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GENETIC DETERMINANTS OF HYPERHOMOCYSTEINEMIA IN ATHEROSCLEROSIS

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ABSTRACT

Hyperhomocysteinemia (Hhcy) is an independent risk factor for the development of atherosclerosis. The mechanisms by which HHcy promotes cardiovascular disease may be due to activation of pro-inflammatory factors, endoplasmic reticulum (ER) stress and oxidative stress. We aimed to study (i) gene mutations that cause HHcy. (ii) Estimation of inflammatory marker like ultrasensitive C-reactive proteins (hs-CRP) and total antioxidant levels (iii) determination of Hcy– dependent gene expression in vivo*. 25 HHcy patients and 25 healthy controls were taken for this study. Mutation detection in MTHFR, CBS, MS and eNOS gene was by PCR-based restriction enzyme analysis and subsequently expression study was carried out by Reverse Transcriptase PCR and cloning technique. A significant association of HHcy with MTHFR (C677T) and MS (A2756G) genotype was observed (p< 0.05). There was no association of Hhcy and eNOS genotype. The Hhcy patients, showed no expression of the ER stress gene, GRP78 in lymphocytes. Our study showed no effect of Hcy on the CD18 gene (pro- inflammatory pathway) expression, but a significant association of tHcy and hs-CRP levels in HHcy grp (t=2.28, p< 0.05). This shows that HHcy induces inflammatory response, which could lead to tissue injury in the pathogenesis of the atherosclerotic process. Our findings show higher mRNA expression of manganese superoxide dismutase (Mn SOD) in HHcy group as compared to the control group .The Total Antioxidant Status (TAS) estimated was found to be significantly lower in the HHcy group as compared to healthy normals (t=4.8, p<0.01). Taken together these findings strongly suggest that the adverse effects of homocysteine are at least partly mediated by oxidative stress. Our study supports the hypothesis that Hcy evokes adverse vascular effects by promoting oxidative damage to endothelial cells.*

KEY WORDS

Hyperhomocysteinemia, Atherosclerosis, MTHFR, MS, eNOS, CBS

INTRODUCTION

Atherosclerotic cardiovascular disease is a major cause of mortality & morbidity. (1) An elevated plasma level of the amino acid homocysteine (hcy) has been identified as an independent risk factor for atherosclerosis (2). A plasma hcy concentration exceeding 15 mmol per L is now termed as hyperhomocysteinemia (Hhcy) (3)

The genetic factor predisposing to Hhcy are the common thermolabile variant of methylene tetrahydrofolate reductase (MTHFR) gene, mutations in Cystathionine b Synthase (CBS), methionine synthase (MS) and the polymorphisms in endothelial Nitric oxide synthase (eNOS) gene. (4,5)

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There are reports that the 677C T mutation at the MTHFR gene is associated with Hhcy in young population, and older patients with Coronary Artery disease. The one most prevalent mutation at the MS gene, which lead to deficient MS enzyme activity and markedly elevated hcy levels is that of the A 2756 G transition. (4). The two polymorphisms in the eNOS3 gene that have been studied in association with Coronary Artery disease are, Glu298 Asp and T786C. Taken together, the Published reports of modulation of hcy metabolism by Nitric Oxide and the possible association of the eNOS3 polymorphism with vascular disease suggests that Glu298 Asp genotype may be a genetic determinant of tHcy concentration. (5)

Several hypothesis have been proposed to explain the cellular mechanisms by which HHcy promote cardiovascular disease, including the activation of pro- inflammatory factors, endoplasmic reticulum (ER) stress and oxidative stress. *In vitro* and animal models studies have demonstrated a

relationship between HHcy, endothelial dysfunction and accelerated atherosclerosis. Experimental evidence for these mechanisms has been obtained from studies of endothelial cells exposed to exogenous hcy *in vitro*, but the mechanisms have not been examined *in vivo (6,7)*. We carried out studies to see whether these hcy- dependent changes in gene expression are seen in *vivo* in Hhcy patients. Till date no study has been carried out in India to check for gene expression of the hcy – respondent genes.

