Association of Aberrations in One Carbon Metabolism with Intimal Medial Thickening in Patients with Type 2 Diabetes Mellitus

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Abstract The present work was aimed to study the association of one carbon genetic variants, hyperhomocysteinemia and oxidative stress markers, i.e., serum nitrite, plasma malondialdehyde (MDA) and glutathione (GSH) on intimal medial thickening (IMT) in patients with type 2 diabetes mellitus (T2D). A total number of 76 subjects from ACS Medical College and Hospital, Chennai, India were included in the study, i.e., Group I ($n = 42$) of T2D and Group II ($n = 34$) of age- and sex matched healthy controls. The glycated haemoglobin was measured by ion-exchange resin method; plasma homocysteine by Enzyme Linked Immunosorbant Assay method; serum nitrite (nitric oxide, NO), plasma MDA and GSH by spectrophotometric methods; the IMT by high frequency ultrasound. The polymorphisms of one carbon genetic variants were genotyped using polymerase chain reaction-restriction fragment length polymorphism and amplified fragment length polymorphism methods. Results indicate that methyltetrahydrofolate homocysteine methyl transferase (MTR) A2756G allele was found to be protective in T2D and the other variants were not significantly associated with T2D. Glutamate carboxypeptidase II (GCP II) C1561T ($r = 0.34$; $p = 0.05$) and methylene tetrahydrofolate

reductase (MTHFR) C677T ($r = 0.35$; 0.04) showed positive correlation with plasma homocysteine in T2D cases. In this study, MTR A2756G allele was found to be protective in T2D; GCP II C1561T and MTHFR C677T showed positive association with plasma homocysteine in T2D cases. Among all the genetic variants, MTR A2756G was found influence IMT. RFC 1 G80A and TYMS 5'-UTR 2R3R showed synergistically interact with MTR A2756G in influencing increase in IMT.

Keywords Diabetes mellitus · One carbon metabolism · IMT · Homocysteine · Oxidative stress

Introduction

Cardiovascular complications are the leading cause of death among the diabetic patients. Individuals with pre-diabetes, undiagnosed T2D and long-lasting T2D are at high risk of all complications of macrovascular disease, coronary heart disease, stroke and peripheral vascular disease. More than 70 % of patients with T2D die of cardiovascular causes [1]. The assessment of intimal medial thickening (IMT) of common carotid artery is important for evaluating the atherosclerotic risk in people with diabetes and can further be used to facilitate better use of various treatment strategies. Several studies including our own study, have shown that along with hyperglycemia, other metabolic factors associated with diabetes that are known to increase cardiovascular risk, including obesity, insulin resistance, hypertension, hyperlipidemia and increased inflammatory state may contribute to progression of IMT in subjects with diabetes [2–5]. In our recent study, we found that IMT was significantly increased in T2D patients, as compared with non-diabetic healthy controls [5]. Also,

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IMT increased with the increase in the age of T2D patients, however, no significant increase was observed in healthy subjects. It was supported by a study in which the influence of age was found in patients with various cardiovascular-risk factors, including diabetes [6].

Functional polymorphisms in genes regulating one-carbon metabolism and deficiency of micronutrients such as folate, B₁₂, B₆ etc., perturbate one-carbon metabolism. Hence, they are associated with elevated plasma homocysteine (HCY) levels and decreased functional folate, S-adenosyl methionine (SAM) and glutathione (GSH) [7]. Elevated HCY acts as pro-oxidant and found to generate free radicals by auto-oxidation, inducing lipid peroxidation [8], causes endothelial cell damage and also perturbing hemostasis [9]. Decreased levels of folate are known to be associated with defective synthesis of purines and uracil misincorporation in DNA [10]. Decreased SAM with increased S-adenosylhomocysteine (SAH) blocks methyltransferases, further leading to decreased cellular methylation [11] and is associated with altered gene expression i.e., up regulation of monocyte chemo attractant protein-1 (MCP-1) [12], platelet derived growth factor (PDGF) [13], down regulation of SOD [14]. All the above events are likely to trigger the cascade of events leading to atherosclerosis and arterial thrombosis. Several SNPs were reported in genes regulating the one-carbon metabolic pathway such as glutamate carboxypeptidase II (GCP2 C1561T), reduced folate carrier 1 (RFC G80A), serine hydroxyl methyl transferase 1 (cSHMT C1420T), thymidylate synthase (TYMS 5'-UTR) 28 bp tandem repeat, methylenetetrahydrofolate reductase (MTHFR C677T) and 5-methyltetrahydrofolate homocysteine S-methyl transferase (MTR A2756G) interfere with the uptake of folate, folate carrier, DNA synthesis, synthesis of methyltetrahydrofolate, remethylation of HCY and reductive methylation of cobalamin, whereas the presence of 2R allele of functional polymorphism in TYMS, i.e., 28 bp tandem repeat in 5'-UTR (transcription enhancer element) was found to affect transcription process compared to 3R allele and might modulate folate flux. Among the different polymorphisms of one-carbon metabolism, GCP2 C1561T [15]; MTHFR C677T [16], and MTR A2756G are studied extensively in CAD cases [17]. MTHFR C677T was observed to be associated with independent risk for CAD in the subjects with low folate status [18]. In view of limited data available on different polymorphisms of one-carbon metabolism in relation IMT in T2D, this study was conducted to elucidate the role of majority of functional polymorphisms regulating one-carbon metabolism in relation to IMT in T2D patients and controls in order to explore the possible association between one carbon genetic variants, oxidative stress and IMT.

