Assessment of gene Polymorphism of Methylenetetrahydrofolate Reductase (MTHFR) A1298C and Complications among cases with Type 2 Diabetes

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Abstract

Background: Type 2 diabetes mellitus (T2DM) is a major public health problem around the world. MTHFR gene, located on chromosome 1 (1p36.3), encodes for methylenetetrahydrofolatereductase enzyme. one of the most investigated polymorphisms in the MTHFR gene is A1298C (rs1801131). This polymorphism have been reported to be associated with T2DM and its complications. This study is a case control study which was performed to clarify the association between polymorphism in this gene and T2DM among Egyptians

Patients and Methods: A whole number of 102 individuals were selected, classified into two groups: group (1) were 51 healthy subjects, group (2) were 51 diabetic patients. This group was further subdivided into: Diabetic group without complications and Diabetic with complications . MTHFR gene polymorphism(rs1801131) was genotyped with restriction fragment length polymorphism method(PCR-RFLP), followed by enzymatic digestion with HinfI.

Results: A1298C genetic polymorphisms conveyed an increase in T2DM risk (OR = 2.2, 95% CI = 0.7–6.9, p = 0.004). Additionally, MTHFR 1298 AC+CC genotypes were associated with increased risk (2 fold) for diabetic complications.

Conclusion: our data suggest that MTHFR A1298C polymorphisms is risk factor for T2DM and its complications in Egyptian patients.Furthermore, because the size of the examined population was very small, large-scale prospective investigations are required to validate these findings.

Keywords: Type 2 diabetes mellitus, gene polymorphism, MTHFR ,Complications.

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Introduction

DM is regarded as a global epidemic, with more than 400 million people suffering from the condition worldwide. (1) Despite the fact that diabetes is possibly treatable, it is nevertheless the 9th biggest cause of mortality, with a 90 percent rise in burden between 1990 and 2010. (2) The International Diabetes Federation (IDF) estimates that T2DM will reach 18.7 million people by 2025. (3) By 2045, the number of people living with diabetes is anticipated to rise to 578 million (10.2 percent) and 700 million (10.9 percent). (4) In Egypt, the prevalence of diabetes mellitus is estimated to be at 15.56 percent in people aged 20 to 79. (5)

Diabetes mellitus (DM) is a condition marked by high blood glucose levels. (DM) is a condition in which blood glucose levels are abnormally high. It becomes unmanageable over time, allowing other complex metabolic illnesses such diabetic neuropathy (DN), diabetic retinopathy, diabetic foot, and cardiovascular problems to develop. (6)

Functional polymorphism in genes involved in the folate metabolic pathway has been associated with low level of folate and a high level of homocysteine (Hcy) [7-9]. Methylenetetrahydrofolate reductase (MTHFR) is the key enzyme that plays an important role in folate metabolism. The MTHFR enzyme, encoded by the MTHFR gene, is responsible for catalyzing the irreversible reaction of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate which is the primary circulating form of folate [10]. The gene encoding MTHFR is located at chromosome 1p36.3. Single-nucleotide polymorphism (SNP) in MTHFR A1298C (rs1801131) leads to glutamate to alanine substitution within the C-terminal regulatory domain of the enzyme due to an A to C transversion that occurs in exon 7, that results in a decrease in MTHFR activity [11]. Low MTHFR activity reduces DNA methylation [12], thereby results in elevated plasma homocysteine (Hcy) [13]. Several studies have also shown that elevated levels of Hcy may induce DNA damage either by an increased production of ROS or by biological mechanisms directly associated with an excessive misincorporation of uracil in DNA and the process of DNA methylation [14-16] .The present study was designed to find the association of MTHFR gene A1298C polymorphism with the risk of type 2 diabetes and also to find out the effect of this polymorphism on complications .paper

Subjects and Methods

This study was done in Medical Biochemistry and Internal Medicine Departments, Faculty of Medicine, Zagazig University. This study included 102 subjects aged between 35-55 years old: classified into two groups: group (1) were 51 healthy subjects, group (2) were 51 diabetic patients. This group was further subdivided into: Diabetic without complications and Diabetic with complications. Complications included in the study were coronary heart disease (18%), peripheral vascular disease (16%), stroke (1%), neuropathy (56%), retinopathy (1%), nephropathy (6%), and foot ulcer (2%). Both patients and controls are enlisted from outpatient clinics of the Endocrinology Unit of Internal Medicine Department; Zagazig University