AIMS AND OBJECTIVE

A) To screen the study population for the mutations C677T in MTHFR, T833C in CBS and A2756G in MS genes that cause HHcy.

B) To investigate the association between HHcy and the Glu 298 Asp polymorphism alone and in combination with T786C polymorphism in the 5' flanking region of the eNOS gene.

C) Estimation of atleast one inflammation marker (high sensitive C – reactive proteins) and the Total Antioxidant Status (TAS) related state in HHcy.

E) To determine whether the hcy -dependent changes in gene expression are seen *in vivo.*

MATERIALS AND METHODS

Subjects: Blood samples were collected in sodium citrate and heparin tubes from 25 HHcy patients and equal number of healthy individuals for normal controls. Blood samples were collected from individuals attending the health check clinic at this hospital, after an overnight fast.

(A) **Determination of hcy levels by HPLC**: Plasma samples were analysed for tHcy by HPLC using Ashavaid *et al* method (8)

(B) **Lipid, lipoprotein and hs-CRP measurements:** Lipid profile and hs - CRP measurements were done on Synchron Cx7 Beckman auto analyzer with standard enzymatic methods (Beckman,USA).TAS was determined spectrophotometrically.

(C) **Determination of known mutations in genes involved in Hhcy**

The major enzymes involved in hcy metabolism are: MTFHR, CBS and MS. Certain known mutations viz: **C677T**, **T833C** and **A2756G** in the MTHFR, CBS and MS gene respectively are associated with Hhcy. These mutations are such that they alter restriction enzymes sites. Therefore, polymerase chain reaction (PCR)-based restriction enzyme (RE) analysis was used for their detection.

(i) DNA Isolation: Genomic DNA was isolated from whole blood using salting out procedure of Miller et al (9).

(ii) a) PCR-Amplification of MTHFR, CBS and MS Genes: Genomic DNA was PCR-amplified using specific oligonucleotide primers and conditions as described by Frosst et al (10), Tsai et al (11), and Leclerc et al (12). The amplified DNA was detected by 1.5% agarose gel electrophoresis to confirm the presence of 198 bp, 159 bp and 189 bp of MTHFR, CBS and MS genes respectively.

b) PCR-Amplification of eNOS gene: Genomic DNA for Glu298Asp and T786C polymorphisms was amplified using specific oligonucleotide primers and conditions as described by Colombo et al (13) The amplified DNA was detected by 1.5% agarose gel electrophoresis to confirm the presence of 203bp and 236 bp of genes respectively.

(iii) PCR-based RE analysis: The amplified DNA for all the above genes were digested with the specific Restriction Enzyme and resolved on Polyacrylamide gel and visualized for digested fragments by ethidium bromide staining.

Statistical Analysis: Biochemical levels were expressed as mean +/- SD. Allele frequencies were deduced from genotype frequencies and the difference between groups were tested by Chi square analysis. A value of $p<0.05$ was considered to be statistically significant.

(D) **Gene expression analysis**

(1) RNA Preparation : Total cellular RNA extraction was done by using Trizol reagent (GIBCO BRL, Life Technologies).

.(2) Reverse Transcriptase PCR (RT-PCR) :
We performed RT-PCR method as described by Kokame et al (7) .The conversion of RNA into first – strand cDNA was done using oligo-deoxythymidylate: 5'-T_{11} C – 3' by reverse trancriptase enzyme. The 3' dinucleotide sequence functioned as an anchor, ensuring that the primer base pairs at the 5' –most end of the

poly $(A)^+$ tail. This was followed by amplification of the products of the first strand cDNA using primers 5'-T_{18} C – 3'and an arbitrary decanucleotide. These products were subsequently analyzed by polyacrylamide gel electrophoresis and the product intensities were compared directly to the other samples under investigation i.e. controls vs patients for over expression.

