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Biochemical Profile and Genetic Polymorphism of MTHFRC677T in Risk of Type 2 Diabetes Mellituss

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Abstract: Diabetes mellitus (DM) is a common endocrine metabolic disorder and a leading cause of death worldwide Diabetes type-2 is a multicausal disease which develops slowly and in a stepwise order. Our study showed there was no significant difference in serum high density lipoprotein (HDL) and Tri glyceride (TG) of patients and controls (0.90 ± 0.59 vs 1.15 ± 0.39 p>0.05) and (1.19 ± 0.70 vs 1.01 ± 0.52 p>0.060) respectively. Low density lipoprotein (LDL) and total cholesterol (TC) are significantly higher in patients than control group (4.09 ± 1.14 vs 3.01 ± 1.02 p<0.0002) and (4.21 ± 1.28 vs 3.78 ± 1.29 p<0.05). However, HDL/TC ratio is significantly higher in patients than controls (0.21 ± 0.91 vs 0.30 ± 0.99 p<0.05). Serum levels of all liver enzymes (ALT, AST, and ALP) analyzed are significantly higher in patients than controls (12.69 ± 10.80 vs 4.95 ± 2.66, p<0.0002), (15.99 ± 10.70 vs 6.95 ± 3.84, p<0.0002) and 68.29 ± 27.78 vs 21.27 ± 7.77, p<0.0001) respectively. On genetic level the role of MTHFR C677T polymorphisms our results showed 63% of the cases showed homozygous mutant condition. The allelic association of polymorphism of controls with cases was found to be significant (P=0.007). Homozygous mutant condition of MTHFRC677T gene was found to be certainly higher in Diabetes Mellitus 2 Cases of above 60 years of age (80%), than ages below 60 years and in controls (16.6%) and was significant as p=0.005, compared to below 60 years of age (33.3%) and in controls (0%) and association was insignificant as p=0.4667. Our data suggest that there is an important role of LDL, TC, HDL/TC, ALT, AST, and ALP in type-2 Diabetes, also gene polymorphisms of MTHFR C677T gene may act synergistically to increase the risk of type 2 diabetes.

Keywords: Diabetes Mellitus-2, Biochemical Parameters, MTHFR Gene Polymorphism

1. Introduction

Diabetes mellitus (DM) is a common endocrine metabolic disorder and a leading cause of death worldwide. Diabetes type-2 is a multicausal disease which develops slowly and in a stepwise order. Initially, it commences with insulin resistance, which progress gradually with the passage of time. Secondary hyperinsulinism develops to counter it, but it too at one point of time fails to maintain glucose homeostasis resulting in glucose intolerance [1]. This high blood sugar produces the symptoms of frequent urination, increased thirst, and increased hunger. Untreated, diabetes can cause many complications. Acute complications include diabetic ketoacidosis and nonketotic hyperosmolar coma. Serious long-term complications include heart disease, kidney failure, and damage to the eyes. This high blood sugar produces the symptoms of frequent urination, increased thirst, and increased hunger. Untreated, diabetes can cause many complications. Acute

complications include diabetic ketoacidosis and nonketotic hyperosmolar coma. Serious long-term complications include heart disease, kidney failure, and damage to the eyes. Prevention and treatment often involve a healthy diet, physical exercise, not using tobacco, and being a normal body weight. Globally, as of 2013, an estimated 382 million people have diabetes worldwide [2], with type 2 making up about 90% of the cases [3]. This is equal to 3.3% of the population with equal rates in both women and men [4]. In 2011 it resulted in 1.4 million deaths worldwide making it the 8th leading cause of death [5].