Materials and Methods

A total number of 76 subjects from ACS Medical College & Hospital, Chennai were included for the study, i.e., Group I ($n = 42$) of T2D and Group II ($n = 34$) of age- and sex matched healthy controls. The T2D patients with fasting blood glucose of ≥ 126 mg/dL and glycated haemoglobin (HbA1c) of 7.0–10.0 % were included in the study. The T2D patients with the history of alcohol consumption, smoking, hypertension, cancer, cardiac, liver and kidney diseases, and women with oral contraceptives or on hormone replacement therapy were excluded from the study. The ethical clearance was obtained from the Institutional Ethics Committee of ACS Medical College and Hospital, Chennai. The informed consent was obtained from all the subjects before the enrolment in the study. The blood pressure, body mass index (BMI), family history, medical history was recorded. The blood samples in fasting state were collected from all the subjects for biochemical analysis.

Biochemical Analysis

The fasting blood glucose was measured by Statfax semi-automated analyser using the kit supplied by Bayer Diagnostics. HbA1c was estimated by ion-exchange resin method using the kit supplied by Diatek. The plasma homocysteine was determined by commercially available ELISA Kit supplied by Dialab. Serum nitrite (NO), plasma MDA and GSH levels were determined by the spectrophotometric method [19].

Intimal Medial Thickness (IMT) Measurement

The IMT of carotid arteries was determined by using high frequency linear ultrasound as the distance from the leading edge of first ecogenic line, which represents the lumen intimal interface and to second ecogenic line, which represents the collagen containing upper layer of the intimal adventitia [20].

Extraction of Genomic DNA

Whole blood samples were collected from all the subjects in EDTA vacutainers. Genomic DNA was isolated using standard DNA extraction method [21]. To check the quantity and quality of the extracted genomic DNA, DNA was diluted 1:200 (5 μ l of DNA in 1 ml) in TE buffer and incubated at 65 °C for 45 min. Absorbance was taken at 260 nm and 280 nm. DNA free from contaminant protein/phenol/RNA showed an A260/A280 ratio 1.8. 50 ng/ μ l of double-stranded DNA gives an optical density of 1.00.

Table 1 Reaction conditions for PCR based genetic analysis

Variant	Primers (5'.....0.3')	RE	±	PCR conditions
Study of genetic polymorphisms				
GCPII C1561T	CTG GTA GGA ATT TAG CAG TGG CCT CAA GAA AAC ATC ATT TG	AccI	+	D: 94°/1 min A: 60°/45 s E: 72°/1 min X: 35
RFC1 G80A	AGT GTC ACC TTC GTC CCC TC CTC CCG CGT GAA GTT CTT	HhaI	–	D: 95°/1 min A: 59°/1 min E: 72°/1 min X: 30
TYMS 5'-UTR (AFLP)	CGT GGC TCC TGC GTT TCC GAG CCG GCC ACA GGC AT	...	...	D: 95°/30 s A: 62°/30 s E: 72°/30 sX: 35
MTHFR C677T	TTTGAGGCTGACCTGAAGCACTTGAAGGAG GAGTGGTAGCCCTGGATGGGAAAGATCCCCG	HinfI	+	D: 95°/1 min A: 60°/1 min E: 72°/1 min X: 30
MTR A2756G	TGT TCC CAG CTG TTA GAT GAA AAT C GAT CCA AAG CCT TTT ACA CTC CTC	HaeIII	+	D: 95°/30 s A: 60°/30 s E: 72°/30 s X: 40