(2) Validation of amplified fragment

(a) Elution and reamplification of fragment of interest was done and this amplified–fragment was further subjected to cloning and sequence.

(b) Cloning: The amplified DNA fragment was cloned into the plasmid vector pcrII using the TA cloning technique.

(c) Sequencing: Sequencing reaction was performed in sequencing plate containing template DNA, 5X Sequencing buffer, terminator Ready-Reaction mix Primer and H_2O. The reaction was subjected to a PCR programme. Comparison of DNA homology with the Gen Bank and the EMBL databases was performed using BLAST software.

RESULTS AND DISCUSSION

Mutational study

Table 1 Shows the profile of all variables in the Hhcy patients and control groups. In our study, homocysteine was not related to any of the lipid levels. Although the majority of cases of HHcy are thought to be caused by interplay between dietary and genetic factors, the genetic disorders are associated with the highest plasma levels of hcy, with inherited deficiency of several enzymes. The most common being Methylene tetrahydrofolate reductase (MTHFR), Cystathionine B - Synthase (CBS) and Methionine Synthase (MS).

Table 1
Profile of all variables in the Hhcy patients and control group

Variables	Patients (n=25)	Controls (n=25)
Age (yrs)	43.78 ± 8.1	39.2 ± 7.2
Homocysteine(μmol/L)	52.23 ± 22.14 *	10.67 ± 4.1
Cholesterol (mg/dl)	184.56 ± 33.2	172.6 ± 26.57
Triglyceride(mg/dl)	142.74 ± 42.41	110.04± 29.53
HDL – C (mg/dl)	32.12 ± 8.5	43.45 ± 8.9
LDL – C (mg/dl)	124.30 ± 29.9	118.16 ± 19.1
Apo A_1 (mg/dl)	0.98 ± 0.19	1.25 ± 0.2
Apo B (mg/dl)	1.0 ± 0.31	.90 ± 0.2
hs CRP (mg/dl)	0.46± 0.49 *	0.15 ± 0.12
TAS (mmol/L)	1.0 ± 0.17 **	1.5 ± 0.2

**P <0.001. Values are expressed as Mean ± S.D; *P <0.05.

As reported by Frosst et al (10), the 3 MTHFR genotypes for alanine to valine mutation (A/A, A/V and V/V) were diagnosed by digestion of the 198bp-amplified DNA with Hinf I. (Fig 1) In our present study, the A/V heterozygous genotype was found in 48 % (12/25) of the Hhcy patient as compared to 12% (3/25) of control (Table 2). The difference was statistically significant (x^2 =5.6, p< 0.05). In our previous study (14) the heterozygous MTHFR mutation was found to be 54.5% in patients with Hhcy and hence heterozygosity for the thermolabile MTHFR mutation was found to be associated with Hhcy.

Kluitjmans *et al* have reported a prevalence of the heterozygous MTHFR mutation to be 45.2% in patients with intermediate homocysteine levels (15). The MTHFR mutation has been found to be significantly associated with CHD in a Japanese population (16). The prevalence of heterozygous MTHFR mutations was found to be 52% in patients with CHD in that population. Frosst *et al* have also reported a similar finding in the French Canadian population with the prevalence of heterozygous MTHFR to be 51% in patients with CHD. (10).

It is established that inhibition of Methionine synthase results in reduced intracellular homocysteine re-methylation and increased export of homocysteine from cells to plasma. It has been shown that there are large inter- individual differences in homocysteine clearances and it is postulated that this can be explained by variable adaptation to impaired Methionine synthase function, leading to increased homocysteine flux through alternate metabolic pathways. The recently discovered mutation in the MS gene is a 2756A G change, resulting in a glycine for aspartic acid substitution (D919G), which would result in the reduced activity of MS enzyme.