hospital. According to the 2017 The American Diabetes Association (ADA) (9) standards for the diagnosis of diabetes ,patients were diagnosed T2DM if they have one of the next standards: (i) fasting plasma glucose (FPG) level of 126 mg/dL (7 mmol/L) or higher; fasting is defined as no caloric intake for at least 8 hours, (ii) a 2-hours plasma glucose level of 200 mg/dL (11.1 mmol/L) or higher during a 75-g oral glucose tolerance test (OGTT),or (iii) a random plasma glucose of 200 mg/dL (11.1 mmol/L) or more in a individuals with typical symptoms of hyperglycaemia (polyuria, polydipsia, polyphagia, weight loss) or hyperglycemic crisis or (iiii) hemoglobin A1c (HbA1c) level of 6.5% or more. (17). All patients were non-smoker, normotensive, non-obese, no history of coronary artery disease (CAD), liver, cancer, autoimmune disorders, and inflammatory diseases and type-1 DM.

The entire included groups were exposed to whole history taking, full clinical anthropometric dimensions. Estimation of body mass index (BMI) was done through dividing body weight in kilograms by (height in square meters) (18). Laboratory tests including fasting and 2 hours post prandial blood glucose levels, HbA1c%, lipid profile [serum total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-c) and calculation of low density lipoprotein cholesterol (LDL-c)] . Estimation of urea and creatinin level was done.All participants contracted an acquainted written agreement before registration in our research and the study design was permitted by the Ethical Committee of Faculty of Medicine, Zagazig University.

1- After an overnight fasting,6 ml of intravenous blood were taken from every individual by sterile vein-puncture and separated into three samples: The first sample was 2ml of blood were collected in an EDTA containing tubes and were divided into two aliquots; for colorimetric estimation of glycated haemoglobin as percent of total haemoglobin by Stanbio Laboratory, Boerne, Texas, USA (19) and the reamaining part was stored at -20 °C for total DNA and RNA extraction. The second sample was 1 ml blood into sodium fluoride tube, for measurement of FBG and 2 hours postprandial blood glucose via enzymatic colorimetric technique by Spinreact kit,Girona,Spain (20).The remaining blood was moved into a plain tube, allowed to clot at 37°C, centrifuged for 15 minutes at 4000 r.p.m. Total cholesterol and triglyceride levels were tested by routine enzymatic techniques (Spinreact, Girona, Spain) (21),(22). HDL cholesterol concentration was detected after precipitation of the apoB-containing lipoproteins (23). The LDL cholesterol level was calculated with the Friedewald formula (24). Urea and creatinin were estimated by using commercial kits from (Spinreact, Spain) (25),(26).

Detection of MTHFR A1298C genetic polymorphism

DNA Extraction:

Genomic DNA was extracted from white blood cell pellets by salting out extraction method [27] using a wizard genomic DNA extraction kit from Promega. Red blood cell lysis was done by using red cell lysis buffer (20 mM tris–HCL pH 7.6) followed by centrifugation. Nuclei

lysis was carried out by cell lysis buffer (10 mM tris–HCl pH 8.0, 1 mM EDTA pH 8.0, 0.1% (w/v) SDS) and proteinase K (20 mg/mL) followed by centrifugation. Protein was precipitated by proteinprecipitation solution (60 mL of 5 M potassium acetate,11.5 mL of glacial acetic acid, 28.5 mL of water) followed by centrifugation. Finally DNA was precipitated by isopropanol and then ethanol 70% and rehydrated in Tris EDTA buffer (10 mM tris, 1 mM EDTA pH 8.0) and stored at 20 C. DNA purity and concentration were determined by the spectrophotometer measurement of absorbance at 260 and 280 nm.

Detection of MTHFR A1298C genetic polymorphism by restriction fragment length polymerase chain reaction