RE restriction enzyme;
D denaturation; A annealing;
E extension; X number of
cycles; + restriction site
created; - restriction site
abolished

Analysis of Single Nucleotide Polymorphisms

From the extracted DNA, the analysis of SNPs in genes of one carbon metabolism such as GCPII C1561T, RFC1 G80A, MTHFR C677T and MTR A2756G genetic polymorphisms were studied by restriction fragment length polymorphism approach. TYMS 5'-UTR 28 bp tandem repeat (2R3R) polymorphism was studied by amplified fragment length polymorphism technique as shown in Table 1 [22].

Statistical Analysis

Unpaired Student's *t* test and Fisher's exact test were performed on continuous and categorical variables respectively. Student *t* test was used for studying the statistical significant differences in the continuous variables between two groups. The possibility of genotyping errors was confirmed by applying Hardy–Weinberg equilibrium. Fisher exact test was used for the analysis of contingency tables to obtain crude odds ratio (OR) and 95 % confidence interval (CI) by plotting 2 × 2 contingency tables based on the presence or absence of the variable in cases and controls. Independent genetic effects were evaluated by controlling for confounding factors using multivariate logistic regression. Analysis of variance (ANOVA) was used to

study the relationship between the categorical variables and continuous variables. For all other statistical analysis, www.statpages.org was used. A “*p*” value of <0.05 was considered statistically significant. Multifactor dimensionality reduction (MDR) Analysis (version: 2.0, beta 6) was used to explore gene–gene interactions. For this analysis, genotype data were computed as 0, 1 and 2 based on number of variant alleles; and biochemical values as 0 and 1 based on normal and higher levels in blood was considered. All the predictor variables were labeled as X1, X2, X3, X4,..... and disease outcome was labeled as Class.

Results

The distributions of all the polymorphisms were in accordance with Hardy–Weinberg equilibrium in cases as well as controls (*p* > 0.17). The allele frequency distribution of one-carbon genetic variants in T2D cases and healthy controls were as follows: GCPII C1561T (4 vs. 5 %), RFC G80A (36 vs. 30 %), cSHMT C1420T (56 vs. 63 %), TYMS 2R 3R (36 vs. 39 %), MTHFR C677T (13 vs. 13 %) and MTR A2756G (30 vs. 47 %). Among the six genetic variants MTR A2756G allele was found to have protective role in T2D (OR 0.35; 95 % CI 0.14–0.86), whereas the other variants were not significantly associated with T2D (Table 2).

Table 2 Genotype and allele frequency distribution of one carbon genetic variants in T2D patients and controls

SNPs	Genotypes			pHWE	Minor allele frequency	Adjusted OR (95 % CI)	p value
GCP II C1561T	CC	CT	TT			1.27 (0.23–6.96)	0.78
Cases	33	3	0	1.00	3/72 (4 %)		
Controls	31	1	1	0.76	3/66 (5 %)		
RFC I G80A	GG	GA	AA			1.47 (0.68–3.19)	0.32
Cases	19	16	7	0.65	30/84 (36 %)		
Controls	17	8	5	0.23	18/60 (30 %)		
SHMT C1420T	CC	CT	TT			0.87 (0.31–2.47)	0.80
Cases	4	26	9	0.15	34/78 (56 %)		
Controls	2	17	9	0.27	21/56 (63 %)		
TYMS 5'-UTR	3R3R	2R3R	2R2R			0.66 (0.31–1.44)	0.30
Cases	19	16	7	0.65	30/84 (36 %)		
Controls	13	14	6	0.77	26/66 (39 %)		
MTHFR C677T	CC	CT	TT			1.61 (0.44–5.93)	0.48
Cases	30	10	0	0.93	10/80 (13 %)		
Controls	25	9	0	0.92	9/68 (13 %)		
MTR A2756G	AA	AG	GG			0.35 (0.14–0.86)	0.02*
Cases	21	17	4	1.00	25/84 (30 %)		
Controls	8	18	6	0.78	30/64 (47 %)		

pHWE p value of Hardy–Weinberg equilibrium, adjusted OR odds ratio adjusted for conventional risk factor, i.e., age; CI confidence interval

