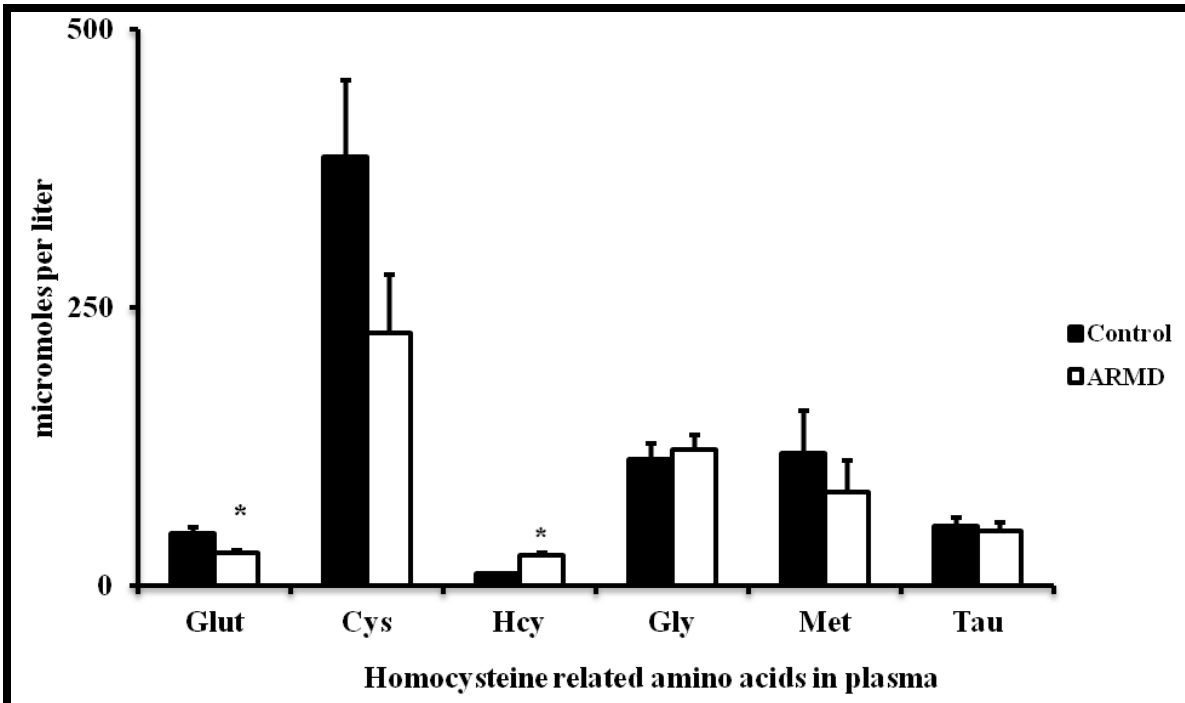

Figure 26: The Bar diagram of plasma amino acids in controls versus ARMD

It was observed that there is an increased level of homocysteine in the ED and ARMD, when compared to the controls by this method, similar results for homocysteine in both ED and ARMD were obtained by ELISA method also. Homocysteine is a sulphur containing amino acids formed from methionine. Homocysteine is a risk factor associated with cardiovascular diseases, cerebrovascular diseases, with ocular complications [149]. The ELISA values of homocysteine in ED and ARMD are given in the table 19. Both the methods ELISA and determination of homocysteine by HPLC using FLD detector are correlated well.

Table 19: ELISA levels of plasma Hcy in controls versus diseases (ED and ARMD)

S.NO	Homocysteine (µM)	Controls	Diseases	P valve
1	ED (n = 20)	11.2 ± 0.64	18.6 ± 1.77	0.001
2	ARMD (n = 16)	10.5 ± 0.6	16.7 ± 1.2	0.01

4.2. Structural damage to proteins due to Hcy and its metabolite HcyTL in ED and ARMD

Metabolites of homocysteine, namely mixed disulfides and homocysteine-thiolactone (HcyTL) are toxic, since they alter the structure and function of proteins [150] . Besides, increased levels of Hcy result in abnormal chelation of the trace element copper, leading to the possible loss of this element, a leading cause for cardiac diseases [151]. Increased levels of HcyTL in ED and ARMD are observed and increased levels may be due to increased Hcy in the patients [152]. Increased Hcy and HcyTL leads to protein alterations and diminishes the antioxidant capacity observed in patients with ED and ARMD. The levels of HcyTL in ED and ARMD are given in the table 20.

Table 20: The levels of plasma HcyTL in controls versus diseases (ED and ARMD)

S.NO	HcyTL (nmole/L)	Controls	Diseases	P valve
1	ED (n = 20)	7.1 ± 0.94	45.3 ± 6.8	0.00002
2	ARMD (n = 16)	8.9 ± 2.1	18.9 ± 3.0	0.01

4.2.1 Analysis of protein modification, namely protein-Cys bound Hcy and protein-Lys bound Hcy by HPLC using FLD in ED

The atherogenic effect of Hyperhomocysteinemia may be accounted for HcyTL. HcyTL is formed from Hcy as a result of an error editing in the formation of methionyl-t-RNA. It was observed that increased HcyTL in ED may be due to increased Hcy. HcyTL has the ability to form isopeptide bonds with protein lysine and cysteine residues, which impairs or alters protein functions. We estimated lysine and cysteine bound Hcy in the plasma proteins of study subjects by HPLC.

It was found that protein-Cys bound Hcy (S-Cys) and protein-Lys bound Hcy (N-Lys) were increased from 2.6 to 3.8 µM ($p = 0.030$) and 2.4 to 3.1 µM ($p = 0.038$) in ED were shown in figure 27. Thus, apart from the damage through the reactive oxygen species, the proteins are further modified through S-Cys and N-Lys by Hcy and HcyTL.

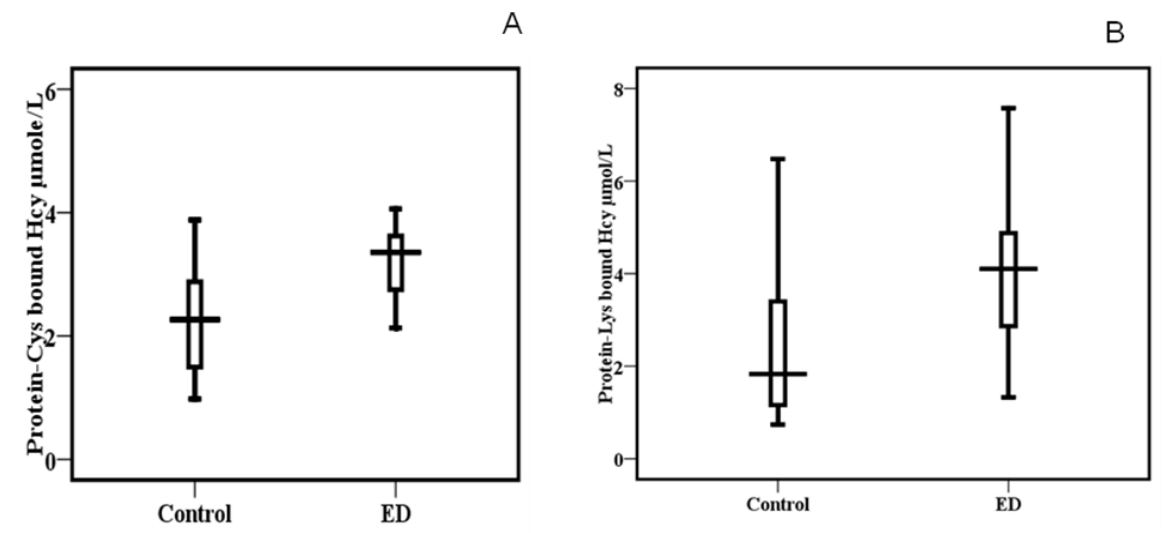

Figure 27: The protein-Cys bound Hcy and protein-Lys bound Hcy in plasma of ED sample compare to controls A) Protein-Cys bound Hcy and B) Protein-Lys bound Hcy.

4.2.2 Analysis of protein modification such as protein-Cys bound Hcy and protein-Lys bound Hcy by HPLC using FLD in ARMD

We found that protein-Cys bound Hcy (S-Cys) and protein-Lys bound Hcy (N-Lys) were increased from 1.4 to 2.6 µM ($p = 0.001$) and 1.7 to 2.7 µM ($p = 0.02$) in ARMD. The results are presented in figure.28. The levels of protein homocysteinylation via lysine and cysteine residues, in the plasma proteins of study subjects were estimated by HPLC and that the increased levels of protein-Cys bound Hcy and protein-Lys bound Hcy in ARMD compared to controls as shown in figure.28. Protein homocysteinylation also affects the normal function of LDL. It is interesting to note that multiple retinal degeneration is caused by injecting HcyTL in an animal eye [153].

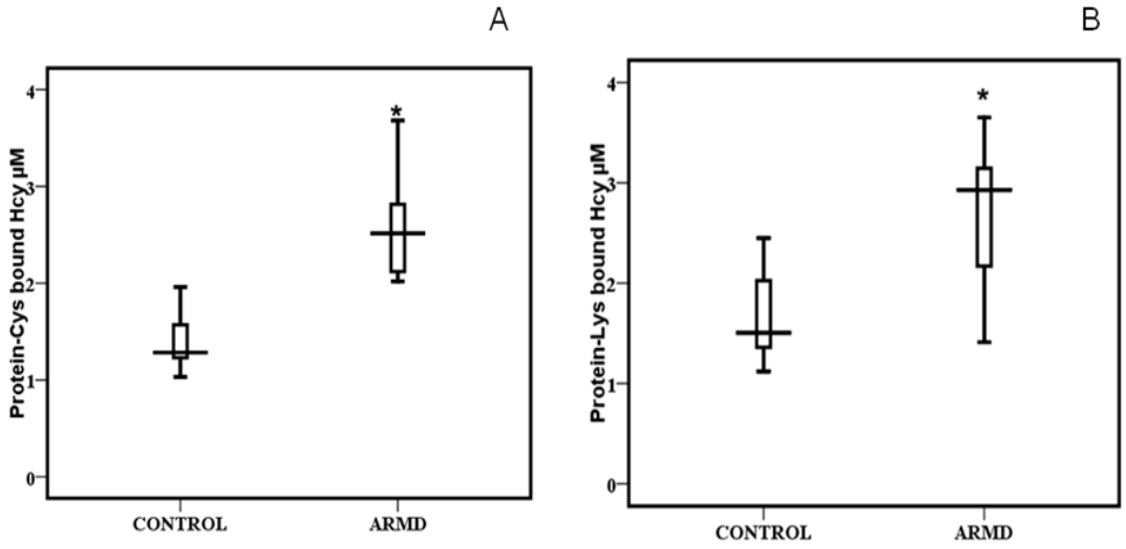

Figure 28: The protein-Cys bound Hcy and protein-Lys bound Hcy in plasma of ARMD sample compare to controls A) protein-cys bound Hcy and B) protein-Lys bound Hcy.

4.3 Increased homocysteine associated with iron metabolism

Erythrocytes (Red blood cells) are responsible for oxygen transport. Oxygen transport is mainly dependent on the presence of iron in haemoglobin. Both oxygen and iron are easily oxidized mainly due to free radicals produced by reactive oxygen species and reactive nitrogen species. All the above elements together can damage the red blood cells as well as vascular endothelium. Free radicals are generated by oxygen saturation, superoxide detoxification, atherosclerosis and bacterial infections. Hcy is known to be a pro-oxidizing agents [154]. Vitamins B_6, B_{12} and folic acid and enzymes MTHFR and CBS are involved in the synthesis of Hcy. Due to the deficiency in any of these vitamins or enzymes can alter the morphology of erythrocytes. To understand, how HHcy is associated with changes in RBC in terms of free and bound iron, Hb, heme, heme synthesizing enzymes, the following analysis were done in samples derived from patients with ED.

4.3.1. Levels of serum haemoglobin, Iron and Total Iron binding capacity

The Serum levels of haemoglobin, iron and total iron binding capacity in ED patients and control subjects are given in table 21 and figure 29. There was a significant increased level of serum haemoglobin and serum total iron binding capacity in ED patients compared to the control subjects ($p < 0.002$ and $p < 0.01$). There was no significant difference between serum iron in ED and control subjects. Thus serum

haemoglobin was positively correlated with plasma Hcy as shown in figure 30 A and plasma Hcy was positively correlated with serum TIBC as shown in figure 30 B.

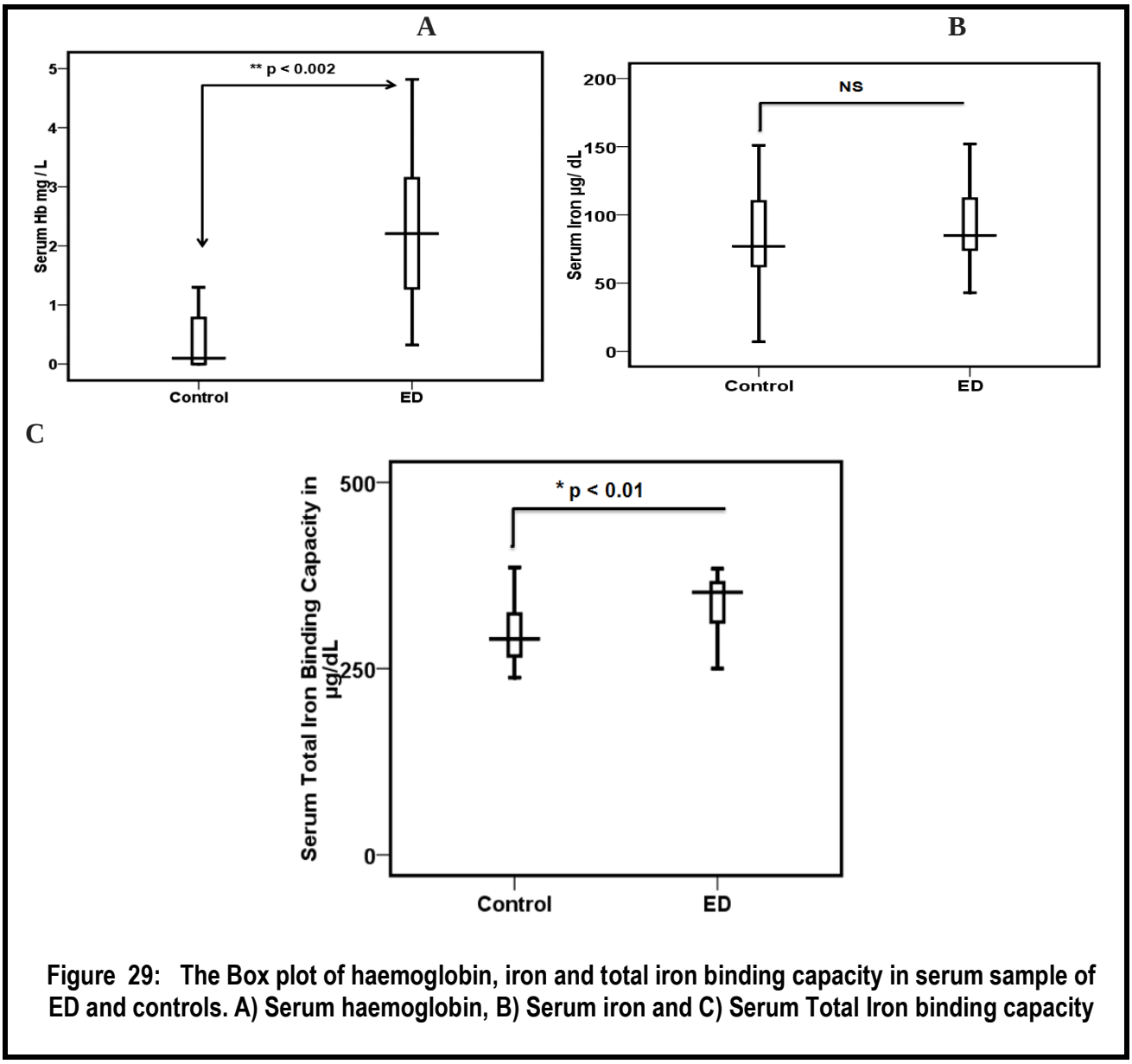

Figure 29: The Box plot of haemoglobin, iron and total iron binding capacity in serum sample of ED and controls. A) Serum haemoglobin, B) Serum iron and C) Serum Total Iron binding capacity