Individuals with type 2 diabetes have a higher incidence of liver function test abnormalities than individuals who do not have diabetes. Mild chronic elevations of transaminases often reflect underlying insulin resistance. Elevation of transaminases within three times the upper limits of normal is not a contraindication for starting oral antidiabetic or lipid modifying therapy in contrast; antidiabetic agents have generally been shown to decrease alanine aminotransferase levels as tighter blood glucose levels are achieved. The most common LFTs include the serum aminotransferases, alkaline phosphatase, bilirubin, albumin, and prothrombin time. Aminotransferases, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST), measure the concentration of intracellular hepatic enzymes that have leaked into the circulation and serve as a marker of hepatocyte injury. Alkaline phosphatase (AP), glutamyltranspeptidase (GGT), and bilirubin act as markers of biliary function and cholestasis. Albumin and prothrombin reflect liver synthetic function. The aminotransferases AST and ALT are normally < 30–40 units/l. Elevations of aminotransferases greater than eight times the upper limit of normal reflect either acute viral hepatitis, ischemic hepatitis, or drug- or toxin-induced liver injury. Much more common than patients with acute hepatitis, however, are patients with chronic mild elevation of aminotransferases, or AST and ALT < 250 units/l for > 6 months. Chronic mild elevation of transaminases are frequently found in type 2 diabetic patients [6].

Plasma Creatinine and Urea are useful clinical tools in assessing the type 2 diabetes. Plasma Creatinine and Urea concentrations are significantly higher in patients with T2DM both males and females. However no significant difference exists between male and female subjects in these test parameters [7]. Type 2 diabetes is associated with a cluster of interrelated plasma lipid and lipoprotein abnormalities, including reduced HDL cholesterol, a predominance of small dense LDL particles, and elevated triglycerides (American Diabetes Association) [8]. These abnormalities occur in many patients despite normal LDL cholesterol levels. These changes are also a feature of the insulin resistance syndrome (also known as the metabolic syndrome), which underlies many cases of type 2 diabetes. In fact, pre-diabetic individuals often exhibit an atherogenic pattern of risk factors that includes higher levels of total cholesterol, LDL cholesterol, and triglycerides and lower levels of HDL cholesterol than individuals who do not develop diabetes [9]. Insulin resistance has striking effects on lipoprotein size and subclass particle concentrations for VLDL, LDL, and HDL [10].

The enzyme is coded by the gene with the symbol MTHFR on chromosome 1 location p36.3 in humans [11]. There is DNA sequence variants (genetic polymorphisms) associated with this gene. Two of the most investigated are C677T (rs1801133) and A1298C (rs1801131) single nucleotide polymorphisms (SNP). C677T SNP (Ala222Val): The MTHFR nucleotide at position 677 in the gene has two possibilities: C (cytosine) or T (thymine). C at position 677 (leading to an alanine at amino acid 222) is the normal allele. The 677T allele (leading to a valine substitution at amino acid 222) encodes a thermolabile enzyme with reduced activity. Individual with two copies of 677C (677CC) have the "normal" or "wildtype" genotype. 677TT individuals (homozygous) are said to have mild MTHFR deficiency. 677CT individuals (heterozygotes) are almost the same as normal individuals because the normal MTHFR can make up for the thermolabile MTHFR. Individuals of 677TT are predisposed to mild hyperhomocysteinemia (high blood homocysteine levels), because they have less active MTHFR available to produce 5-methyltetrahydrofolate (which is used to decrease homocysteine [12].

2. Material and Method

Sample Collection: The blood samples of T2DM patients were collected in EDTA and plane clot activator vials from the Department of Biochemistry, Government Medical College, Srinagar. EDTA vials were transported to laboratory on ice and were stored at -20°C for molecular analysis and from clot activator vials serum was separated in appendroff tubes by centrifugation for biochemical analysis. The information regarding gender, age and residence were collected from the record file of patients present in the hospital.

Molecular Analysis: DNA was extracted from all the blood samples with Phenol-Chloroform K Method [13]. With some modifications. The DNA samples were alliqouted in to four tubes and stored at -20°C for future use.

Qualitative and quantitative analysis of genomic DNA.