The 3 MS genotypes for the aspartic acid to glycine mutation (DD, DG, and GG) were diagnosed by digestion of the 189-bp PCR products by Hae II as shown in the previous report by Leclerc et al (12). (Fig 2) In our study, the D/G heterozygous genotype was found in 44 % (11 /25) of the Hhcy patient as compared to16 % (4 /25) of control. (Table 2). The difference between the Hhcy patient group and controls was statistically significant (x^2 = 7.6,p Ð 0.05) in our study.

In the present study, we describe that the MTHFR and the MS mutation are determinant of plasma levels of homocysteine

Table 2
Percentage of MTHFR, MS and CBS genotype in Hhcy patients and controls

Genotype	Patients (n=25) (%)	Control (n=25) (%)
MTHFR		
A/V (heterozygous)	12 (48)	3 (12)
A/A (Normal)	23 (52)	22(88)
V/V (Homozygous)	0(0)	0 (0)
MS		
A/G (heterozygous)	11 (44)	4(16)
A/A (Normal)	12(48)	22(84)
G/G (Homozygous)	2 (8)	0(0)
CBS		
I/T(heterozygous)	0(0)	0(0)
I/I (Normal)	25(100)	25 (100)
G/G (Homozygous)	0(0)	0(0)

Table 3
Percentage of G298A and T786C polymorphisms of eNOS gene in Hhcy patients and controls

Genotype	Patients (n=25) (%)	Control (n=25) (%)
G298A		
G/G (normal)	12 (48)	18 (72)
A/A (heterozygous)	9 (36)	6 (24)
V/V (homozygous)	4 (16)	1 (1)
T786C		
T/T (normal)	16 (64)	21 (84)
T/C (heterozygous)	7 (28)	4 (16)
C/C (homozygous)	2 (8)	0 (0)

and that the genetic variation of both MTHFR and MS have an influence on the remethylation pathway.

Endothelial function, of which decreased vasodilator activity of Nitric oxide (NO) is a hallmark (19), and which is a component of early atherogenesis, including CAD, has recently been shown to be of prognostic significance (20). Furthermore, endothelial dysfunction is found in the presence of vascular risk, despite the absence of obvious vascular disease, as in hypercholesterolemia and cigarette smoking (21). Hence, factors that influence NO availability are likely to be of considerable clinical importance. The synthesis of endothelial NO from L – arginine is regulated by the enzyme, nitric oxide synthase (eNOS) and a number of polymorphisms in the eNOS gene sequence have been identified (22), of which 2 are studied here.

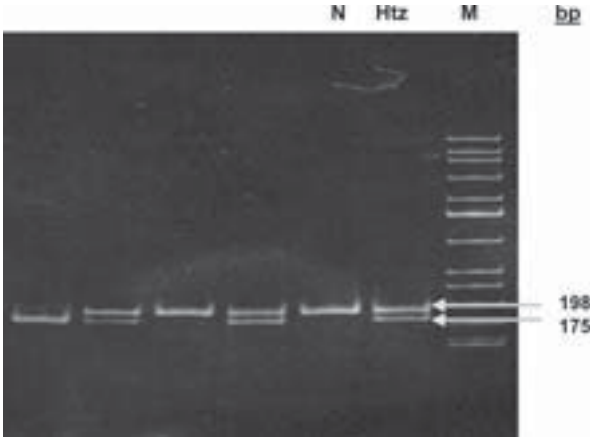

Figure 1: MTHFR polymorphism C677T.Analysis of the MTHFR mutation on 8% polyacrlyamide gel after digestion with Hinf I.Lane M – molecular weight marker, Lane Htz – patient with heterozygous mutation, lane N – normal control.