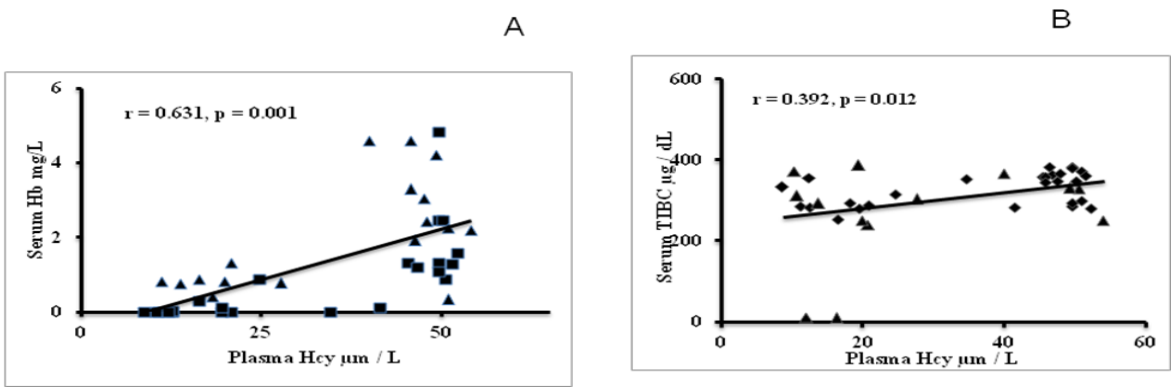

Figure 30: Correlation between the Hcy, serum haemoglobin and serum total iron binding capacity in ED and control samples. A) Positive correlation between plasma Hcy and Serum haemoglobin, B) positive correlation between plasma Hcy and Serum TIBC.

4.3.2. Levels of Ferritin, Transferrin and Serum transferrin receptor

Since Hcy alters the membrane structure of erythrocytes and leads to intravascular and extravascular haemolysis of RBCs, it was felt important to know what happens to the iron storage and iron status in diseased conditions of ED subjects compared to controls. The serum levels of ferritin, transferrin, and serum transferrin receptor are given in table 21 and figure 31. There was a significant increased of serum total iron binding capacity in the ED, we need to check the status of iron. Therefore, to know the iron binding proteins status; ferritin, transferrin, and serum transferrin receptor levels were measured in the study subjects. There was a significant increase in the level of serum and intracellular (PBMC) ferritin (table 22) in ED patients compared to the controls ($p< 0.002$, $p < 0.001$) and there was a significantly decrease in serum transferrin levels in ED patients compared to the controls ($p < 0.02$). There was a significant negative correlation between the serum ferritin and serum transferrin in ED patients (figure 31 D). There was a significant positive correlation between serum ferritin and plasma Hcy in both the subjects together in figure 32 A (ED and controls), ($r = 0.640$, $p = 0.0001$), similar results were observed by Tamura et.al., in the year 2011 in cardiovascular diseases, and there was a significant negative correlation between serum transferrin and plasma Hcy in both the subjects together in figure 32 B (ED and controls), ($r = - 0.345$, $p = 0.029$). Thus, the above results indicate that, both iron (ferritin) and homocysteine are associated and have role in vascular eye diseases.

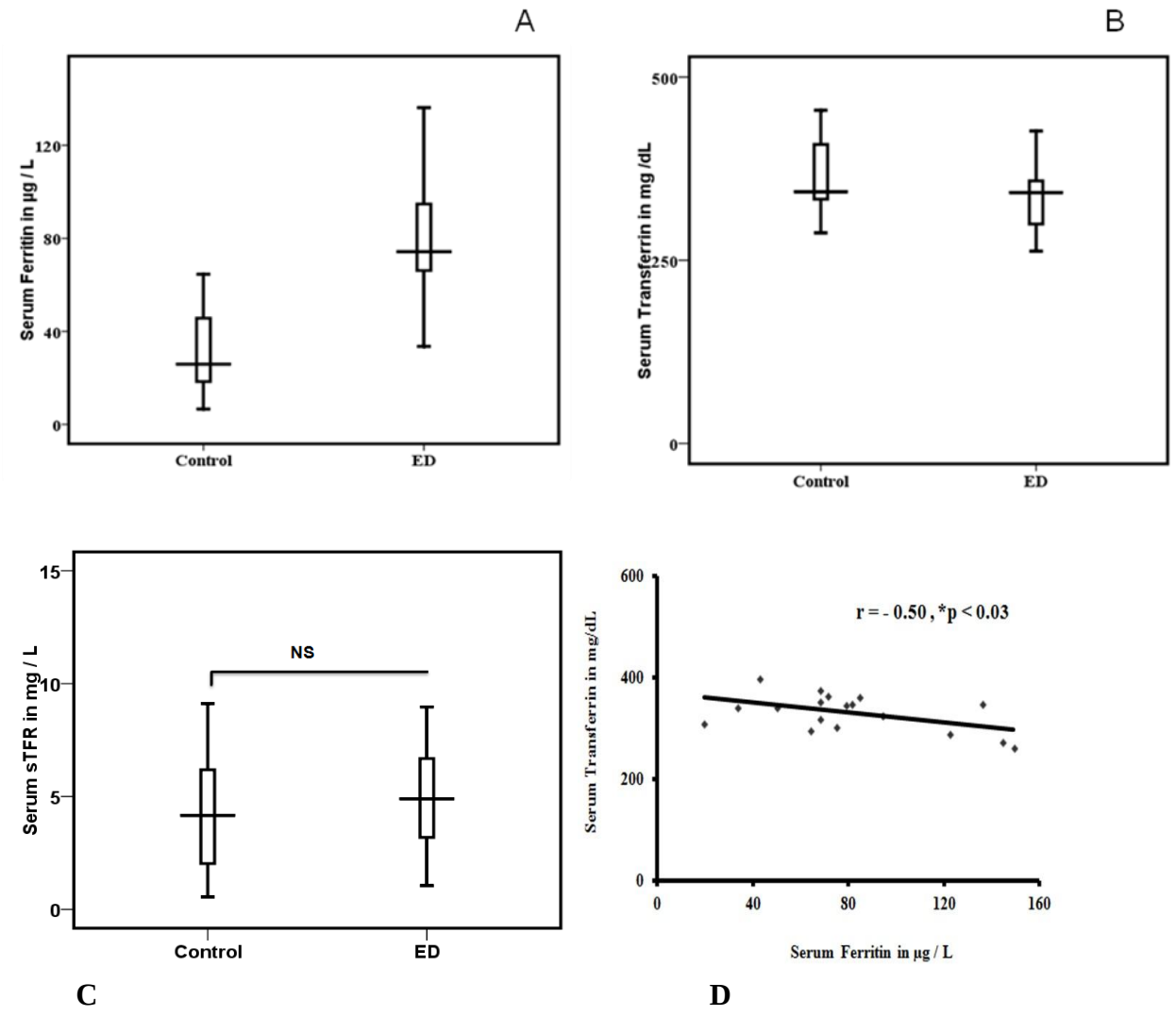

Figure 31: The level of ferritin, Transferrin and serum transferrin receptor in serum samples of ED compared to controls. Correlation between serum ferritin and serum transferrin.
A) Serum Ferritin, B) Serum Transferrin, C) Serum Transferrin receptor (s TFR),
D) Negative correlation between Serum Ferritin and Serum Transferrin.

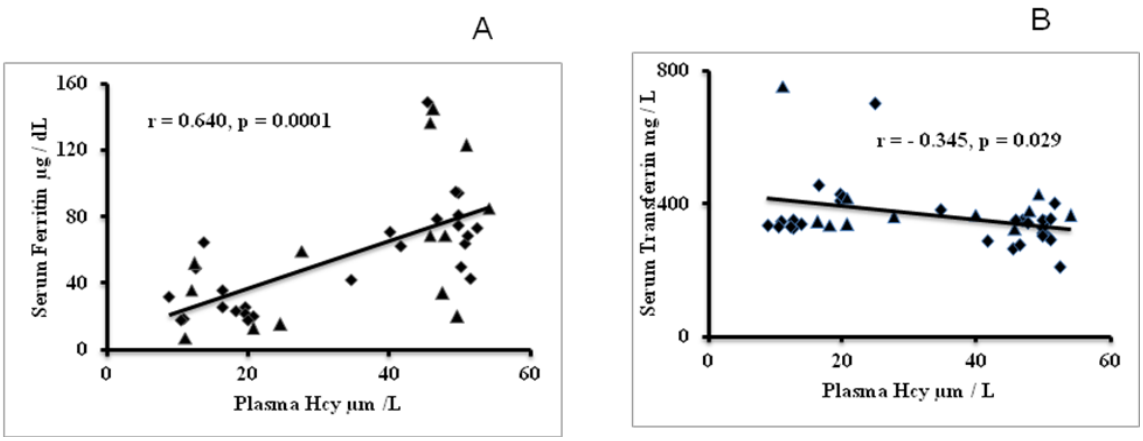

Figure 32: Correlation between the plasma Hcy, serum ferritin and tranferrin in the ED and control samples, A) Positive correlation between plasma Hcy and serum ferritin and B) Negative correlation between plasma Hcy and Serum Transferrin.

4.3.3. Levels of Aminolevulinic acid synthase (ALAS), Heme and Heme oxygenase

Heme and vitamin B_6 along with an enzyme CBS act as a co-factor for the synthesis of glutathione via cysteine. Since the serum haemoglobin level is increased, to know any changes happen in the synthesis or breakdown of haemoglobin. The serum and intracellular levels of ALAS, Heme and Heme oxygenase are given in table 21, 22 and figure 33. Since serum haemoglobin was more in the ED, because of excess production or breakdown of haemoglobin. This could be addressed by measuring ALAS, the rate limiting enzyme of heme synthesis and heme oxygenase the breakdown of heme into iron, carbon monoxide and bilirubin. There was a, significant increase in the level of serum heme, ALAS and HO in ED patients compared to the control subjects ($p < 0.013$, $p < 0.0001$ and $p < 0.0001$). Also significant increase in the levels of PBMC heme, ALAS and HO in ED patients compared to the control subjects ($p < 0.001$, $p < 0.001$ and $p < 0.001$ respectively).There was a significant positive correlation between the serum heme and serum ALAS in ED patients (figure 33 E) and also a significant positive correlation between serum ALAS and HO in both subjects (such as ED and control) ($r = 0.647$, $p < 0.001$). In PBMC it was observed that there was a significant positive correlation between the heme and ALAS in ED patients ($r = 0.514$, $p<0.01$). There was a significant positive correlation between serum heme and plasma Hcy in both the subjects together in figure 34 A (ED and controls), ($r = 0.379$, $p = 0.016$), and significant positive correlation between serum ALAS and plasma Hcy in both the subjects together in figure 34 B (ED and control), ($r = 0.540$, $p = 0.0001$), significant

positive correlation between serum HO and plasma Hcy in both the subjects together in figure 34 C, (r = 0.587, p = 0.0001).

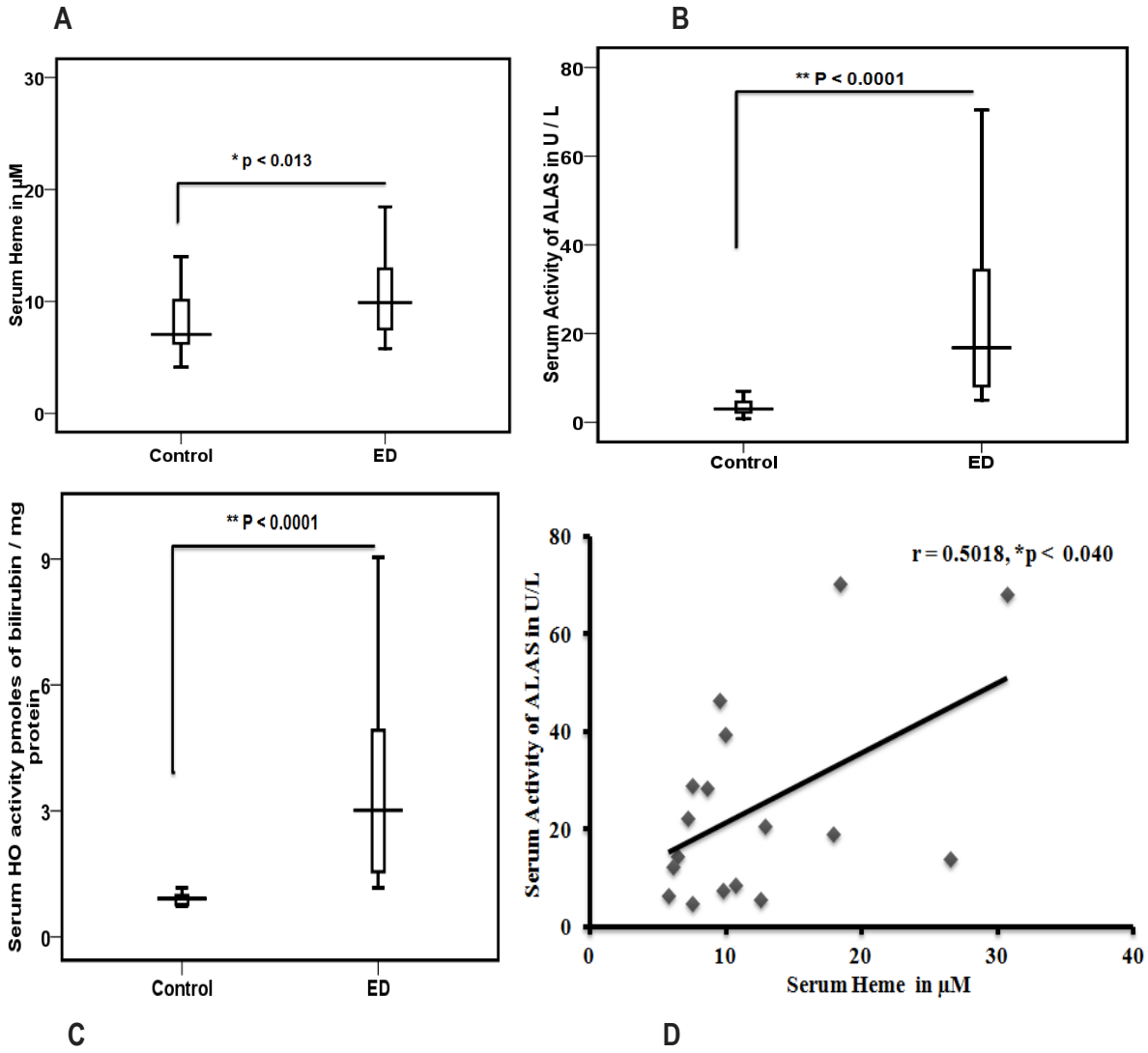

Figure 33: Heme, ALAS and Heme oxygenase in serum sample of ED compared to controls. A) Serum Heme, B) Serum Activity of ALAS, C) Serum Heme oxygenase activity and D) Positive correlation between serum Heme and Serum ALAS in ED