0.5–2.0 lg of human genomic DNA was amplified by polymerase chain reaction on Gene Amp PCR System 9700 thermocycler (Applied Biosystems), Genotyping was based on the methods described by Frosset et al., [28] and Van der Put et al., [29] as the target genes were amplified by PCR followed by restriction digestion with the endonuclease. For MTHFR A1298C polymorphism, Primer sequences were: Forward primer 5-CTT TGG GGA GCT GAA GGA CTA CTA C-3 and the reverse primer 5-CAC TTTGTGACCATTCCGGTTTG-3. 10 mM TrisÆHCl, 50 mM of KCl, 1 mg/ml of gelatin, 3.0 mM of MgCl2, 200 lM each of dNTP, and 1.25 units of DNA Taq polymerase (Sigma). PCR conditions were optimized for an initial 2-min denaturation cycle at 92 C followed by 35 cycles of denaturation at 92 C for 1 min, annealing at 60 C for 1 min, extension at 72 C for 30 s followed by a 7-min final extension at 72 C. 12.5 lL of PCR product was digested with 2.5 ll of MboII buffer and 2.5 units of MboII restriction enzyme (Promega, UK). RFLP products was separated on 2% agarose gel and visualized by ethidium bromide staining. Wild types (1298AA) produced five fragments of 56, 31, 30, 28, and 18 bp, heterozygotes (1298AC) produced six fragments of 84, 56, 31, 30, 28, and 18 bp, and the homozygous mutants (1298CC) produced four fragments of 84, 31, 30, and 18 bp. The major visible bands were those of 84 and 56 bp

Statistical analysis

All data were collected, tabulated and statistically analyzed using SPSS 20.0 for windows (SPSS Inc., Chicago, IL, USA). Quantitative data were expressed as the mean ± SD & median (range), and qualitative data were expressed as absolute frequencies (number) & relative frequencies (percentage). Independent samples Student's t-test was used to compare between two groups of normally distributed variables while Mann Whitney U test was used for non- normally distributed variables. Kruskal Wallis test was used to compare between more than two groups of non- normally distributed variables. Percent of categorical variables were compared using Chi-square test or Fisher's exact test when appropriate. Spearman's rank correlation coefficient was calculated to assess relationship between various study variables, (+) sign indicate direct correlation & (-) sign indicate inverse correlation, also values near to 1 indicate strong correlation & values near 0

indicate weak correlation. All tests were two sided. p-value < 0.05 was considered statistically significant (S), p-value ≥ 0.05 was considered statistically insignificant (NS).

Results

Clinical and laboratory data of studied groups are summarized in (Table 1). Compared to healthy controls, individuals who developed diabetes had significantly higher mean values of fasting glucose, HbA1c and triglyceride levels.

Comparing the frequency of different genotypes among patients and controls.

Individuals homozygous for MTHFR A1298C gene mutation (C/C homotype) had an increased risk of T2DM (OR = 1.97, 95% CI = 0.6–6.35, p = 0.255). This risk increase was also observed with A/C heterotype (OR = 2.5, 95% CI = 1.11– 5.79, p = 0.026). A1298C C allele carry 2.4 fold risk for development of T2DM (OR = 2.37, 95% CI =1.3 - 4.33, p < 0.001)

Regarding A1298C locus, when both groups were tested for the HWE the frequencies of AA, AC and CC genotypes of the A1298C gene inT2DM patients were 33.33%, 49% and 17.64% respectively**,** and in control subjects were 62.74%, 27.45% and 9.8% respectively. No deviation from HWE was detected in T2DM group (p2 = 0.3346, 2pq = 0.487 and q2 = 0.177) or in control group (p2 = 0.584, 2pq = 0.359 and q2 = 0.055).

3.3 Comparing MTHFR(rs1801131)polymorphism between diabetic patients without and with diabetic complications (n=51).

Significant differences were found when type 2 diabetic patients (with and without complications) and controls were compared according to AA genotype versus AC+CC genotypes of the MTHFR A1298C gene (patients with complications: p=0.027, OR=3.79 , 95% CI=1.38-10.38 & patients without complications: p=0.007 , OR=2.99, 95% CI=1.11-8.09). The results revealed that the MTHFR A1298C gene polymorphism is associated with increased risk (4 fold) for diabetes and its complications**.(Table 2)**

Parameters	**Controls (n =51)**	**Type-2 DM patients (n =51)**	**P value***
Age (years) Range Mean ± SD	 40– 53 46.07±5.36	 40 – 50 44.92±3.20	 0.19135
FBG (mg/dl) Range Mean ± SD	 75 –106 89.42±7.51	 146 –205 165.72±14.25	 <0.001
PPBG (mg/dl) Range Mean ± SD	 129 – 141 135.12 ±4.11	 238 – 390 **297.02±39.49**	 <0.001
HbA_{1c} (%) Range Mean ± SD	 4 –5.1 4.45±0.35	 6 – 8.4 7.28±0.64	 <0.001
Total cholesterol (mg/dl) Range Mean ± SD	 155 – 177 166.02±5.83	 165 –250 204.30±24.33	 <0.001
Triglycerides (mg/dl) Range Mean ± SD	 71–128 95.63±14.54	 89 – 208 166.63±35.08	 <0.001
LDL-C (mg/dl) Range Mean ± SD	 62.6 –98.6 83.25± 8.91	 103–184 144.2±40.08	 <0.001
HDL-C (mg/dl) Range Mean ± SD	 42 – 58 51.47±4.86	 31 – 52 38.5±5.99	 <0.001
Homocysteine (mmol/L)	(4.12_14.82) 10.41±2.89	(12-20.86) 14.73±2.46	<0.001