The integrity of the genomic DNA was examined by gel electrophoresis using 1% agarose gel. The quantity of the DNA was determined by measuring optical density at 260nm and 280 nm by double beam spectrophotometer (HITACHI-U-1800 made in Japan). The ratio of 260/280nm was calculated and the DNA samples for which the ratio was 1.7-1.9 were considered for the future use. DNA was alliqouted into three to four tubes so as to protect damage from freeze thawing and stored in -20°C freezer for longer duration of time.

PCR-RFLPMTHFR C677T genotypes were determined with a PCR-RFLP method. The primers for analysis were:

5'-TGAAGGAGAAGGTGTCTGCGGGA-3' (Forward)
5'-AGGACGGTGCGGTGAGAGTG-3' (Reverse).

Table 1. *Volume and concentrations of different reagents used in PCR.*

S. No.	REAGENTS	CONCENTRATION	VOLUME
1	PCR Master Mix		12.5 µl
2	Forward Primer	10 pm/µl	0.5 µl
3	Reverse Primer	10 pm/µl	0.5 µl
4	DNA	10 ng	1.5 µl
5	Nuclease Free Water		10 µl
Total			25 µl

PCR Standardization Conditions: The reaction mixture was initially denatured at 93°C for 2 min, followed by 35 cycles of 93°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min, with final extention at 72°C for 10 mins. The PCR products were digested by the Hinf1 restriction endonucleases, respectively.

Table 2. *Volume of different reagents used in RFLP.*

S. No.	REAGENTS	VOLUME
1	DNA Amplified	10 µl
2	Nuclease Free Water	18 µl
3	10 x Buffer	2 µl
4	Hinf1	1.5 µl

Than reaction mixture was incubated at 37°C for 4 hours. The DNA fragment generated were separated using 2% agarose gel for detecting RFLP pattern.

3. Results

Biochemical analysis: From separated serum TC, HDL, LDL, TG, AST, ALT, ALP, were estimated on the same day by semi automated analyzer (Photometer V5+, Berlin) while rest sample volume was stored at -20°C. The samples were allowed to thaw properly prior to assay, mixed thoroughly. Hemolysed and lipemic samples were rejected.

Results obtained were analysed with the help of SPSS soft were setting of significant level at $p \leq 0.05$.
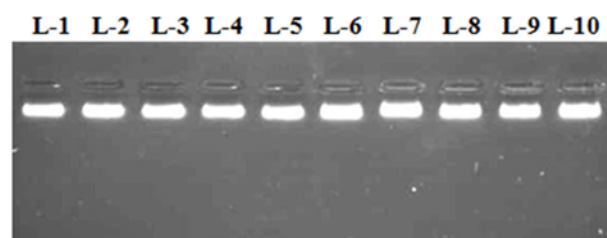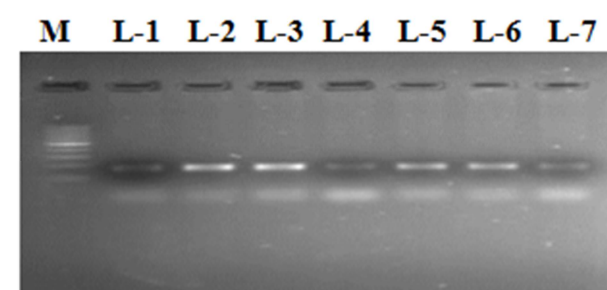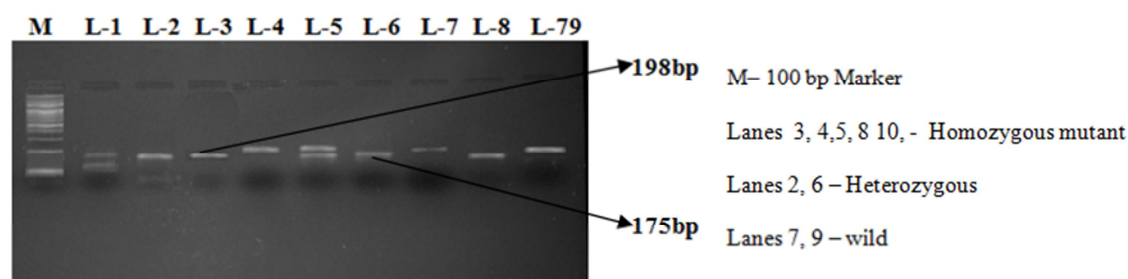
There was no significant difference in serum HDL and TG of patients and controls (0.90±0.59 vs 1.15±0.39 p>0.05) and (1.19±0.70 vs 1.01±0.52 p>0.060) respectively. LDL and TC are significantly higher in patients than control group (4.09±1.14 vs 3.01±1.02 p<0.0002) and (4.21±1.28 vs 3.78±1.29 p<0.05). However, HDL/TC ratio is significantly higher in patients than controls (0.21±0.91 vs 0.30±0.99