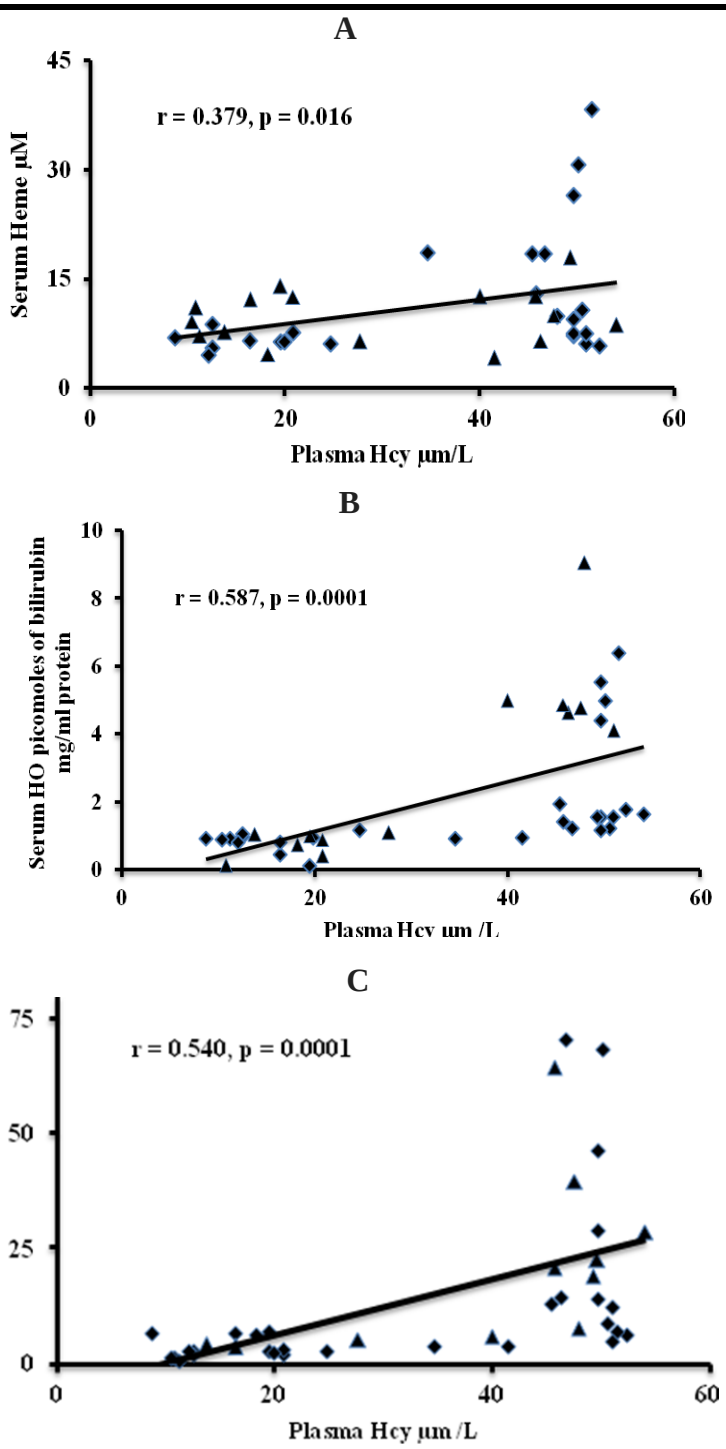

Figure 34: Correlation between the plasma Hcy, serum Heme, ALAS, Heme oxygenase and in ED and control samples. A) Positive correlation between plasma Hcy and Serum Heme in ED and control, B) Positive correlation between plasma Hcy and Serum Heme oxygenase in ED and control and C) Positive correlation between plasma Hcy and serum ALAS in ED and control.

4.3.4. Levels of VEGF in ED

VEGF growth factor is also referred as a survival factor for endothelial. HO and VEGF are co-expressed protein during the hypoxic condition. In retina micro capillary endothelium is expressing hypoxic environment in any vascular lesion. ED is associated with retinal vascular phlebitis and venous occlusions. Retinal neovascularization is a common phenomenon in most vascular disorders of retina. To further understand the link between HO and VEGF, the latter was estimated in both serum and PBMC cells. Even though, the disease is localized in the eye, it is expected that, the circulatory cells have an impact in the micro vascular endothelium. The serum and PBMC level of VEGF in ED and controls is given in table 21, 22 and figure 35. HO may also regulate the synthesis of VEGF [136]. There was a significant increase in the level of serum VEGF in ED patients compared to the control subjects ($p < 0.001$). The level of VEGF was also increased in PBMC of ED compared to control subjects ($p< 0.002$). There was a significant positive correlation between the serum ALAS and VEGF, serum HO and VEGF. In PBMC, there was a significant positive correlation between the HO and VEGF ($r = 0.543$, $p < 0.001$). It was observed that there was a significant positive correlation between serum VEGF and plasma Hcy. Results are presented in the figure 36 ($r = 0.652$, $p = 0.001$).

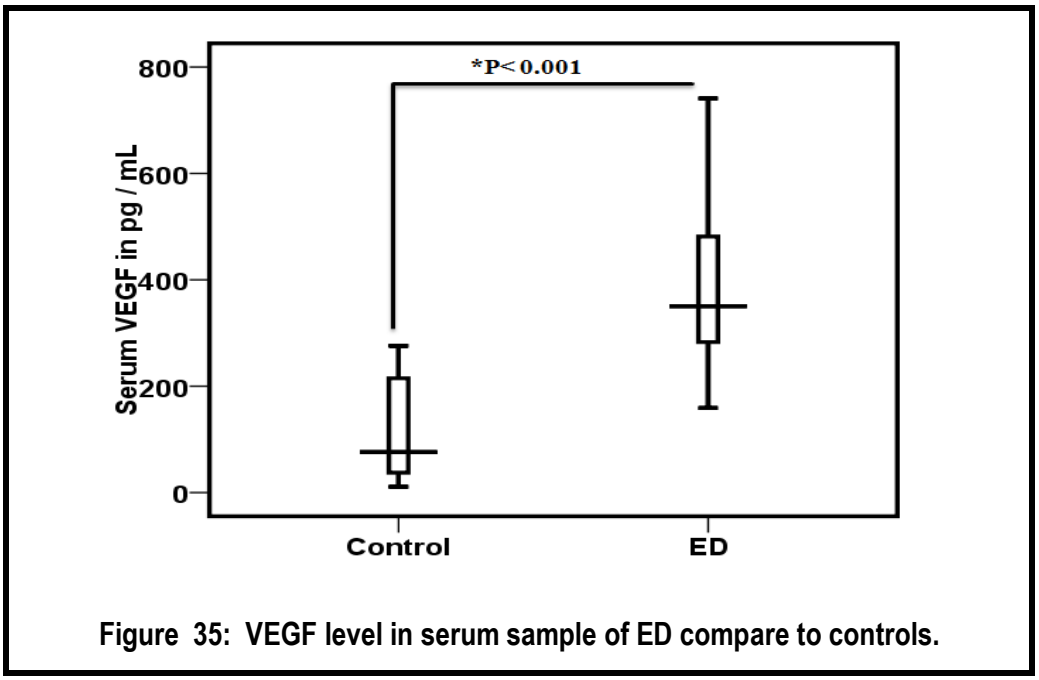

Figure 35: VEGF level in serum sample of ED compare to controls.

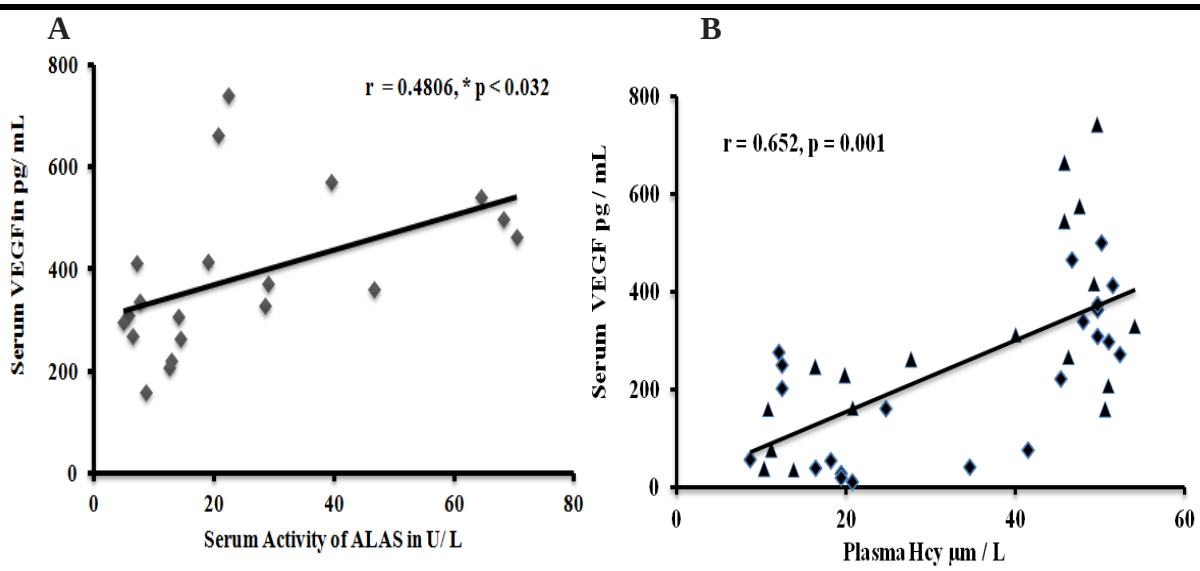

Figure 36: Correlation between the serum ALAS, VEGF and Hcy in ED and control samples. A) Positive correlation between Serum ALAS and Serum VEGF in ED and B) Positive correlation between plasma Hcy and Serum VEGF in ED and control.

4.3.5. mRNA expression of Iron regulators

There is an increased intracellular ferritin levels, in ED subjects, provoking interest in knowing the status of iron regulators, their role in the disease and hence to measure the mRNA expression of hepcidin an iron sensor and ferroportin an iron exporter. The mRNA expression of hepcidin was up regulated and ferroportin was down regulated in ED patients when compared to the controls (figure 37). Thus, the result shows a potential role for that hepcidin in ED. It is known that hepcidin can bind to ferroportin, triggering its internalization and degradation resulting in the accumulation of intracellular iron in the form of ferritin. This increase in the expression of hepcidin may be due to increased iron, inflammation or infection.

4.3.6. Over expression of Hypoxia inducible factors (HIF1 and HIF2)

Since, both VEGF and HO are elevated in ED; it is of interest to know further on the status of hypoxia inducible proteins. Hence, the expressions of hypoxia (HIF1 and HIF2) were measured. The mRNA expression of HIF1 and HIF 2 was up regulated when compared to the controls, whereas HIF1 expression was significantly increased in ED as shown in figure 37. A previous report says that hypoxia is a negative regulator of hepcidin expression, but we observed that HIF 1 and HIF 2 expression

were increased in the ED may be due to inflammation. It is already reported, IL-6 is elevated in ED and it is known for its association with hypoxia

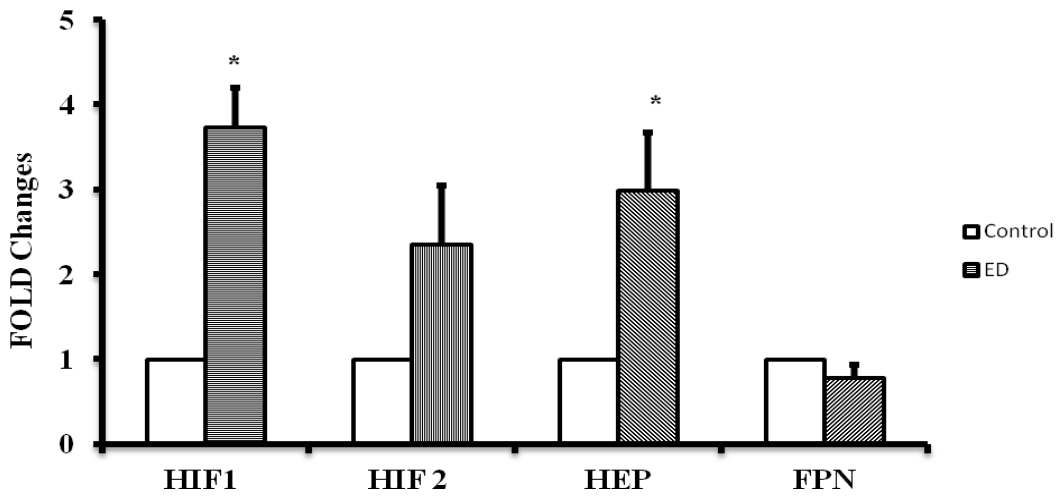

Figure 37: The mRNA expression hepcidin, ferroportin and hypoxia inducible factor – HIF 1 and HIF 2

4.3.7. Protein Expression of Iron regulators

The protein expression of Hepcidin was significantly increased in ED when compared to the controls, whereas the protein expression of ferroportin was significantly decreased in ED compared to the controls (figure 38). The protein expression of HIF 1 was slightly increased in ED compared to the controls and the protein expression was quantified by densitometry and the densitogram was given (figure39 A and 39 B).

Figure 38: The protein expression of hepcidin and ferroportin in ED compare to controls in PBMC normalized with β - actin.