* $p < 0.05$ statistically significant

The GCP II C1561T ($r = 0.34$; $p = 0.05$) and MTHFR C677T ($r = 0.35$; $p = 0.04$) showed positive correlation with plasma homocysteine in T2D cases. In healthy controls, RFC I G80A ($r = 0.52$; $p = 0.005$) showed positive correlation with plasma nitrite, whereas it showed negative correlation ($r = -0.38$; $p = 0.05$) with MDA. On the other hand, in T2D cases, GCP II C1561T ($r = 0.38$; $p = 0.02$) showed positive correlation with MDA. RFC I G80A ($r = 0.34$; $p = 0.05$), cSHMT C1420T ($r = 0.44$; $p = 0.009$) in T2D cases and RFC I ($r = 0.50$; $p = 0.007$) in healthy controls showed positive correlation with GSH (Table 3). The IMT was negatively correlated with RFC I G80A ($r = 0.63$; $p = 0.05$) in healthy controls (Table 4).

The functional relevance of MTR A2756G genotypes AA, AG and GG with IMT in T2D patients and controls is shown in Fig. 1. GG (mean $\pm$ SD 0.92 ± 0.06 mm) showed insignificant increase in IMT than the genotypes AA (mean $\pm$ SD 0.90 ± 0.1 mm) and AG (mean $\pm$ SD 0.91 ± 0.04 mm). The IMT values (mean $\pm$ SD) in the genotypes of controls are as follows: AA: 0.74 ± 0.02 ; AG: 0.75 ± 0.03 mm and GG: 0.73 ± 0.06 mm respectively.

The functional relevance of MTR A2756G, RFC I G80A, TYMS 5'-UTR genotype with IMT in T2D patients is shown in Fig. 2. The mean $\pm$ SD of IMT among the wild genotypes were as follows: AA of MTR A2756G: 0.90 ± 0.07 mm, GG of RFC I G80A: 0.91 ± 0.05 mm and 3R3R of TYMS 5'-UTR: 0.91 ± 0.05 mm; among the heterozygous: AG of MTR A2756G: 0.91 ± 0.04 , GA of RFC I G80A: 0.89 ± 0.06 mm and 2R3R of TYMS 5'-UTR: 0.89 ± 0.07 mm; among the homozygous mutants:

GG of MTR A2756G: 0.92 ± 0.06 mm, AA of RFC I G80A: 0.92 ± 0.06 mm and 2R2R of TYMS 5'-UTR: 0.93 ± 0.05 mm respectively.

The MDR analysis indicated the following gene–gene interactions: out of the polymorphisms tested, MTR A2756G was found to influence IMT. The RFC I G80A and TYMS 5'-UTR 2R3R were observed to act synergistically with MTR A2756G in influencing IMT. cSHMT C1420T had weak modulatory effect on other genetic variants in determining IMT

Discussion

The measurement of carotid IMT is an excellent non-invasive technique to assess early cardiovascular risk and is also used to examine the stages of atherosclerosis [23]. In the current study, the association of one carbon metabolic genetic variants and oxidative stress markers on IMT in T2D was investigated. Among the six genetic variants of one carbon metabolism, MTR A2756G allele was found to play a protective role in T2D. One-carbon genetic variants such as GCPII C1561T and MTHFR C677T were found to be risk factors for CAD, whereas cSHMT C1420T and TYMS 5'-UTR 28 bp tandem repeat were found to confer protection against CAD [24]. In another study from our group, it was reported that the polymorphisms, i.e., cSHMT C1420T, TYMS 28 bp tandem repeat were associated with reduced risk for CAD [19]. MTHFR 677 TT-genotype was demonstrated to be a risk factor for CAD [25], and in

Table 3 Correlation of one-carbon genetic variants with oxidative stress markers and homocysteine

Variants	NO		MDA		GSH		HCY	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
GCP IIC1561T								
Cases	−0.19	0.28	0.38	0.02*	−0.03	0.86	0.34	0.05*
Controls	−0.31	0.11	0.009	0.97	−0.04	0.83	0.39	0.85
RFC I G80A								
Cases	−0.07	0.68	0.07	0.71	0.34	0.05*	−0.18	0.30
Controls	0.52	0.005*	−0.38	0.05*	0.50	0.007*	0.06	0.75
SHMT C1420T								
Cases	−0.003	0.99	−0.08	0.63	0.44	0.009*	0.05	0.80
Controls	−0.14	0.48	0.04	0.82	−0.10	0.63	0.04	0.85
TYMS 5′-UTR								
Cases	0.09	0.59	0.10	0.59	0.19	0.28	0.05	0.77
Controls	−0.20	0.32	0.40	0.04	−0.31	0.11	0.26	0.20
MTHFR C677T								
Cases	−0.19	0.29	0.36	0.04	0.07	0.70	0.35	0.04*
Controls	0.06	0.76	−0.16	0.43	0.21	0.29	0.01	0.95
MTR A2756G								
Cases	0.18	0.31	0.04	0.82	−0.03	0.86	0.02	0.90
Controls	−0.10	0.63	0.08	0.69	0.12	0.54	0.29	0.15