p<0.05). Serum levels of all liver enzymes (ALT, AST, and ALP) analysed are significantly higher in patients than controls (12.69±10.80 vs 4.95±2.66 p<0.0002), (15.99±10.70 vs 6.95±3.84 p<0.0002) and 68.29±27.78 vs 21.27±7.77 p<0.0001) respectively as shown in table-3 below.

Table 3. *Showing biochemical parameters among diabetic patients and controls.*

Parameter	Diabetic Patients (30)	Controls (20)	P. Value
TC (mmol/l)	4.21±1.28	3.78±1.29	0.0301
HDL (mmol/l)	0.90±0.59	1.15±0.39	0.069
LDL (mmol/l)	4.09±1.14	3.01±1.02	0.0002
TG (mmol/l)	1.19±0.70	1.01±0.52	0.060
ALT (IU/L)	12.69±10.80	4.95±2.66	0.0002
AST (IU/L)	15.99±10.70	6.95±3.84	0.0002
HDL/TC	0.21±0.91	0.30±0.99	0.0401
ALP (IU/L)	68.29±27.78	21.27±7.77	0.0001

Genetic analysis

Figure 1. *Lane 1-10 showing the isolated DNA of case samples, run on 1% agarose gel.*

Figure 2. *PCR Amplified products of Diabetes Mellitus 2 samples run on 2% agarose gel.*

Figure 3. *RFLP products run on 2% agarose gel.*

MTHER (C677T) Genotype Analysis The polymorphism of MTHFR GENE (C677T) was determined using polymerase chain reaction and restriction fragment length polymorphism. Lane 1 – M: 100 bp Marker, Lane – 2, 6 Heterozygous, Lane – 3, 4, 5, 8, 10 Homozygous mutant, Lane – 7, 9 Homozygous normal.

The genotypes were designated pp, Pp and PP (figure-3) corresponding to 198bp, 175 bp and 23 bp. The prevalence of Type 2 Diabeties Mellitus was highest among homozygous pp patients and lowest in homozygous PP patients. The prevalence of Diabeties Mellitus 2 in heterozygous subjects was intermediate between those of the two homozygous patient groups.

Restriction digestion was done to examine the genetic polymorphism in the MTHFRC677T gene as shown in Figure 4. 63% (19/30) of the Diabetes mellitus 2 cases showed homozygous and 26% (8/30) of the cases however showed heterozygous condition. Almost all 80% (16/20) of the normal samples showed heterozygous condition except only in four cases where MTHFRC677T gene was found to be Homozygous Mutant two & Homozygous normal two. The allelic association of this polymorphism with Diabetes mellitus 2 was evaluated by χ^2 (Chi square) test and was found to be significant (P=0.007) as shown in table 4 below

Table 4. *Representing genetic polymorphism in the MTHFRC677T gene among diabetic patients and controls.*