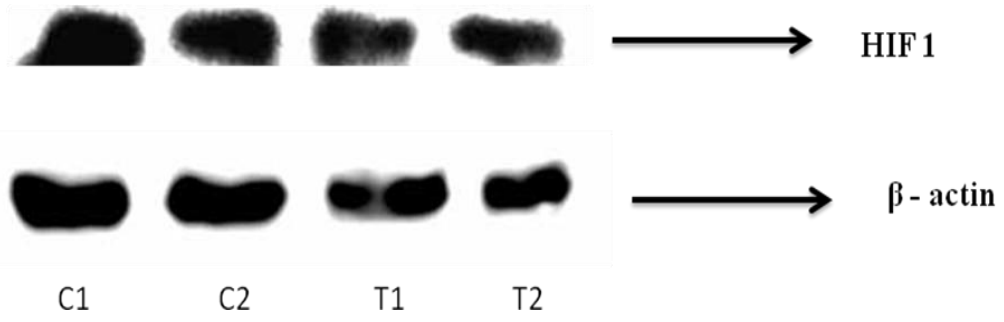

Figure 39 A: The protein expression of HIF 1 in ED compare to controls in PBMC normalized with β-actin.

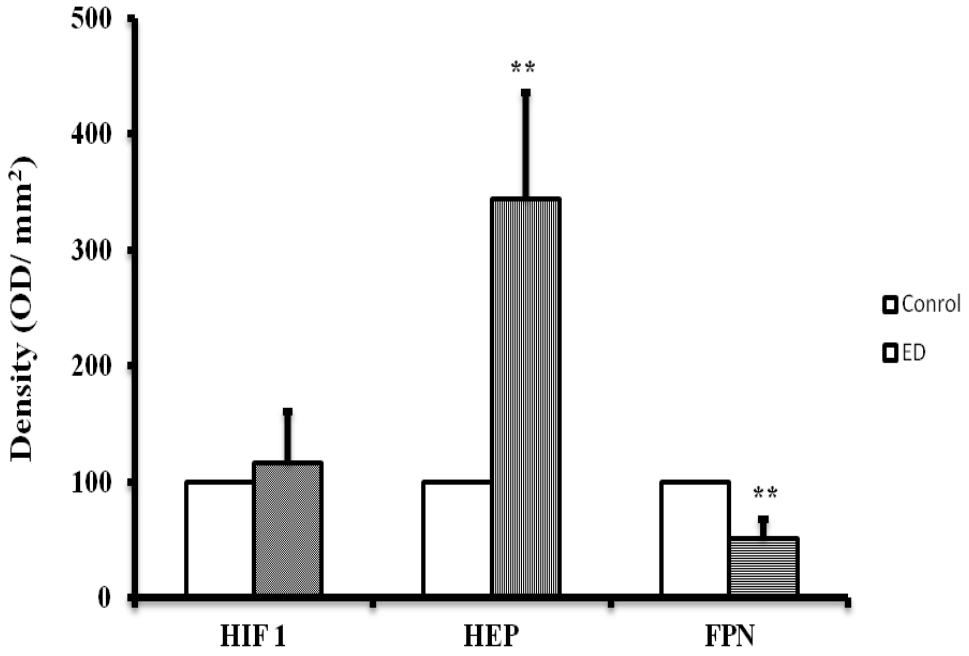

Figure 39 B: The protein expression of hepcidin, ferroportin and hypoxia inducible factor – HIF 1

Consolidated table showing the levels of various biochemical parameters related to iron.

Table 21: Levels of iron related parameter in controls versus ED serum sample

Biochemical Parameters	Controls (n = 20)	ED (n = 20)	P value
Serum Haemoglobin (mg / L)	0.35 ± 0.1	2.0 ± 0.3	0.002
Serum Iron (µg / dL)	82 ± 8	93 ± 7	0.298
Serum Total iron binding capacity (µg / dL)	274 ± 22	340 ± 9	0.01
Serum Ferritin (µg / L)	31.9 ± 3.9	81.1 ± 7.8	0.002
Serum Transferrin (mg / dL)	397.9 ± 26.8	330.2 ± 11.0	0.02
Serum transferrin receptor (mg / L)	4.37 ± 0.57	4.92 ± 0.52	0.47
Serum Heme (µM)	8.3 ± 0.8	13.9 ± 2.0	0.013
Serum Heme oxygenase (pmoles of bilirubin / mg protein)	0.8 ± 0.1	3.4 ± 0.5	0.0001
Serum Aminolevulinic acid synthase (U / L)	3.6 ± 0.4	25.2 ± 4.8	0.0001
Serum Vascular endothelial growth factor (pg /mL)	120.6 ± 21.2	387.7 ± 34.3	0.001

All values are expressed as mean ± SEM

Table 22: Intracellular levels of iron related parameter in controls versus ED

Biochemical Parameters	Controls (n = 20)	ED (n = 20)	P value
Ferritin (µg / mg of proteins)	20.9 ± 1.4	65.7 ± 2.5	0.001
Heme (µm / mg of proteins)	8.9 ± 1.7	27.2 ± 5.1	0.001
Heme oxygenase (pmoles of bilirubin / mg protein)	1.5 ± 0.2	6.4 ± 0.5	0.001
Aminolevulinic acid synthase (U / mg of proteins)	2.4 ± 0.2	12.5 ± 3.0	0.001
Vascular endothelial growth factor (pg/mg of proteins)	29.8 ± 4.0	147.5 ± 40.2	0.005

All values are expressed as mean ± SEM

4.4. Analysis of glutathione by spectrofluorometer

To measure the glutathione synthesis (GSH level) in the plasma sample of ED patients and controls. It was observed , that levels of plasma glutathione (5.9 ± 0.44 µM) in ED patients are lowered when compared to the controls (8.1± 0.41 µM), shows in figure 40 and it was negatively correlated with plasma Hcy, shows in figure 41. Decreased synthesis of GSH in ED may be due to the decreased levels of glutamic acid and cysteine. Here, GSH level may be decreased due to the in available of glycine, because the glycine are utilized for the synthesis of heme.

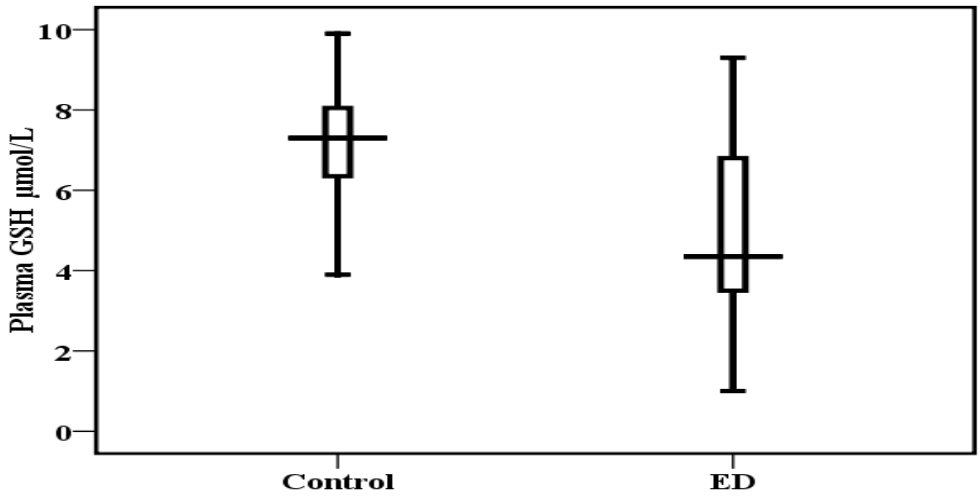

Figure 40: Glutathione levels in plasma samples of ED compared to controls.

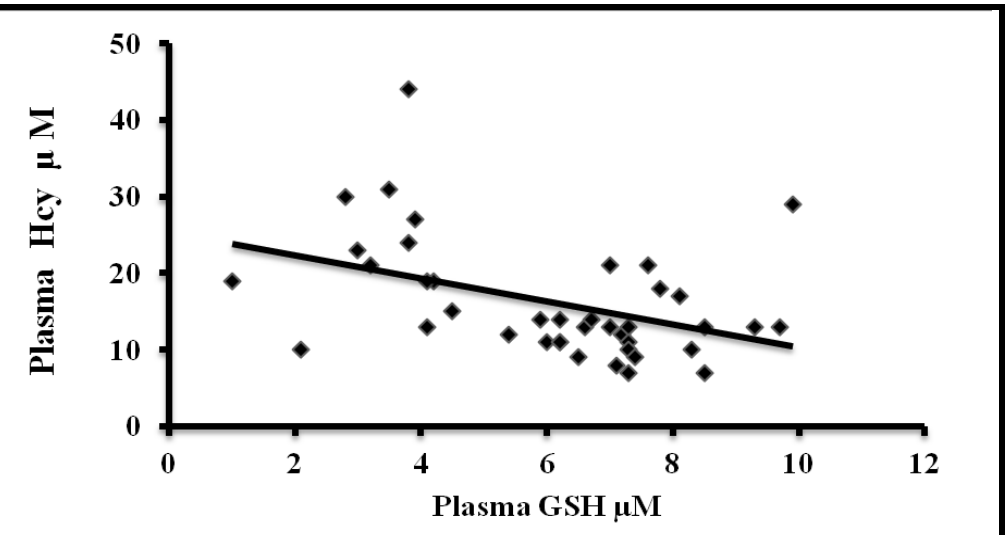

Figure 41: Correlation between Plasma Hcy and Plasma GSH

4.4.1 Analysis of reduced and oxidized glutathione by HPLC using ECD

Both reduced and oxidized glutathione are measured in plasma samples of ED patients. The GSH (reduced) and GSSG (oxidized glutathione) plays an important role in cell homeostasis and also in signaling processes. Analyzing the levels of GSH and GSSG are the valid tools for the assessment of the function of redox system. It was observed that there is a decreased level of both GSH and GSSG level in plasma samples of ED patients when compared to the controls as shown in table 23 and figure 42. The levels of both GSH and GSSG were decreased significantly in ED patients compared to the controls. The results indicate, that homocysteine may have a role in altering the glutathione synthesis and/or utilization, which need to addressed. Attempts have been made to further understand the link between GSH and Hcy at the level of enzymes involved in GSH synthesis.

Table 23: Levels of reduced and oxidized glutathione in controls versus ED of plasma samples

S.NO	Plasma Glutathione	Controls (n = 15) µmole/L	ED (n = 15) µmole/L
1	Reduced	5.0 ± 1.4	3.0 ± 0.9
2	Oxidized	2.4 ± 0.4	1.2 ± 0.4

All values are expressed as mean ± SEM.

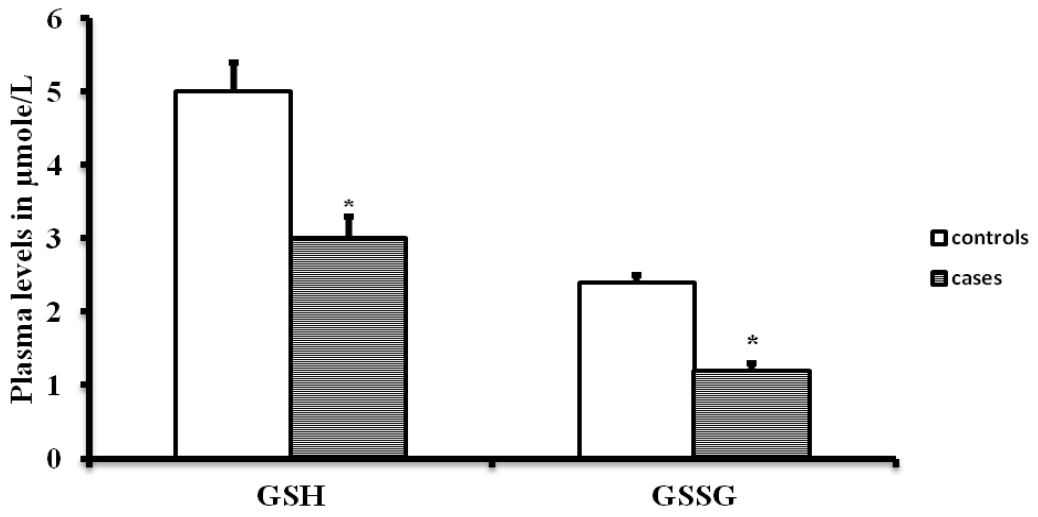

Figure 42: reduced and oxidized plasma glutathione in controls versus ED of plasma sample

4.4.2 Analysis of ϒ-glutamate-cysteine ligase by spectrofluorometer

To evaluate whether homocysteine affected the rate limiting enzyme GCL (ϒ-glutamate-cysteine ligase) involved in GSH synthesis. To measure an enzyme activity of ϒ-glutamate-cysteine ligase (GCL) levels in both serum and peripheral blood mononuclear cells of ED patients when compared to the controls by spectrofluorometer. ϒ-glutamate-cysteine ligase is the rate limiting enzyme in the synthesis of GSH. The level of ϒ-glutamate-cysteine ligase was decreased in the ED when compared to the controls in serum (table 24 and figure 43) as well as in peripheral blood mononuclear cells (figure 44). It shows that the homocysteine altered the synthesis of GSH by inhibiting an enzyme via decreasing the binding constant of glutamic acid to an enzyme GCL. Binding constant of glutamic acid is lowered, means the inhibitory constant of GSH is increased.

Table 24: Levels of ϒ-glutamate-cysteine ligase in serum and peripheral blood mononuclear cells in controls versus ED

S.NO	ϒ-glutamate-cysteine ligase (GCL) (µmoles / 10 min)	Controls (n = 20)	ED (n = 20)	P value
1	Serum	13 ± 2.7	4.6 ± 1.0	0.007
2	Peripheral blood mononuclear cells	2.6 ± 0.3	0.57 ± 0.08	0.01

All values are expressed as mean ± SEM.