Association of Glu298Asp polymorphism of the eNOS gene was not significantly associated with the Hhcy in our group ($x^2 = 3.6$, $p = 0.10$). As for the T786 C polymorphism in the 5' flanking region of the eNOS gene was also not significantly associated with the presence of HHcy in our patients as well ($x^2 = 2.46$, $p = 0.10$). (fig 3a and b; Table 3)

The MS- inhibition hypothesis predicts that the Glu298 Asp should be associated with unchanged or lower thcy concentration rather than higher concentrations observed. Our results do not support the hypothesis that NO modulates thcy concentration by inhibiting MS. Reports to date suggest that the Asp298 form of NOS3 has either unchanged or reduced activity relative to the Glu 298 enzyme. (22,23).

GENE EXPRESSION STUDY

Kokame et al (7) have demonstrated in cultured human vascular endothelial cells (EC) that hcy induces expression and synthesis of GRP 78, a stress response gene. Since these studies are only carried out *in vitro*. We wanted to carry out *in vivo* studies and provide a first step in identifying the gene pathways by which hcy alters EC function. Outinen et al have done similar work on a cell culture system (6). We used freshly isolated lymphocytes from patients with Hhcy to examine the expression of these genes *in vivo*.

We have compared mRNA expression pattern of lymphocytes from 25 controls and 25 Hhcy patients with different levels of Hcy, the lowest level being 25mmol/L and highest being 103 mmol/L .The cDNA synthesis and RT-PCR protocol was performed using Kokame et al method (7). A total of 4 decamer

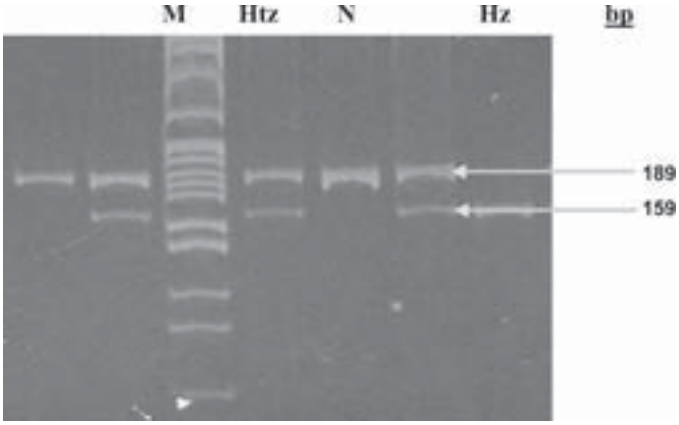

Figure 2: MS polymorphism 2756 AG. Analysis of the MS mutation on 8% polyacrlyamide gel after digestion with Hae III.Lane M – molecular weight marker, Lane Htz – patient with heterozygous mutation, lane N – normal control. Lane Hz-pateint with homozygous mutation.

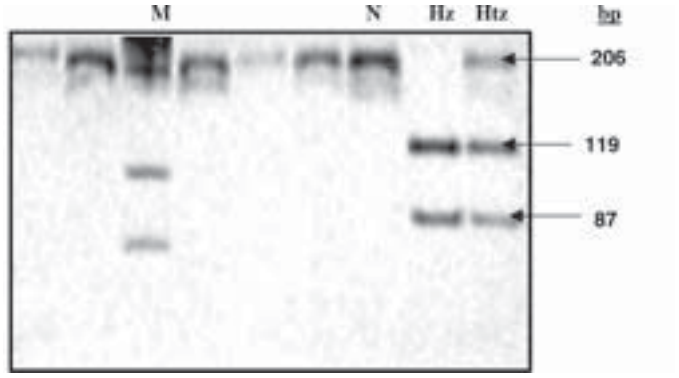

Figure 3 a: eNOS polymorphism Glu 298- Asp . Analysis on 8% polyacrlyamide gel after digestion with Mbo1.Lane M – molecular weight marker, lane N – normal control. Lane Hz-pateint with homozygous mutation., Lane Htz – patient with heterozygous mutation.