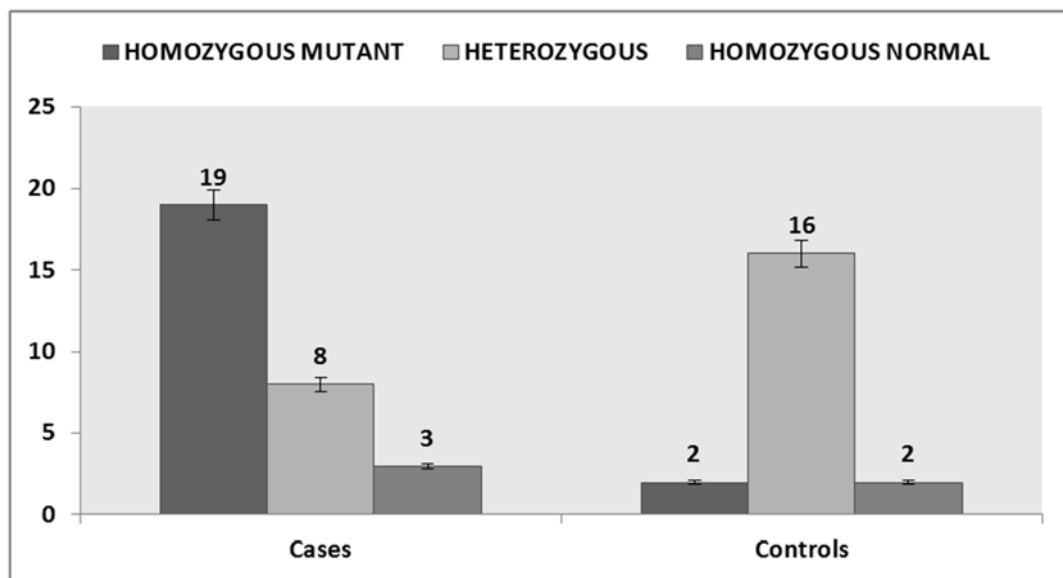| CASES – 30	GENOTYPE	FREQUENCY
19	Homozygous Mutant	63% (19/30)
8	Heterozygous	26.6% (8/30)
3	Homozygous normal	10% (3/30)
CONTROLS – 20		
2	Homozygous Mutant	10% (2/20)
16	Heterozygous	80% (16/20)
2	Homozygous normal	10% (2/20)

Figure 4. *Histogram representing restriction conditions of cases of Diabetiesmellits-2 and normal controls.*
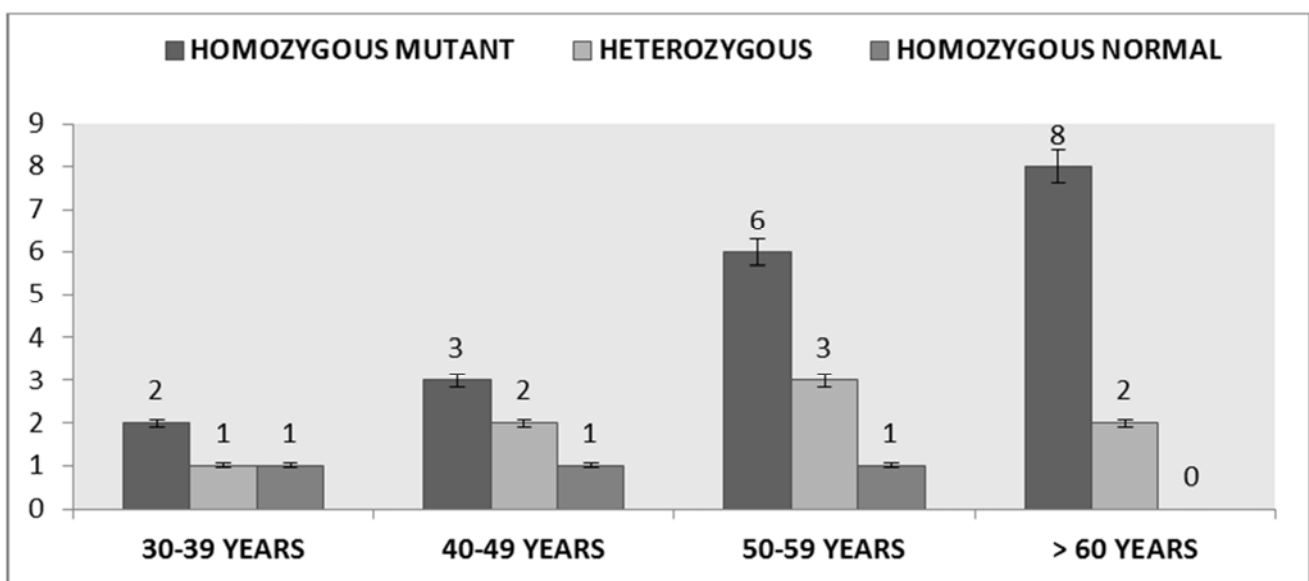