r Pearson's correlation coefficient

* *p* < 0.05 statistically significant

Table 4 Correlation of IMT with one carbon genetic variants

Variants	<i>r</i>	<i>p</i>
GCP II C1561T		
Cases	−0.01	0.94
Controls	0.10	0.61
RFC I G80A		
Cases	−0.02	0.89
Controls	−0.63	0.05*
SHMT C1420T		
Cases	0.05	0.78
Controls	0.10	0.61
TYMS 5′-UTR		
Cases	0.04	0.80
Controls	−0.36	0.07
MTHFR C677T		
Cases	−0.02	0.89
Controls	0.09	0.65
MTHFR C677T		
Cases	0.13	0.45
Controls	−0.11	0.57

r Pearson's correlation coefficient

p < 0.05 statistically significant

Fig. 1 Functional relevance of MTR A2756G in controls and T2D patients (Cases) X axis: MTR genotypes; Y axis: IMT values in mm. A AA genotype, B AG genotype, C GG genotype

another study it was observed that MTHFR polymorphism (TT and CT genotypes together) in females was associated with CAD [26]. In contrast, there was no association of MTR A2756G polymorphism with CAD risk was reported [27].

In our recent study in CAD cases, increased oxidative stress was observed in subjects with cSHMT C1420T and TYMS 5′UTR 28 bp tandem repeat polymorphisms. In the

same study, it was observed that GCP II C1561T, MTHFR C677T and MTRR A66G polymorphisms showed a positive association with plasma homocysteine, whereas inverse relationship with cSHMT C1420T was demonstrated [19]. In the current study, GCP II C1561T and MTHFR C677T showed positive association with plasma homocysteine in T2D cases. Studies have shown hyperhomocysteinemia as a significant risk factor for CAD

Fig. 2 Functional relevance genotypes of MTR A2756G, RFC 1 G80A, TYMS 5'-UTR in T2D patients (Cases) X axis: genotypes; Y axis: IMT values in mm. A: Wild genotypes of MTR A2756G, RFC 1 G80A, TYMS 5'-UTR B: Heterozygous genotypes of MTR A2756G, RFC 1 G80A, TYMS 5'-UTR C: Homozygous mutant genotypes of MTR A2756G, RFC 1 G80A, TYMS 5'-UTR

among the Indians [25, 28]. In contrary, other studies showed the lack of such association [29, 30]. The elevated plasma homocysteine levels were found to be associated with reduced activity of dimethyl arginine dimethyl aminohydrolase (DDAH), thus increasing asymmetric dimethylarginine which in turn reduces the bioavailability of NO [31]. The low levels 5-MTHF (due to MTHFR C677T) and reduced levels of NO may alter the endothelium dependent vasomotor response [32].

In the current study, the association between the oxidative stress and variant alleles of GCPII C1561T, RFC I G80A and cSHMT C1420T indicate that the GCP II C1561T polymorphism showed positive correlation with MDA; RFC I G80A and cSHMT C1420T showed positive correlation with GSH in T2D patients. The increased oxidative stress in subjects with GCPII 1561T variant might be mediated through the elevated homocysteine levels as observed in the current study. These findings are in agreement with other studies in demonstrating increased oxidative stress in CAD cases [19, 33].

In our recent study, we observed the decreased levels of NO in T2D subjects [5]. This may be due to the presence of eNOS G894T variant in T2D subjects (unpublished data). Studies have provided evidence for reduced NO bioavailability and impaired endothelial function in diabetes subjects, probably due to polymorphisms in the eNOS gene [34]. Several studies have shown that the reduced bioavailability of NO in subjects with hyperhomocysteinemia, [35, 36]. Decreased bioavailability of NO may result in endothelial dysfunction by increasing IMT. In the current study, all the variant of one carbon metabolism did not

show significant association with NO in T2D subjects. In one study, it was reported that eNOS Glu298Asp polymorphism as a genetic susceptibility factor for hyperinsulinemia, insulin resistance and T2D [37]. Evidence suggests that Glu298Asp polymorphism may be associated with CAD in the presence of other risk factors [38].