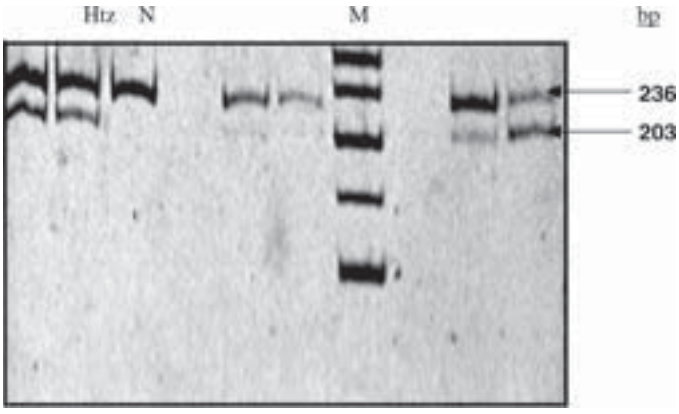

Figure 3b: eNOS polymorphism T- 786 C. Analysis on 10% polyacrlyamide gel after digestion with *Pdi* I. Lane Htz – patient with heterozygous mutation. Lane N – normal control, Lane M – molecular weight marker.

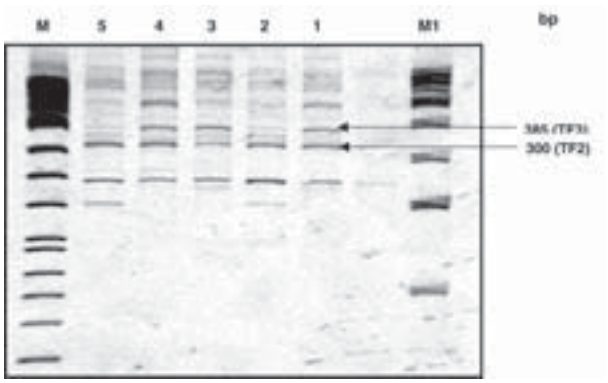

Figure 4: Representative band patterns for hcy respondent genes on analysis showing PCR fragments in hyperhomocysteinemic patient lymphocytes. A non-denaturing 6% PAGE post stained with Etbr is shown.

M- PhiX 174 DNA/Hinf I Digest
M1 -100bp DNA ladder
Lane 1- patient with > 50 u M Hcy
Lane 2 -control with 10 u M Hcy
Lane 3 -patient with > 50 u M Hcy
Lane 4 - patient with > 50 u M Hcy
Lane 5- control with 10 u M Hcy

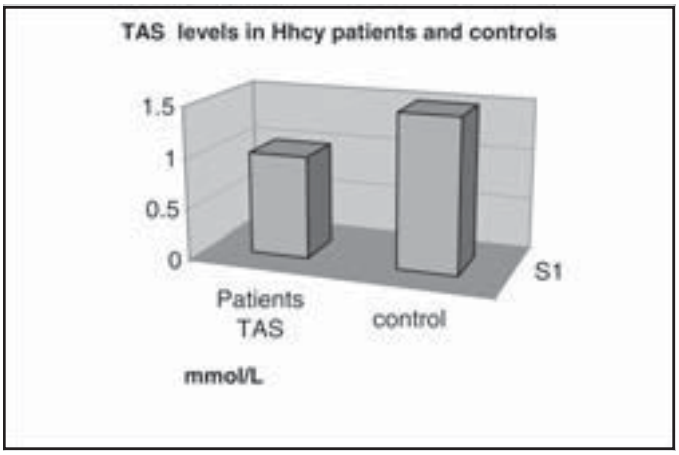

Figure 5 : TAS levels in Hhcy patients and controls.