Figure 5. *Histogram representing age group polymorphism of MTHFRC677T gene.*

Table 5. *Representing genetic polymorphism in the MTHFRC677T gene within different age groups among diabetic patients.*

TOTAL NUMBER OF CASES (30)			
AGE GROUP (years)	CASES	GENOTYPE	FREQUENCY
30-39	04	2-Homozygous Mutant	50% (2/4)
		1-Heterozygous	25% (1/4)
		1-Homozygous normal	25% (1/4)
40-49	06	3-Homozygous Mutant	50%(3/6)
		2-Heterozygous	33.3%(2/6)
		1-Homozygous normal	16.6%(1/6)
50-59	10	6-Homozygous Mutant	60%(6/10)
		3-Heterozygous	30%(3/10)
		1-Homozygous normal	10%(1/10)
Above-60	10	8-Homozygous Mutant	80%(8/10)
		2-Heterozygous	20%(2/10)
		0-Homozygous normal	0%(0/10)

Homozygous mutant condition of MTHFRC677T gene was found to be certainly higher in Diabetes mellits 2 Cases of above 60 years of age, 8 out of 10 (80%), than ages below 60 years and in controls 3 out of 18 (16.6%) and was significant as p=0.005, compared to below 60 years of age 1 out of 3 (33.3%) and in controls 0 out of 2 (0%) (Figure 5) and association was insignificant as p=0.4667 as shown in table 5.

4. Discussion and Conclusion

A cluster of interrelated plasma lipid and lipoprotein abnormalities associated with alteration in VLDL metabolism contributes to the risk atherosclerosis and CHD in the majority of patients with T2DM. Each of the lipid abnormalities (low HDL, small dense LDL and elevated TG) is associated with an increased risk of CHD [14]. Individuals with T2DM have a higher incidence of LFT abnormalities than individuals who do not have diabetes. There was no significant difference in serum HDL and TG of patients and controls (0.90±0.59 vs 1.15±0.39 p>0.05) and (1.19±0.70 vs 1.01±0.52 p>0.060) respectively. LDL and TC are significantly higher in patients than control group (4.09±1.14 vs 3.01±1.02 p<0.0002) and (4.21±1.28 vs 3.78±1.29 p<0.05). HDL/TC ratio is also significantly higher in patients than controls (0.21±0.91 vs 0.30±0.99 p<0.05). Serum levels of all liver enzymes (ALT, AST, and ALP) analyzed were significantly higher in patients than controls (12.69±10.80vs 4.95±2.66 p<0.0002), (15.99±10.70 vs 6.95±3.84 p<0.0002) and 68.29±27.78 vs 21.27±7.77p<0.0001) respectively. The results obtained as such are in accordance with [15].