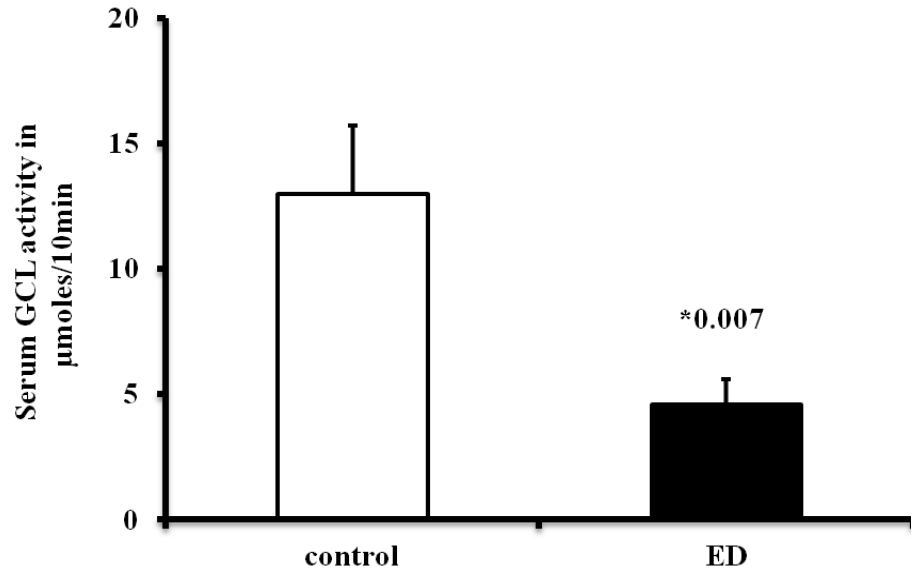

Figure 43: The Bar diagram of ϒ-glutamate-cysteine ligase activity of serum samples in controls versus ED

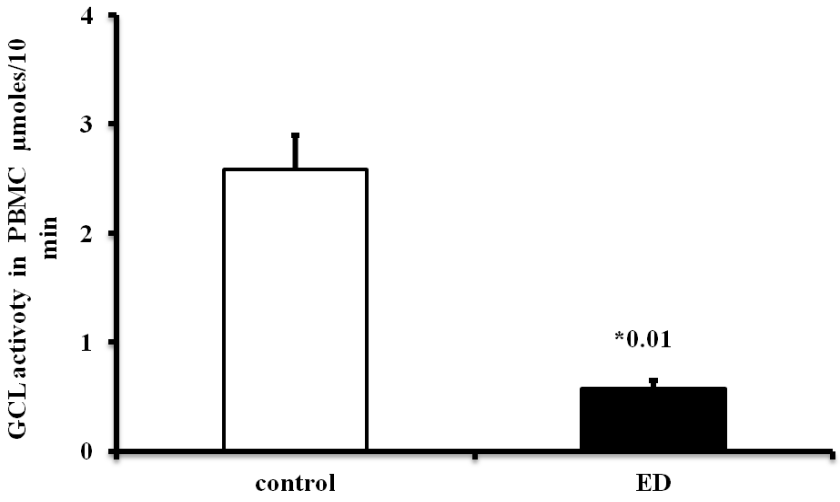

Figure 44: The Bar diagram of ϒ-glutamate-cysteine ligase activity of PBMC in controls versus ED

4.4.3. Expression of ϒ-glutamate-cysteine ligase (Both catalytic unit and modifier unit)

To evaluate whether homocysteine alter the mRNA expression of GCLC and GCLM in peripheral blood mononuclear cells of ED and control subjects. To measure the mRNA expression of ϒ-glutamate-cysteine ligase (both catalytic GCLC and Modifier unit GCLM) in peripheral blood mononuclear cells of ED patients. It was observed that GCLC and GCLM mRNA expression is decreased in ED when compare to the controls, whereas the mRNA expression of GCLC is significantly decreased in diseased conditions. It was shown in figure 45. It shows the transcription levels of the rate limiting enzyme GCL (GCLC and GCLM) are altered; it may be due to the effect of homocysteine and oxidative stress.

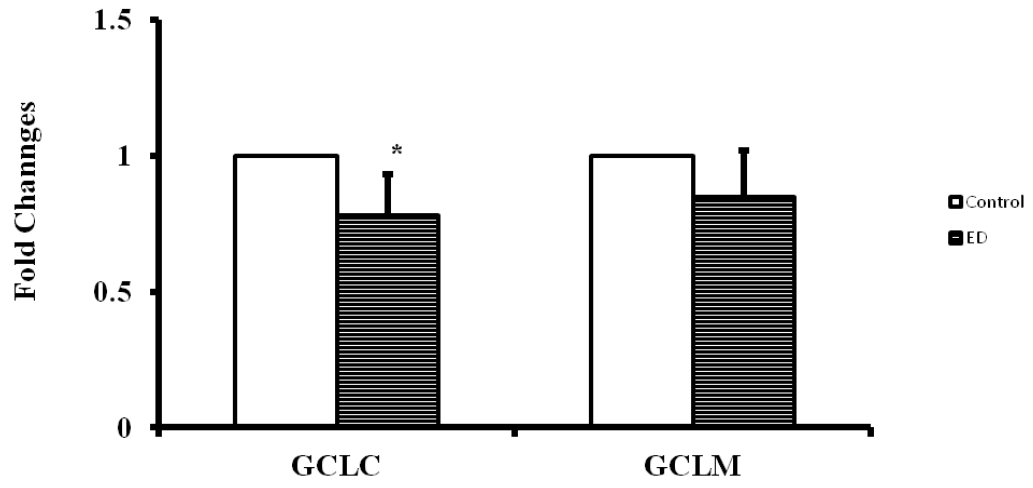

Figure 45: The mRNA expression levels of GCLC and GCLM in peripheral blood mononuclear cells in controls versus ED

4.4.4 Expression of Nuclear erythroid related factor 2 (Nrf2)

Expressions of the catalytic and modifier unit of ϒ-glutamate-cysteine ligase are regulated by the redox-sensitive transcription factor; Nrf2. Nrf2 is one of the defense mechanisms against oxidative stress [155]. The mRNA expression of Nrf2 is significantly decreased in ED when compare to controls in PBMC (peripheral blood

mononuclear cells). Thus the decreased expression of both the catalytic and modifier unit of GCL is due to the lowered levels of Nrf2, this effect may in turn be due to higher levels of Hcy.

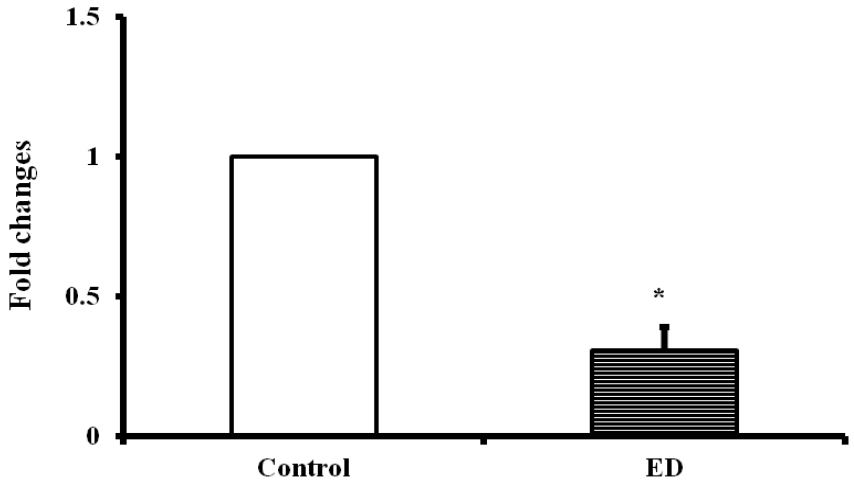

Figure 46: The mRNA expression of nuclear erythroid related factor 2 (Nrf2) in peripheral blood mononuclear cells.

4.5. To understand the effect of homocysteine in glutamate-cysteine ligase by Molecular Dynamics simulation study.

The protein sequence of GCLC (Accession no: P48506) consists of 637 amino acids as per UniProt database. The retrieved sequence was incorporated in BLAST for protein similarity search against the PDB database. The BLAST results revealed Saccharomyces cerevisiae glutamate cysteine ligase in complex with Mg^{2+} and L-glutamate protein as suitable template (3IG5 protein as "PDB ID") which exhibits 42% sequence identity, query coverage of 95% and E-value of 1e-163. The holoenzyme structure of GCLC protein was prepared with Glutamate (Glu), ADP, and three Mg^{+2} ions from the template structure and Cys from the available protein co-ordinate from the PDB database (PDB ID: 2D32) in Modeller9v13. It generated ten possible conformations of the target protein using ligand py module of Modeller. The model structures were further assessed for structural stability by the Discrete Optimized Protein Energy **(**DOPE) and molecular PDF (molpdf) scores. Among the ten models,

the one with lowered DOPE score of -46065.46 kcal/mol and molPDF of 157061. 29 were selected for further studies. Here, it is noticeable that for homocysteine (CSO) complex of GCLC was prepared by addition of the methylene bridge to Cys639 residue in the Cα region.

4.5.1. Structural comparison of GCLC-Cys and GCLC-Cso complex

The modelled structures of GCLC-Cys and GCLC-Cso complexes showed structural similarity, consisting of 5 β-sheets, 10 β-strands and 24 α-helices. The superimposition of these complexes in PyMOL retained the functional Glutamate-cysteine ligase domain with RMSD deviation of 1.316 Å and 1.313 Å in comparison to the template which suggests that the modelled structure is reliable and suitable for further studies. Structural insight into the modelled structure suggests that Cys has better interaction with GCLC protein than Cso. The Cso and Cys interactions with GCLC have been demonstrated in figure 47.

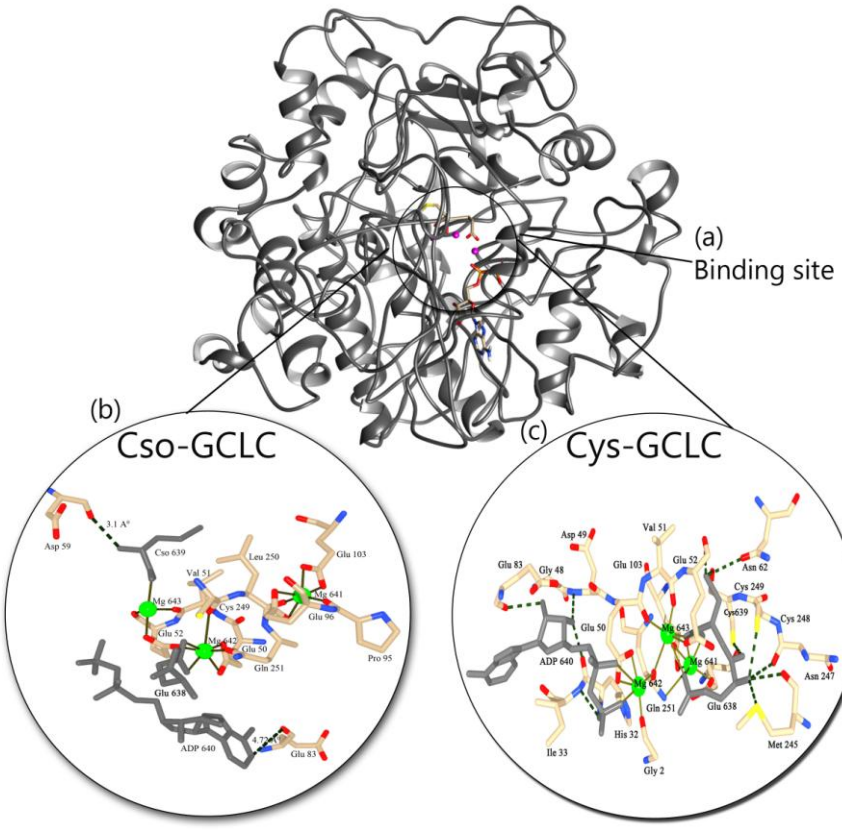

Figure 47: Interactions of Cso, Glu, ADP and 3Mg ions with GCLC protein (a) Binding site of GCLC, (b) Cso-GCLC complex, showing interactions with metal ions with GCLC protein and (c) Cys-GCLC complex, showing interactions with metal ions.

4.5.2. Molecular Dynamics Simulations studies of GCLC-Cys and GCLC-Cso

Protein backbone RMSD: The protein backbone RMSD of both the protein complexes showed stable conformation. Initially, in both the complexes, minor fluctuations were observed up to the 5ns, thereafter complexes showed stability throughout the simulation process. It suggests that backbones of both the protein complexes are stable (figure 48) and binding of Cys/Cso not playing any significant role in the protein backbone conformation.

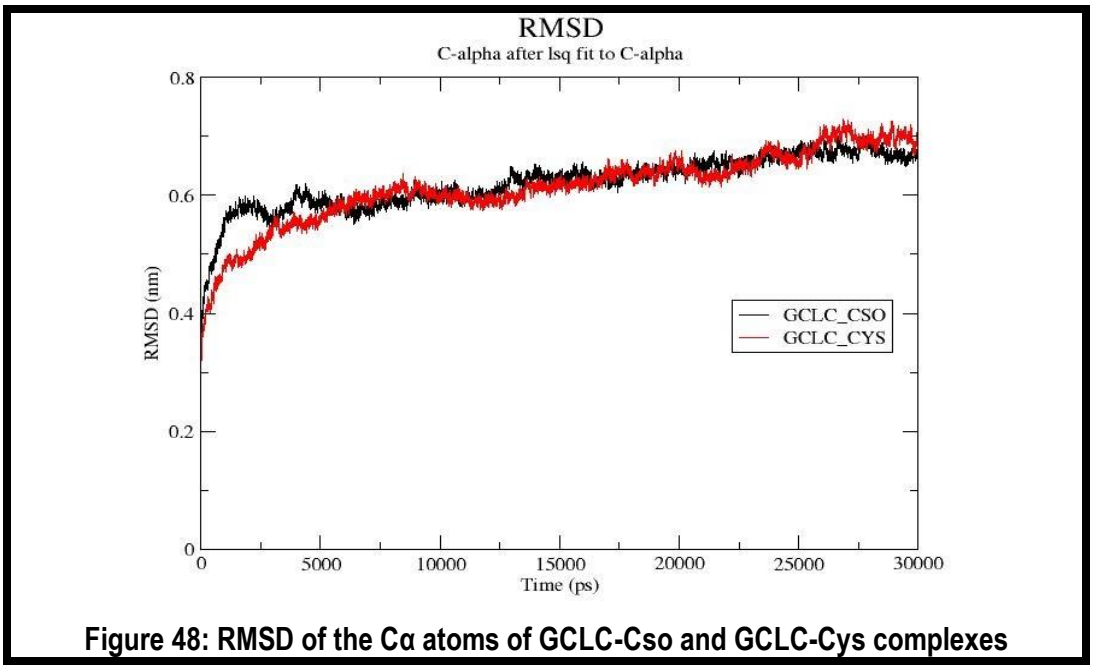

Figure 48: RMSD of the Cα atoms of GCLC-Cso and GCLC-Cys complexes

4.5.3. Ligand Positional RMSD

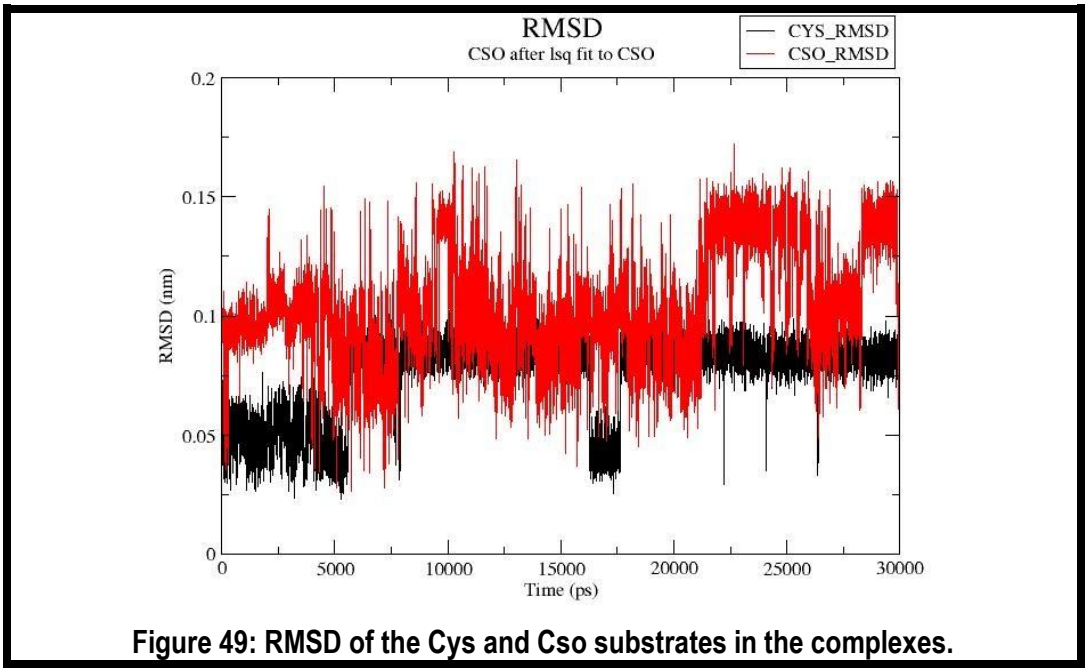

Figure 49: RMSD of the Cys and Cso substrates in the complexes.