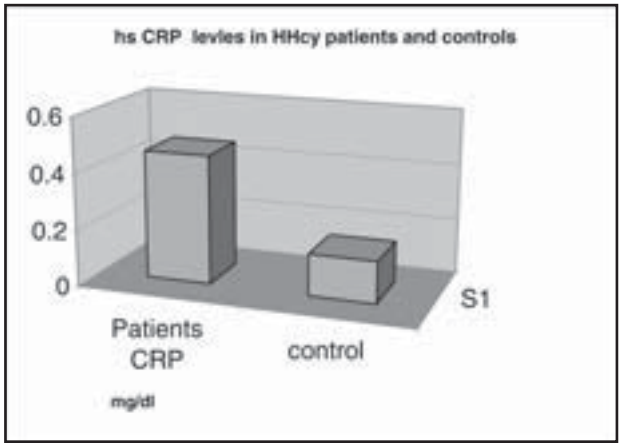

Figure 6 : hs CRP levels in Hhcy patients and controls

primers were used for screening against the cDNA pool prepared by T_{11}CC oligonucleotide primer .For all the 25 Hhcy patients and 25 controls this was carried out. (i.e. 200 PCRs in total were performed amongst all the samples). We analyzed the changes in expression of the stress genes such as GRP78and leukocyte adhesion molecule gene ITGB2/CD18 when hcy levels were high *in vivo* (24,25).

The stress gene, GRP78 did not show bands in the base pair of interest when visualized on native PAGE. Outinen et al (6) have investigated the effects of hcy on expression of stress genes in cell culture. Their results showed that hcy causes reductive stress. The expression of genes coding for ER-resident stress proteins is specifically activated under conditions of ER –stress. The expression of GRP78 mRNA in the cell culture studies (6) indicate that hcy – stimulated cells are under stressful conditions.

A number of experimental studies demonstrated that high concentrations of homocysteine are toxic for endothelial cells. It is important to note that these *in vitro* studies used very high concentration of hcy at levels never observed in patients with Hhcy. Furthermore, most studies compared this high concentration of hcy with cell cultures in a non – physiological hcy – free environment .The clinical importance of these *in vitro* observations remains uncertain. We examined the effects of hcy that occur at physiologically relevant concentration of hcy and confirmed these effects *in vivo* in patients with Hhcy. Our studies on Hhcy patient lymphocytes showed no change in expression in same genes thus showing that the *in vivo* effects of hcy are different from the way it acts *in vitro*.

We identified bands at around 207 bp (TF1 fragment) with the primer combination TACAACGAGG, T_{18}C and band at around 385bp and 300 bp (TF2, TF3 fragment) with the primer combination GATCTGACAC, T_{18}C (Fig 4). To confirm the gene expression pattern observed in the analysis the cDNA fragments (TF1, TF2, TF3) were recovered from gels and reamplified by PCR using the corresponding pair of primers. The reamplified fragment was then cloned into plasmid pcr II.

The cloned cDNA fragments (TF1, TF2, TF3) were sequenced, and the results revealed that the fragments were flanked by the sequence corresponding to the particular oligo (dT) primer and the decanucleotide primer. The nucleotide sequence was then confirmed by searching for homologies against the Genbank / EMBL databases using BLAST software.

The sequence for cDNA fragment TF1 completely matched the bases 2552- 2792 of the human gene Gen Bank accession No. H17426. The nucleotide sequence of the fragment was found to > 99% identical to the ITGB2/ CD18 gene. The leukocyte adhesion molecule gene also known as Integrin Beta 2 (ITGB2/ CD18) has recently been hypothesized to be upregulated *in vivo* which could contribute to acceleration of atherosclerosis, in patients with elevated plasma homocysteine levels (19,20). In both the groups, there was consistency in the expression, and thus showed there was no effect of hcy on the leukocyte adhesion molecule gene, known Integrin Beta 2 (ITGB2/ CD18).

The sequencing analysis of the cDNA fragment (TF2) revealed that it belonged to the Alu family. (iii)The sequences that were cloned from the PCR fragment (TF3) showed increased intensity in the Hhcy patients .The sequencing results as described earlier showed that the nucleotide sequence of the fragment, had a deduced protein sequence of 177 amino residues that was similar to the enzyme, Manganese Superoxide dismutase (Mn SOD)(gi 33186704).