The one-carbon metabolism provides a variety of metabolic intermediates, which react either with pro-oxidants or promote antioxidant defence. 5-methyl tetrahydrofolate (5-MTHF), a product of MTHFR catalysis, is found to be effective scavenger of superoxide with capability to restore NO availability by preventing oxidation of BH_4 to BH_2 [39]. The epidemiologic studies support a positive association between the plasma homocysteine concentrations and CAD risk. Hyperhomocysteinemia resulting from aberrations in one-carbon metabolism was demonstrated to increase superoxide anion production by multiple mechanisms [40], includes: (i) inhibition of the activity of cellular antioxidant enzymes such as cellular glutathione peroxidase or hemeoxygenase-1, (ii) homocysteine autooxidation, (iii) NOS-dependent generation of superoxide anion ($O_2^{\bullet-}$) via uncoupling of eNOS, (iv) decrease of extracellular superoxide dismutase activity and (v) by phosphorylation at $p47^{phox}$ and $p67^{phox}$ subunits of NADPH oxidase, thereby stimulating superoxide generation [39]. A study found that plasma homocysteine concentration and SNPs in the enzyme MTHFR were not significantly associated with carotid IMT [33]. The most crucial end-product of this pathway i.e., SAM, a universal methyl donor, modulates oxidative stress by potentiating SOD and GST activity and restoring GSH [11]. Decreased SAM with subsequent

increase in SAH blocks the methyltransferases resulting in decreased cellular methylation, which in turn is associated with altered gene expression i.e., up regulation of MCP-1 [13], PDGF [14] and down regulation of SOD [41]. Further, long term depletion of folate/methyl from the diet was shown to decrease reduced/oxidized GSH ratio, alter activity of Mn-SOD, catalase and glutathione peroxidase and induce irreparable oxidative DNA damage [18]. These evidences indicate the possible association between the aberrations of one-carbon metabolism and oxidative stress, which may contribute for increased IMT in T2D subjects. The limitation of the current study is its sample size. Future studies on different ethnic groups and populations with large sample size warrant further investigation.

Conclusion

In this study, MTR A2756G allele was found to be protective in T2D; GCP II C1561T and MTHFR C677T showed positive association with plasma homocysteine in T2D cases. GCP II C1561T showed positive association with MDA; RFC I G80A and cSHMT C1420T showed positive correlation with GSH in T2D patients. The increased oxidative stress in subjects with GCP II 1561T variant might be mediated through elevated homocysteine levels. Among all genetic variants, MTR A2756G was found influence IMT. RFC 1 G80A and TYMS 5'-UTR 2R3R were found to act synergistically with MTR A2756G in influencing IMT in T2D subjects.