In order to evaluate the protein backbone stability of Cys and Cso, we estimated the positional RMSD of both the ligands. RMSD plot of Cso revealed continuous fluctuation throughout the trajectory period in the window size of 0.03 to 0.15, whereas the positional RMSD plot of Cys has shown better RMSD. Cys backbone has shown minor fluctuation in between the 16 -17ns thereafter it maintained the backbone stability till the simulation period (figure 49). It suggests that native Cys exhibits stable interactions with GCLC protein while Cso is not able to make stable interaction with protein.

4.5.4. Distance between Cso/Cys and Glu

GCLC ligates the Cys and the Glu residues as a rate determining step during the synthesis of the Glutathione. Cys and Glu residues in GCLC bound form exhibit a constant distance of 1.5Å making the ligation reaction feasible. The Cso substrate lies at a distance greater than 5Å making the bond formation between the two species not feasible (figure 50). In order to investigate the distance between Cys639/Cso639 and Glu638 in GCLC complex, we downloaded the protein co-ordinates and explored the interactions. It reveals 2.63 and 8.20Å distance between the Cys/Cso and Glu638 (figure 51 and 52).

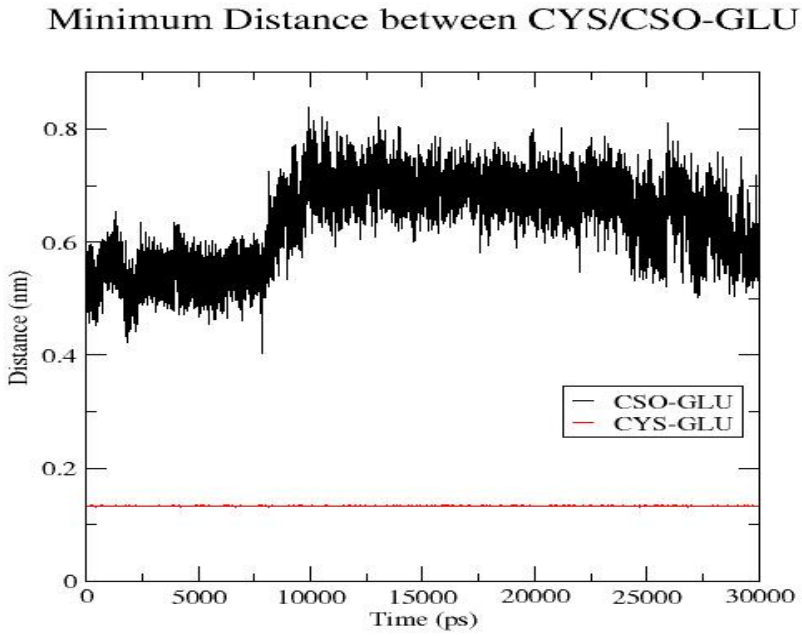

Figure 50: The shortest distance between Cso and Cys residues with Glu residue.

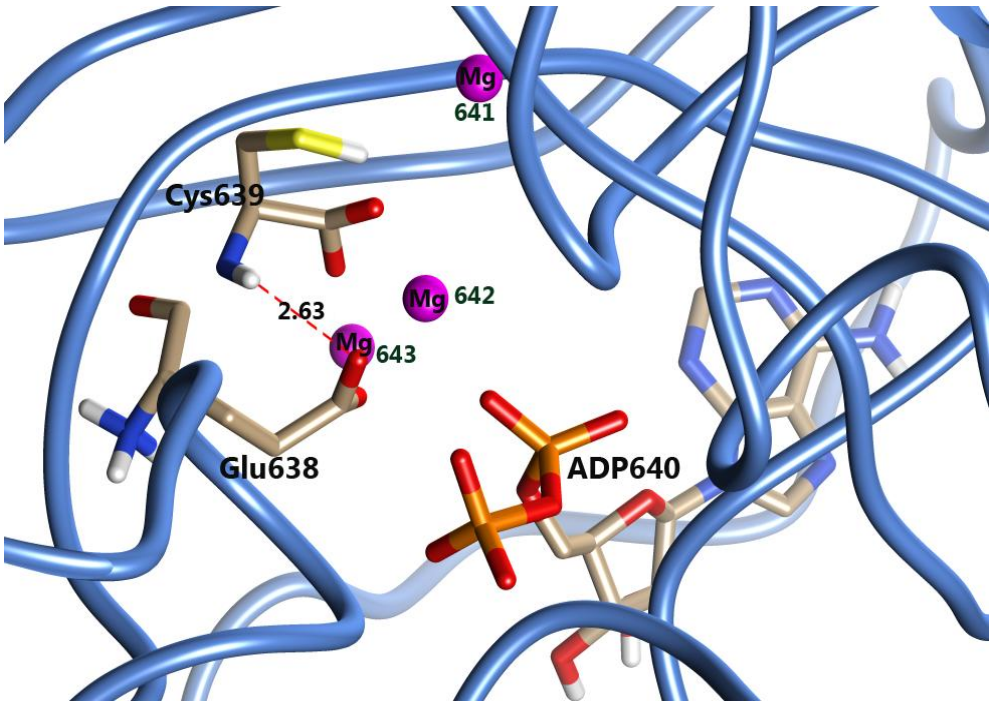

Figure 51: The shortest distance between Cys with Glu residue

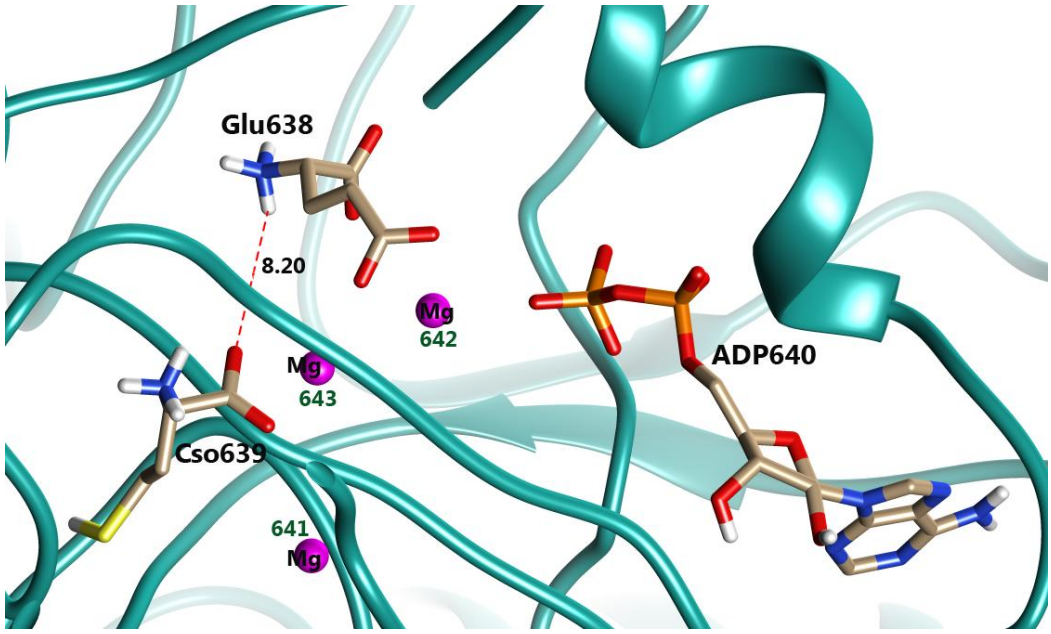

Figure 52: The shortest distance between Cso with Glu residue

4.5.5. Binding pose analysis

In order to identify the binding pose of Cys/Cso and cofactors in the complex form, we downloaded the protein complex coordinates and explored using Chimera software. Thus the present study revealed that Mg^{+} ions are playing a very crucial in cofactors interactions. $Mg^{+}642$ make co-ordination bonds with Glu638 and ADP640 and $Mg^{+}643$ with Cso639 while $Mg^{+}641$ is not taking part in cofactor interactions (figure 28). During the 10 ns interval of time frame observed that Glu638 and Cso639 is maintain larger distance which results inability to make ligation reaction between these two molecules. For Glutathione synthesis the ligation reaction of Glutamate with Cso is an indispensible process. Whereas, in case of Cys-GCLC-Mg and ADP complexes, Mg642 and Mg643 are making co-ordination bonds with all the three cofactors (Cys-Mg and ADP), which hold Glu638 and Cys639 molecule to closure enough to ensure the ligation process for successful glutathione synthesis (figure 53). Thus the present study suggests that in presence of Cys639 is essential for better Glutathione synthesis while Cso obstruct the process.

Thus the modelling and MD studies revealed that GCLC enzyme can bind to the native Cys and Cso (639) forms in the 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 present results suggest 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.

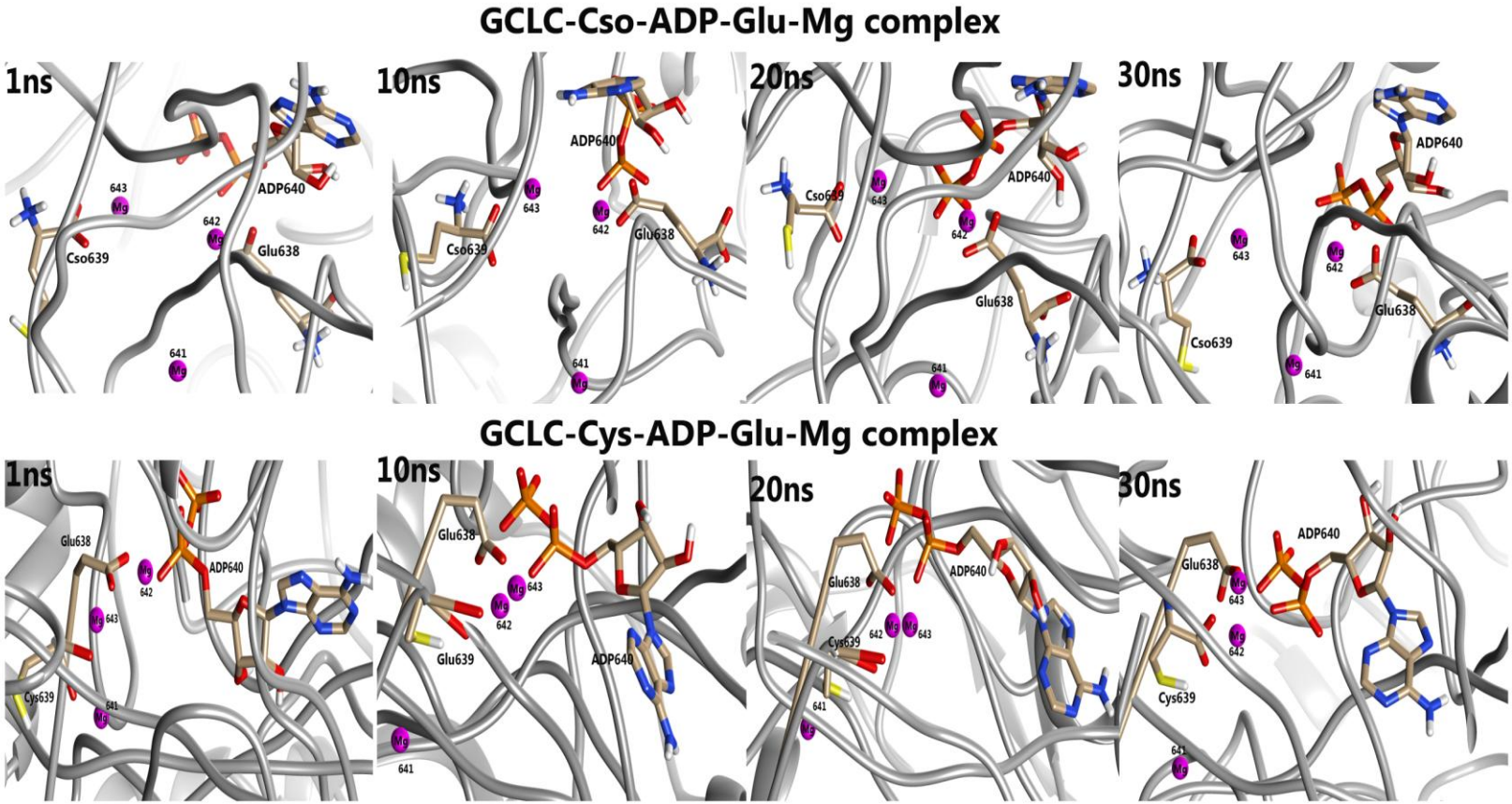

Figure 53: Binding pose of Cso and Cys with cofactors in GCLC complex in the interval of various time frame. Sticky ones are ligands.

4.6. To understand the effect of homocysteine in the expression of ϒ-glutamate-cysteine ligase, nuclear related factor (Nrf2), amino acid levels and intracellular glutathione levels in human ARPE-19 cells as a cell culture model system for ARMD (*In Vitro*).

4.6.1 Cytotoxicity assay

MTT assay was performed to check the cytotoxicity of the Hcy and Cys on ARPE – 19 cells. Concentration ranges from 5 – 100 µM for 1 h time point. Increasing concentration of both Hcy and Cys after 1 h time point shows the toxic effect to the cells (figure 54 A and B).