The role of MTHFR C677T polymorphisms has been widely studied across the world in different populations suggesting a substantial genetic contribution to the susceptibility of type 2 diabetes [16]. A closer association between the MTHFR C677T polymorphism and T2DM have been reported and explained by the fact that C677T polymorphism decreases the enzyme activity more than does A1298C polymorphism: 70% versus 30% respectively [17]. Our results revealed significant difference in the distribution of MTHFR C677T genotypes and mutant T allele (p = 0.007)

between diabetic patients and control subjects. MTHFR C677T/TThomotype was found to be significantly higher in T2DM patients compared to controls, in accordance with [18] who found fourfold risk for developing T2DM in Indian population (OR: 4.0423; 95% CI: 1.8753, 8.7133). Other studies also have confirmed the association of this polymorphism with diabetic complications as nephropathy in a Polish population [19] and coronary heart disease in a Chinese population [20] as well as Egyptian population [21]. C677T/TT genotype was more frequent in T2DM patients than in the healthy controls. Hence, it can be concluded that ethnicity is one of the most important factors that play a role in C677T gene polymorphism and susceptibility to T2DM. The C677T polymorphism of the MTHFR gene has been reported to cause reduced enzyme activity and impaired homocysteine / folate metabolism, leading to moderate hyperhomocysteinemia [22]. This hyperhomocysteinemia may damage the vascular endothelium, which is responsible for vasopressin effects and may cause a status of elevated blood pressure [23].

The MTHFR polymorphisms may play some roles in the pathogenesis and complications associated with type 2-diabetes in Kashmiri patients. Lack of association between the MTHFR genotypes with some diabetic related indexes in our study could be attributed to ethnic variations in MTHFR genotypes, as a given population may have elements in its genetic reservoir that are protective against certain disease despite the high prevalence of disease-susceptible alleles [24]. Restriction digestion was done to examine the genetic polymorphism in the MTHFR C677T gene. As shown in our study, 63% (19/30) of the Diabetes mellitus 2cases showed homozygous and 26% (8/30) of the cases however showed heterozygous condition. Almost all 80% (16/20) of the normal samples showed heterozygous condition except only in four cases where MTHFRC677T gene was found to be Homozygous Mutant two & Homozygous normal two. The allelic association of this polymorphism with Diabetes mellitus 2 was evaluated and found to be significant (P=0.007). Homozygous mutant condition of MTHFRC677T gene was found to be certainly higher in Diabetes mellitus 2 Cases of above 60 years of age, 8 out of 10 (80%), than ages below 60 years and in controls 3 out of 18 (16.6%) and was

significant as p=0.005, compared to below 60 years of age 1 out of 3 (33.3%) and in controls 0 out of 2 (0%) and association was insignificant as p=0.4667.

Combined heterozygosity of MTHFR mutations including C677T is associated with decreased total plasma +homocysteine levels [25]. MTHFR 677 AA combined genotype was significantly higher in the controls compared to patients with decreased risk of T2DM, which may have a protective role against the susceptibility to T2DM. No statistically significant difference could be encountered between the patients and the controls regarding the frequency of the combined genotypes; 677 CC. The genotypes MTHFR 677 TT was not detected in the controls, so the statistical difference between the two groups and the risk estimates for these combined genotypes were not determined. This could be an evidence of increased T2DM risk of this combined genotype. Conclusion: There was no significant difference in serum HDL and TG of patients and controls (p>0.060). LDL and TC are significantly higher in patients than control group (p<0.05). However, HDL/TC ratio is significantly higher in patients than control groups (p<0.05). Serum levels of all liver enzymes (ALT, AST, and ALP) analysed are significantly higher in patients than controls p<0.0002) and p<0.0001) respectively. Our data suggest that the MTHFR C677T risk factor for T2DM in Kashmiri patients and also suggest that the gene polymorphisms may act synergistically to increase the risk of diabetes. Furthermore, it should be noted that the sample size was relatively small in the studied population and so, large-scale prospective studies are to be needed to confirm these findings.

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