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References

1. Laakso M. Cardiovascular disease in type 2 diabetes: challenge for treatment and prevention. *J Intern Med.* 2001;249:225–35.
2. Li C, Engstrom G, Berglund G, Janzon L, Hedblad B. Incidence of ischemic stroke in relation to asymptomatic carotid artery atherosclerosis in subjects with normal blood pressure. A prospective cohort study. *Cerebrovasc Dis.* 2008;26:297–303.
3. Lee EJ, Kim HJ, Bae JM, Kim JC, Han HJ, Park CS, et al. Relevance of common carotid intima media thickness and carotid plaque as risk factors for ischemic stroke in patients with type 2 diabetes mellitus. *Am J Neuroradiol.* 2007;28:916–9.
4. Urbina EM, Kimball TR, McCoy CE, Khoury PR, Daniels SR, Dolan LM. Youth with obesity and obesity-related type 2 diabetes mellitus demonstrate abnormalities in carotid structure and function. *Circulation.* 2009;119:2913–9.
5. Dhananjayan R, Malati T, Brindha G, Vijay Kumar K. Association of family history of type 2 diabetes mellitus with markers of endothelial dysfunction in South Indian population. *Indian J Biochem Biophys.* 2013;50:93–8.
6. Kanters SD, Algra A, Banga JD. Carotid intima media thickness in hyperlipidemia type 1 and type 2 diabetic patients. *Diabetes Care.* 1997;20:276–80.
7. Finkelstein JD. Pathways and regulation of homocysteine metabolism in mammals. *SeminThrombHemost.* 2000;26(3): 219–44.
8. Stamler JS, Osborne JA, Jaraki O, Rabbani LE, Mullins M, Singel D, et al. Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen. *J Clin Invest.* 1993;91(1):308–26.
9. Harker LA, Slichter SJ, Scott CR, Ross R. Homocystinemia. Vascular injury and arterial thrombosis. *N Engl J Med.* 1974; 291(11):537–80.
10. Linhart HG, Troen A, Bell GW, Cantu E, Chao WH, Moran E, et al. Folate deficiency induces genomic uracil misincorporation and hypomethylation but does not increase DNA point mutations. *Gastroenterology.* 2009;136(1):227–35.
11. Loehrer FM, Angst CP, Haefeli WE, Jordan PP, Ritz R, Fowler B. Low whole-blood S-adenosylmethionine and correlation between 5-methyltetrahydrofolate and homocysteine in coronary artery disease. *ArteriosclerThrombVascBiol.* 1996;16(6):727–60.
12. Wang G, Mao JM, Wang X, Zhang FC. Effect of homocysteine on plaque formation and oxidative stress in patients with acute coronary syndromes. *Chin Med J.* 2004;117(11):1650–4.
13. Nishio E, Watanabe Y. Homocysteine as a modulator of platelet-derived growth factor action in vascular smooth muscle cells: a possible role for hydrogen peroxide. *Br J Pharmacol.* 1997; 122(2):269–343.
14. Jiang Y, Jiang J, Xiong J, Cao J, Li N, Li G, et al. Homocysteine-induced extracellular superoxide dismutase and its epigenetic mechanisms in monocytes. *J Exp Biol.* 2008;211:911–31.
15. Vinukonda G, Shaik Mohammad N, Md Nurul Jain J, Prasad Chintakindi K, Rama Devi Akella R. Genetic and environmental influences on total plasma homocysteine and coronary artery disease (CAD) risk among South Indians. *ClinChimActa.* 2009;405:127–31.
16. Laraqui A, Allami A, Carrié A, Raisonnié A, Coiffard AS, Benkouka F, et al. Relation between plasma homocysteine, gene polymorphisms of homocysteine metabolism-related enzymes, and angiographically proven coronary artery disease. *Eur J Intern Med.* 2007;18(6):474–83.
17. Lievers KJA, Kluijtmans LAJ, Boers GHJ, Verhoef P, den Heijer M, Trijbels FJM, et al. Influence of a glutamate Carboxypeptidase II (GCP II) polymorphism (1561C → T) on plasma Homocysteine, folate and vitamin B12 levels and its relationship to cardiovascular risk. *Atherosclerosis.* 2002;164:269–73.
18. Girelli D, Martinelli N, Pizzolo F, Friso S, Olivieri O, Stranieri C, et al. The interaction between MTHFR 677 C→T genotype and folate status is a determinant of coronary atherosclerosis risk. *J Nutr.* 2003;133(5):1281–5.
19. Vijaya Lakshmi SV, Naushad SM, Seshagiri Rao D, Kutala VK. Oxidative stress is associated with genetic polymorphisms in one-carbon metabolism in coronary artery disease. *Cell Biochem Biophys.* 2013;67(2):353–61.
20. Barth JD. An update on carotid ultrasound measurement of intima-media thickness. *Am J Cardiol.* 2002;89:32–8.
21. Luis A, Salazar MHH, Selma A, Cavali, Marcos O, Machado, et al. Optimized procedure for DNA isolation from fresh and cryopreserved clotted blood useful in clinical molecular testing. *Clin Chem.* 1998;44:1748–50.
22. Naushad SM, Pavani A, Raghunatha Rao D, Suryanarayana Raju G, Vijay Kumar K. Epistatic interactions between loci of one-carbon metabolism modulate susceptibility to breast cancer. *Mol Bio Rep.* 2011;38:4893–901.
23. Ross R. Atherosclerosis: an inflammatory disease. *N. Eng J Med.* 1999;340:115–26.