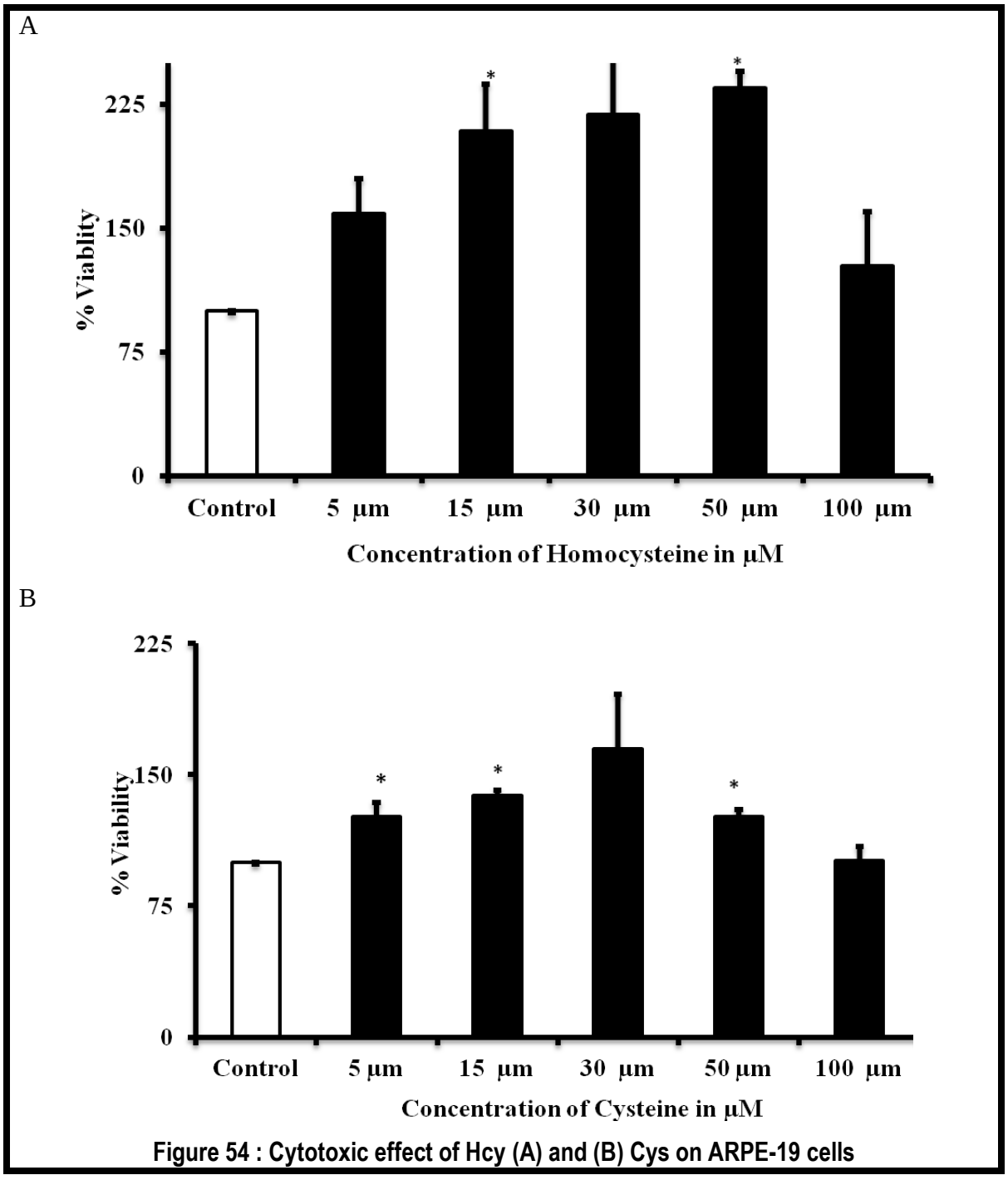

Figure 54 : Cytotoxic effect of Hcy (A) and (B) Cys on ARPE-19 cells

4.6.2 Intracellular GSH levels

To determine whether Hcy decreased the GSH in ARPE-19, cells were treated with increased concentration of Hcy, and or Cysteine (5, 15, 30, 50 and 100 µM) and intracellular GSH concentrations were analyzed after 1 h. The intracellular GSH content was decreased when compare to controls with increase concentration of Hcy, whereas the GSH content was increased when compare to controls with increased concentration of cysteine. Increased Hcy decreases the synthesis of GSH; the GSH level can be retrieved upto 60 % by adding cysteine to the cells (figure 55 and 56).

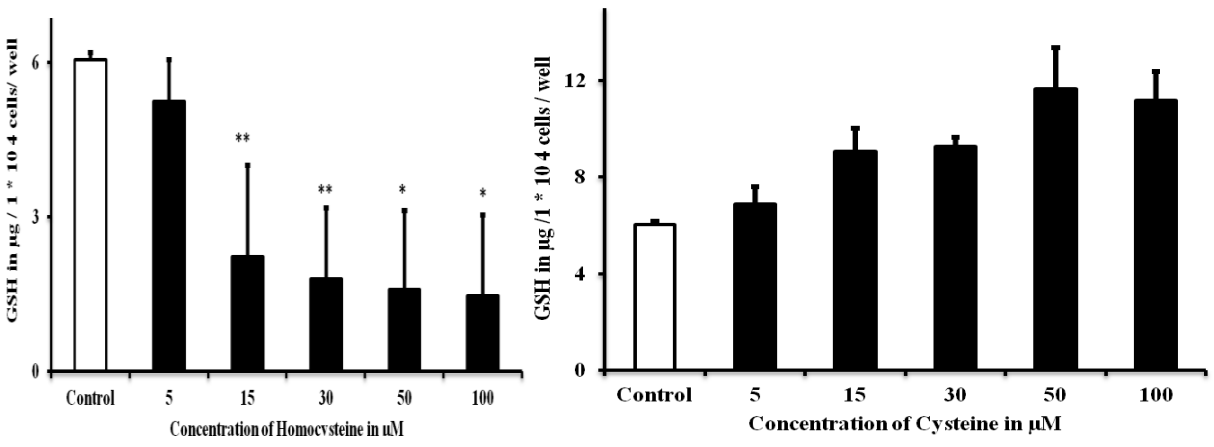

Figure 55: Intracellular Glutathione content A) Hcy and B) Cys on ARPE-19 cells

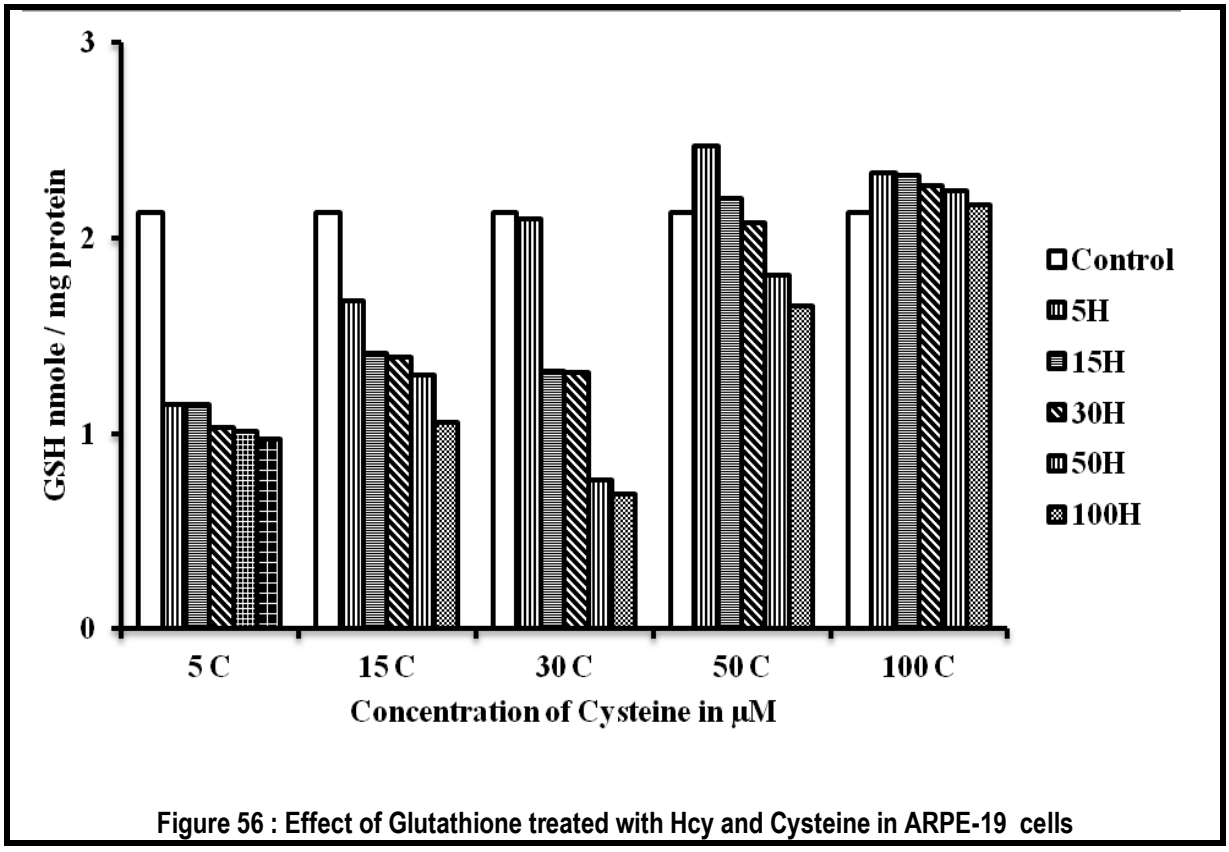

Figure 56 : Effect of Glutathione treated with Hcy and Cysteine in ARPE-19 cells

4.6.3 Homocysteine alters the amino acids level in ARPE-19 cells

Amino acids concentrations were determined in cell extracts by following the same method which was developed in this research for clinical samples. Glutamic acid is decreased when increased the concentration of Hcy, whereas in 5, 15 and 30 µM concentrations of homocysteine not affected the cysteine level, whereas the Hcy concentration of 50 and 100 µM affected the cysteine level. The glycine level also affected while increasing the concentration of Hcy, Taurine level also affected while increasing the concentration of Hcy. But methionine level is increased while increasing the concentration of Hcy. The amino acids such glutamic acid, cysteine and glycine are decreased when increased the concentration of Hcy, this paves the way to study the Hcy is really affecting the synthesis of GSH or utilization of GSH. Glycine is an abundant amino acid 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 [156], justifying the increased levels of glycine in the plasma of people with ARMD. It is shown in figure 57.

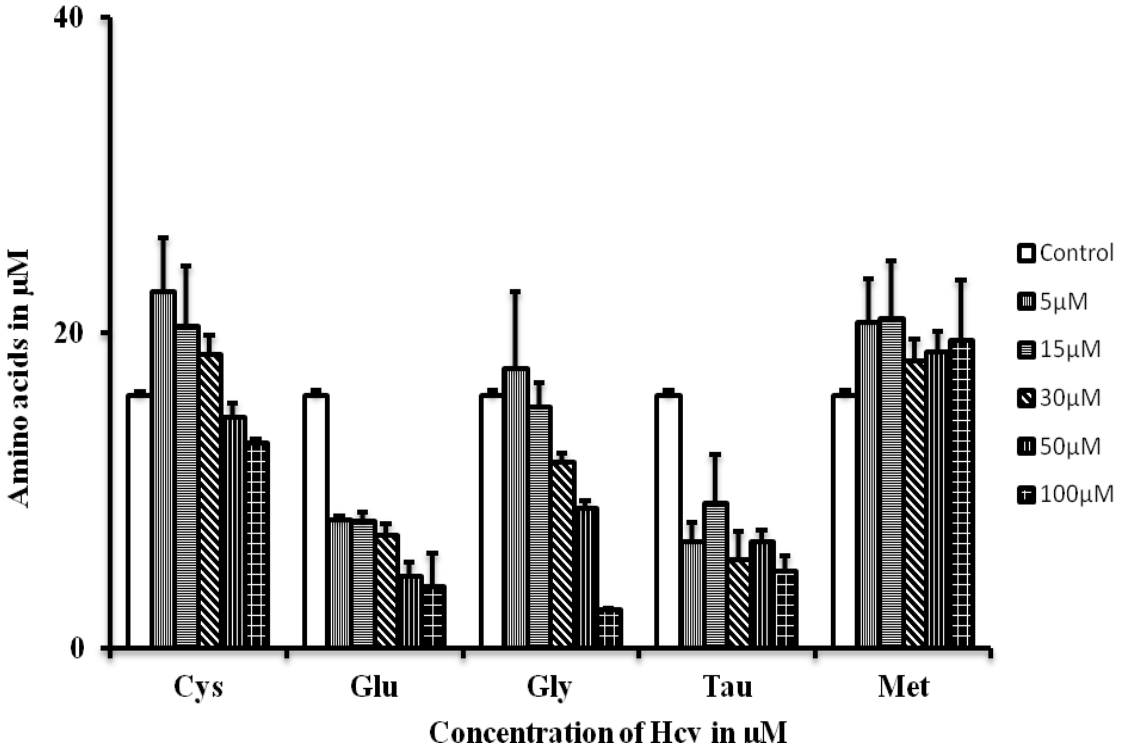

Figure 57: Homocysteine alters the amino acids level in ARPE-19 cells

4.6.4 Homocysteine induces mRNA expression of catalytic unit and the modifier unit of GSH in ARPE-19 cells

Thus, Hcy decreases the amino acids such as glutamic acid, cysteine and glycine and also decreases the intracellular GSH levels; to know what happen to the mRNA expression of rate limiting enzyme GCL (both the catalytic unit – GCLC and modifier unit - GCLM). There is a difference between the expression of both the units GCLC and GCLM in PBMC and ARPE-19 cells. Increased expression of both the units GCLC and GCLM in ARPE-19 cells, with increasing concentrations of Hcy when compared to the controls. Since the GCLM expression level is decreased in 100 µM concentration of Hcy into the cells (figure 58).