Mn SOD coverts superoxide (O_2^-) to produce hydrogen peroxide (H_2O_2) during normal oxygen metabolism and contribute to the redox state of the cell. Mn SOD is located in the mitochondrial matrix. It has been shown that Mn SOD can be induced in the atherosclerotic arteries, may be as a reply to mitochondrial oxidation. (26) Reactive oxygen species mediated mitochondrial – dependent pathways are suggested as major contributing pathomechanisms to nuclear damage, which eventually may result in apoptosis. (26). High levels of homocysteine *in vivo* could also be causing similar effects leading to oxidative stress mechanism.

Although the relationship between the classical CVD risk factors and cardiovascular mortality is widely known, the latest summarized information has shown that less than 50% of cardiac patients present classical risk factor (27). At the same time, prolonged high- grade oxidative stress damage is increasingly recognized as playing an important role in the pathogenesis of CVD. Homocysteine can be viewed as the first risk factor for atherosclerosis believed to exert its effect through a mechanism involving oxidative damage (28). We further investigated tHcy, inflammation (measured by CRP), Total Antioxidant status (TAS) in patients and control group.

In our study, Homocysteine was not related to any of the lipid and lipoprotein levels but the Total Antioxidant Status (TAS) was significantly lower ($t=4.8$, $p< 0.001$) in the Hhcy group compared to the normal group, indicating oxidative stress in the patients. (Fig 3) Low TAS in our study, confirming our gene expression analysis, which shows the induction of the Mn SOD

mRNA, indicates that Hhcy induces oxidant stress *in vivo*. Thus more work is needed to evaluate the biomarkers of oxidative stress, together with the principal biomarkers, when cardiovascular risk is assessed in relation with Hhcy.

It is also widely accepted that the development of CVD has an inflammatory background. Recent studies provided compelling evidence that elevated values to hs-CRP (as inflammatory marker) play a role in the pathogenesis of CVD (29) .We hypothesize that homocysteine causes inflammation. Evidence indicates that inflammation may have an important role in the progression of atherosclerosis (30). HsCRP, an acute phase reactant, is a sensitive marker of inflammation hence we analysed the relation of homocysteine with marker implicated in inflammation. In our study, homocysteine was significantly related of hsCRP levels ($t=2.28$, $p< 0.05$), indicating inflammation in the Hhcy group. (Fig 4)

Homocysteine has been shown to activate Endothelial cells (EC) through up- regulation of components of the inflammatory cascade, including activation of transcription factors such as NF-kB (31). Homocysteine most likely activates NF-kB through creation of oxidative stress by altering the redox thiol status of the cell (31). As a consequence, there is increased production of pro- inflammatory cytokines, and expression of adhesion molecules and chemotactic factors in Hhcy.

CONCLUSION

We found a significant association between the MTHFR and MS genotypes with Hyperhomocysteinemia. Both the mutations of the eNOS gene Glu298 A and T786C studied were not significantly associated with HHcy.

Our *in vivo* study in patients with physiologically relevant concentrations of Hcy, also showed no expression of the ER stress gene, GRP78 in lymphocytes. Although our study showed no effect of Hcy on the CD18 expression (pro-inflammatory pathway), we did observe a significant association of tHcy and hs- CRP levels in HHcy group. This shows atleast partly that HHcy induces inflammatory response, which could lead to tissue injury in the pathogenesis of the atherosclerotic process.

Our findings showed higher mRNA expression of manganese superoxide dismutase (Mn SOD) in HHcy group as compared to the control group. The Total Antioxidant Status (TAS) estimated was found to be significantly lower in the HHcy group as compared to healthy normals. Taken together, these findings strongly suggest that the adverse effects of homocysteine are at least partially mediated by oxidative stress.

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