24. Kim SJ, Kim MG, Kim KS, Song JS, Yim SV, Chung JH. Impact of glutathione S-transferase M1 and T1 gene polymorphisms on the smoking-related coronary artery disease. *J Korean Med Sci.* 2008;23:365–72.
25. Alam MA, Husain SA, Narang R, Chauhan SS, Kabra M, Vasisht S. Association of polymorphism in the thermolabile 5, 10-methylene tetrahydrofolate reductase gene and hyperhomocysteinemia with Coronary artery disease. *Mol Cell Biochem.* 2008;310:111–7.
26. Mukherjee M, Joshi S, Bagadi S, Dalvi M, Rao A, Shetty KR. A low prevalence of the C677T mutation in the methylene tetrahydrofolate reductase gene in Asian Indians. *Clin Genet.* 2002;61:155–9.
27. Vijaya Lakshmi SV, Naushad SM, Rupasree Y, Seshagiri Rao D, Kutala VK. Interactions of 5'-UTR thymidylate synthase polymorphism with 677C → T methylene tetrahydrofolate reductase and 66A → G methyl tetrahydrofolate homocysteine methyl transferase reductase polymorphisms determine susceptibility to coronary artery disease. *J Atheroscler Thromb.* 2011;18(1):56–64.
28. Puri A, Gupta OK, Dwivedi RN, Bharadwaj RP, Narain VS, Singh S. Homocysteine and lipid levels in young patients with coronary artery disease. *J Assoc Phys India.* 2003;51:681–5.
29. Snehalatha C, Ramachandran A, Satyavani K, Sivasankari S, Sathyamurthy I, Viswanathan V. Plasma homocysteine concentration and coronary artery disease in Asian Indians. *J Assoc Phys India.* 2002;50:1229–31.
30. Pandey SN, Vaidya AD, Vaidya RA, Talwalkar S. Hyperhomocysteinemia as a cardiovascular risk factor in Indian women: determinants and directionality. *J Assoc Phys India.* 2006;54:674–769.
31. Stuhlinger MC, Tsao PS, Her JH, Kimoto M, Balint RF, Cooke JP. Homocysteine impairs the nitric oxide synthase pathway: role of asymmetric dimethylarginine. *Circulation.* 2001;104(21):2569–644.
32. Antoniadou C, Shirodaria C, Warrick N, Cai S, de Bono J, Lee J, et al. 5-Methyltetrahydrofolate rapidly improves endothelial function and decreases superoxide production in human vessels: effects on vascular tetrahydrobiopterin availability and endothelial nitric oxide synthase coupling. *Circulation.* 2006;114(11):1193–201.
33. Scaglione L, Gambino R, Rolfo E, Lillaz E, Gai M, Cassader M, et al. Plasma homocysteine, methylene tetrahydrofolate reductase gene polymorphism and carotid intima-media thickness in Italian type 2 diabetic patients. *Eur J Clin Invest.* 2002;32(1):24–8.
34. Channon KM, Guzik TJ. Mechanisms of superoxide production in human blood vessels: relationship to endothelial dysfunction, clinical and genetic risk factors. *J Physiol Pharmacol.* 2002;53:515–24.
35. Stanger O, Weger M. Interactions of homocysteine, nitric oxide, folate and radicals in the progressively damaged endothelium. *ClinChem Lab Med.* 2003;41:1444–54.
36. Dayal S, Arming E, Bottiglieri T, Boger RH, Sigmund CD, Faraci FM, et al. Cerebral vascular dysfunction mediated by superoxide in hyperhomocysteinemia mice. *Stroke.* 2004;35:1957–62.
37. Monti LD, Barlassina C, Citterio L, Galluccio E, Berzuini C, Setola E, et al. Endothelial nitric oxide synthase polymorphisms are associated with type 2 diabetes and the insulin resistance syndrome. *Diabetes.* 2003;52:1270–5.
38. Gardemann A, Lohre J, Cayci S, Katz N, Tillmanns H, Haberbosch W. The T allele of the missense Glu298Asp endothelial nitric oxide synthase gene polymorphism is associated with coronary heart disease in younger individuals with high atherosclerotic risk profile. *Atherosclerosis.* 2002;160:167–75.
39. Siow YL, Au-Yeung KKW, Woo CWH, Karmin O. Homocysteine stimulates phosphorylation of NADPH oxidase p47phox and p67phox subunits in monocytes via protein kinase C β activation. *Biochem J.* 2006;398:73–82.
40. Cavallaro RA, Fusco A, Nicolai V, Scarpa S. S-adenosylmethionine prevents oxidative stress and modulates glutathione metabolism in TgCRND8 mice fed a B-vitamin deficient diet. *J Alzheimers Dis.* 2010;20(4):997–1002.
41. Bagnyukova TV, Powell CL, Pavliv O, Tryndyak VP, Pogribny IP. Induction of oxidative stress and DNA damage in rat brain by a folate/methyl-deficient diet. *Brain Res.* 2008;1237:44–51.