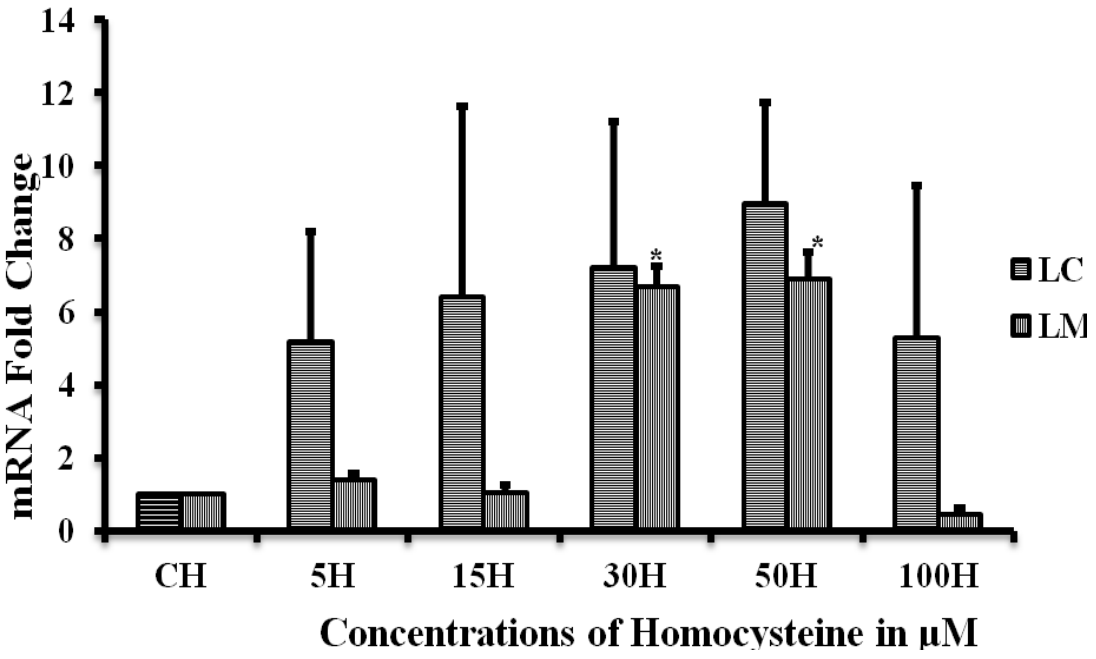

Figure 58: The mRNA expression of GCLC and GCLM after Hcy exposure to ARPE-19 cells

4.6.5 Homocysteine stimulates mRNA expression of Nrf2 in ARPE-19 cells

Nrf2 is an important transcription factor, which contributes towards gene expression. To explore whether Hcy stimulates Nrf2, mRNA expression studies of Nrf2 in ARPE-19 cells were conducted. As shown in figure 59, Hcy increases the mRNA expression of Nrf2 with increased concentration of Hcy exposed to ARPE-19 cells. The results show that Nrf2 play an important role in cellular response against Hcy induced oxidative stress. It is known that, both the units (GCLC and GCLM) in this rate limiting enzyme (GCL of ARPE-19 cells) are regulated through the Nrf2 pathway. Exposure of Hcy depletes the GSH level, but increased the expression of GCLC, GCLM via Nrf2. Homocysteine itself regulates the Nrf2 mediated Glutamate cysteine ligase expression in ARPE-19 cells similar results were observed by Shahnaz Khaghani et.al., in 2013 [157].

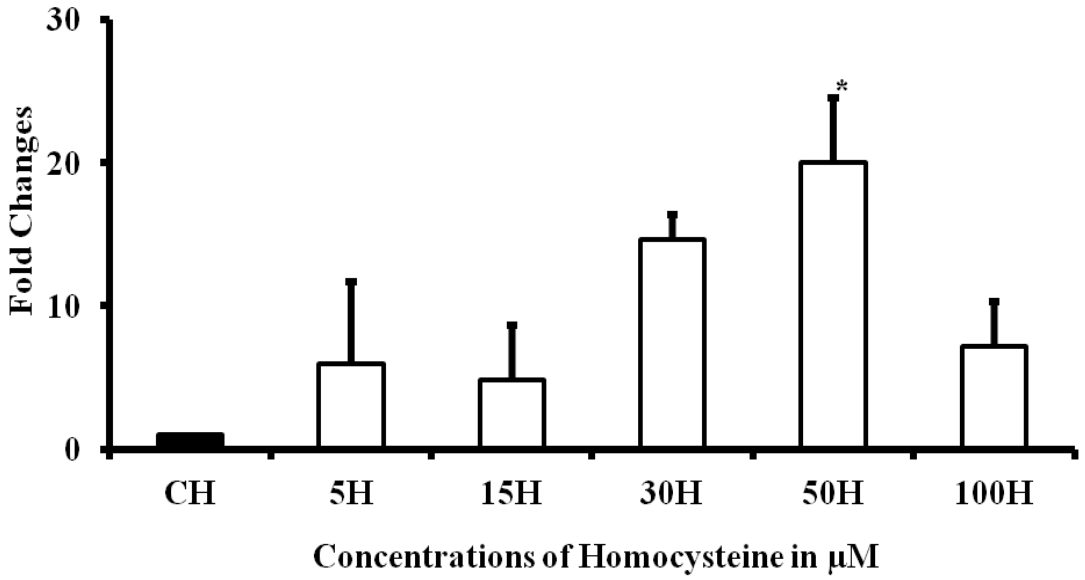

Figure 59: mRNA Expression of Nrf2 after Hcy exposure to ARPE-19 cells

4.7. To understand the effect of homocysteine in their expression of ϒ-glutamate cysteine ligase, and nuclear related factor (Nrf2) in Human umbilical vein endothelial cells (HUVECs) as a cell culture model system for vascular diseases (*In Vitro*).

Homocysteine causes endothelial cell injury when added directly to the cultured endothelial cell [57]. Homocysteine also increases the pro-coagulant activity of endothelial cells by factor V. Endothelial cells undergo apoptosis through stimuli namely tumour necrosis factor α and oxidized lipoprotein. Thus injured endothelial cells impair vascular function. This paves the way to study the role of Homocysteine in HUVECs.

4.7.1 Cytotoxicity Assay

MTT assay was performed to check the cytotoxicity of the Hcy and Cys on HUVEC cells. Concentration ranges from 5 – 100 µM for 1 h time point. Increasing concentration of Hcy after 1 h time point shows the toxic effect to the cells (figure 60).

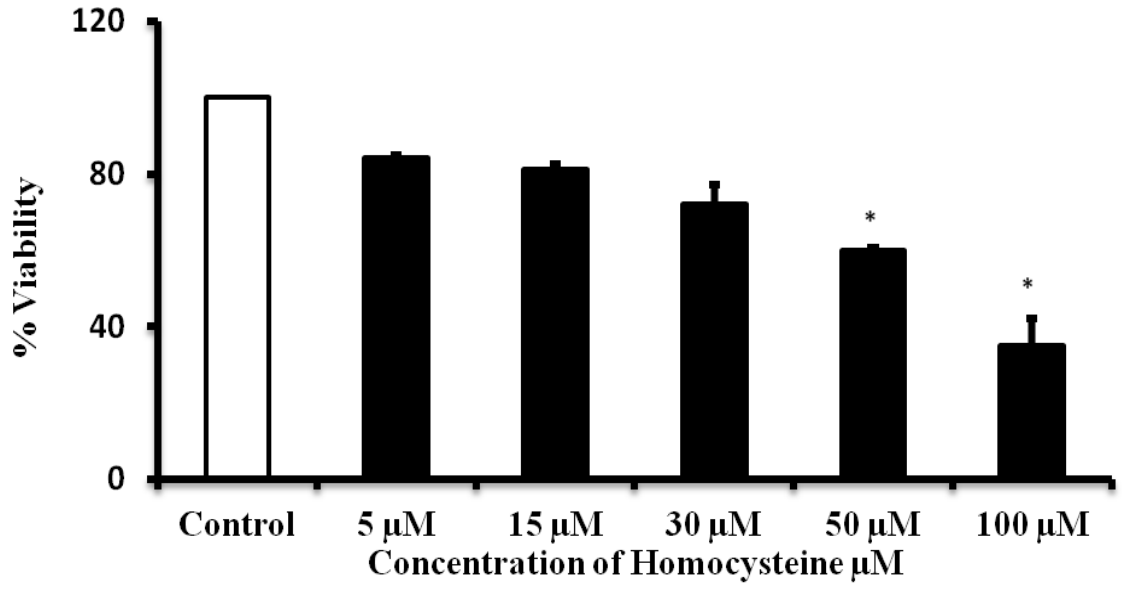

Figure 60: Cytotoxic effect of Hcy on HUVECs

4.7.2 Homocysteine suppressed the mRNA expression of catalytic unit and modifier unit of GSH in HUVECs

Thus Hcy decreases the amino acids such as glutamic acid, cysteine and glycine and also decreases the intracellular GSH levels; to know what happen to the mRNA expression of rate limiting enzyme GCL (both the catalytic unit – GCLC and modifier unit - GCLM). There is a no difference between the expression of both the units GCL in Erythrocytes (Red blood cells) are responsible for oxygen transport. Oxygen transport is mainly dependent on the presence of iron in haemoglobin. Both oxygen and iron are easily oxidized mainly due to free radicals produced by reactive oxygen species and reactive nitrogen species. All the above elements together can damage the red blood cells as well as vascular endothelium. Free radicals are generated by oxygen saturation, superoxide detoxification, atherosclerosis and bacterial infections. Hcy is known to be pro-oxidizing agents [158]. Vitamins B_6, B_{12} and folic acid and enzymes MTHFR and CBS are involved in the synthesis of Hcy. Due to the deficiency in any of these vitamins or enzymes can alter the morphology of erythrocytes and GCLM in PBMC and HUVECs. Increased expression of GCLC and GCLM observed in HUVECs, especially in 5 and 15 µM concentrations of Hcy when compared to the controls. It shows that the normal concentration, i.e., the physiological levels of homocysteine is needed for the synthesis of antioxidants GSH. Since both GCLC and GCLM expression level is significantly decreased in 30, 50 and 100 µM concentration of Hcy in to the HUVECs when compare to

control (figure 61). If Homocysteine levels going beyond the 30 µM concentrations, i.e., in elevated conditions it's affected the vascular cells.

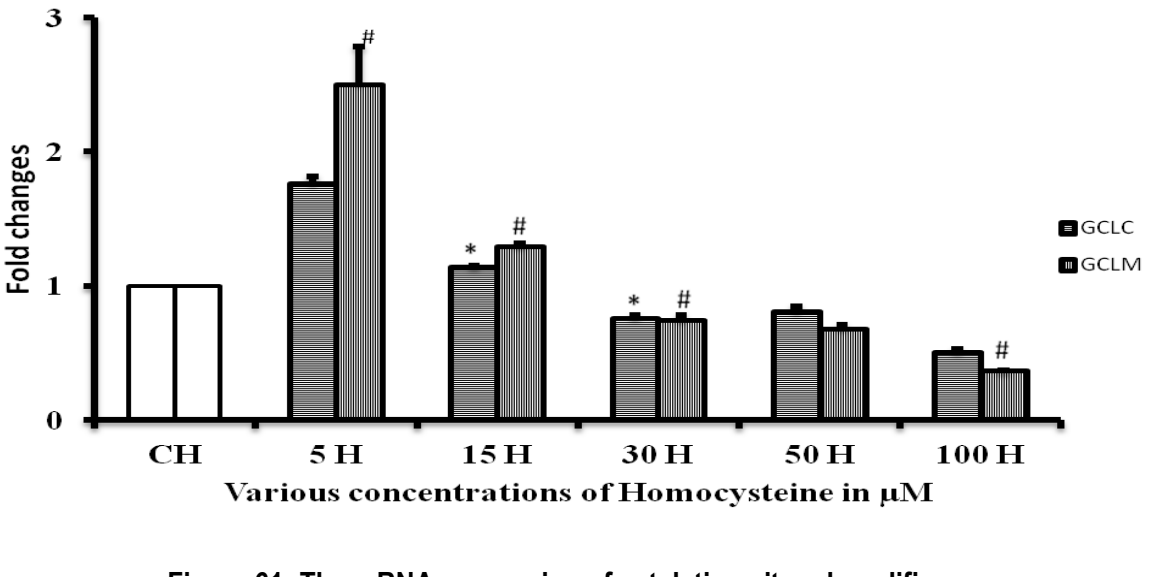

Figure 61: The mRNA expression of catalytic unit and modifier unit of GSH after Hcy exposure to HUVECs

4.7.3 Homocysteine lowers the mRNA expression of Nrf2 in HUVECs

Nrf2 is an important mechanism that contributes towards gene expression. To explore whether Hcy stimulates Nrf2 or decline the Nrf2 levels, mRNA expression of Nrf2 in Huvecs was conducted and as shown in figure 62, Hcy decreases the mRNA expression of Nrf2 with increased concentration of Hcy exposed to Huvecs. The results show that Nrf2 play an important role in cellular response against Hcy induced oxidative stress. Thus the result shows that the expression level of GCLC and GCLM is decreased due to the expression via Nrf2 pathway. Exposure of Hcy depletes the GSH level and also decreased the expression of GCLC, GCLM via Nrf2, this paves the way to study about the homocysteine pathway in enzymatic levels and also it is really a paradox, truly homocysteine is a friend or foe to the vascular diseases.

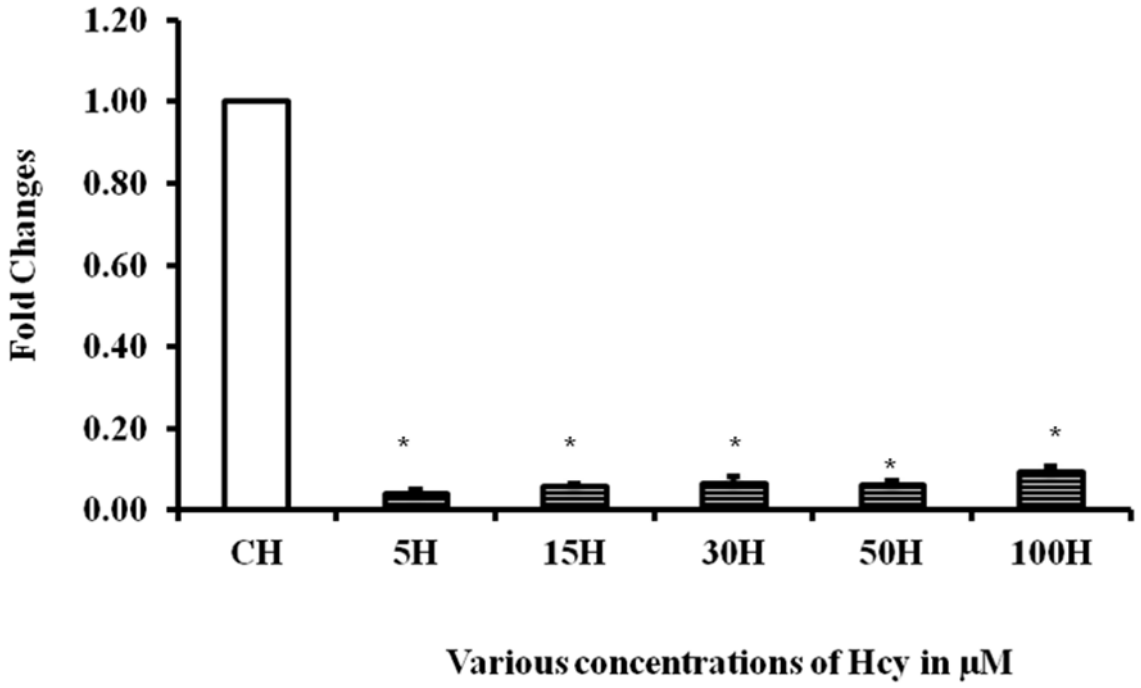

Figure 62: mRNA Expression of Nrf2 after Hcy exposure to HUVECs