MTHFR A1298C Polymorphism	Type-2 DM patients, n (%)	Controls, n (%)	Odds ratio (95% confidence interval)	p value*
Genotype AA	 17 (33.33)	 32 (62.74)	 0.2969 (0.1317 - 0.6694)	 0.0034

AC CC	25 (49) 9 (17.64)	14 (27.45) 5 (9.8)	2.5412(1.1143 - 5.7953) 1.9714(0.6115 - 6.3553)	0.0266 0.2557
Alleles **A** C	**59(57.84)** 43 (42.15)	**78(76.47)** 24 (23.53)	----- 2.3686(1.2959 - 4.3295)	----- 0.005

The chi-square statistic is 8.8373. The p-value is .012051. The result is significant at p < .05.

Table (1): Baseline characteristics of type-2 DM patients versus controls

Table (2): Genotype distributions and allelic frequencies of MTHFR A1298C (rs ******) polymorphism in type-2 DM patients(n=51) and controls(n=51)

HWE

Patients (p2 = 0.3346,

2pq = 0.4876755 and q2 = 0.17766225)

Control (p2 = 0.58476609,

2pq = 0.359867 and q2 = 0.05536609)

(No Deviation)

Table 2: MTHFR A1298C gene polymorphism – Genotype and Allele Frequency in type 2 diabetic patients (with and without vascular complications) and controls

Genotype/Allele	Patients with complications (n=25)	Patients without complications (n=26)	Controls (n=51)
Genotypes			
AA	8	9	32
AC	11	14	14
CC	7	2	5
Alleles			
A	27	32	78
C	25	18	24

Cases versus controls	Odds ratio	95% CI	p value

AA versus AC+CC			
Patients with complications versus Controls	3.79	1.38-10.38	0.027
Patients without complications versus Controls	2.99	1.11-8.09	0.007

Discussion

Diabetes is a serious illness resulting in chronic complications and growing threat to the health of the global world. It has been suggested that the chronic complications could be prevented by initial understanding of the disease and through better diabetes mellitus treatment [30]. Another finding suggests that the level of glycemic control and the presence of complications are associated with quality of life [31]. In the present study, the association of MTHFR gene (A1298C) variant with the risk of type 2 diabetes and its complications were analyzed among egyptians.

The enzyme methylenetetrahydrofolate reductase (MTHFR) methylates homocysteine to generate methionine (32), and its dysfunction can lead to HHcy (33). Therefore numerous studies have investigated whether reduced MTHFR activity is a risk factor for T2DM (34). Paper1

In the current study regarding MTHFR A1298C gene polymorphism, a significant association with T2DM (p 0.005) was evident. The frequency of 1298 CC genotype was higher in the patients compared to controls (17.64%versus 9.8% respectively) in accordance with previous results in Taiwanese [35] and Moroccan populations [36]. Calculation of the risk estimate revealed that 1298CC homozygous and AC heterozygous genotype were associated with 1.97 and 2.5times riskfor T2DM respectively.

Elbaz et al. clearly recognised that the MTHFR A1298C polymorphism may be considered a ge netic risk factor for diabetic nephropathy in Egyptians with type 2 diabetes (37), but Chehadah e t al. discovered that the MTHFR gene A1298C polymorphism is not associated to T2DM in the Emirati population.

In T2DM patients, it can be utilised as a marker for CVA, nephropathy, elevated LDL cholestero l, and triglycerides (38)

Ay et al. also have found that MTHFR A1298C polymorphism may be regarded a genetic risk factor for diabetic nephropathy among individuals with type 2 diabetes in the Turkish community.(39)

Our findings revealed that the MTHFR A1298C polymorphism is more prevalent in Egyptian population and associated with the risk of diabetes and its complications. Regarding diabetic complications, few studies have found the association of this polymorphism with coronary heart disease in Chinese population [40], ischemic stroke in Tunisian population [41], and retinopathy in Egyptian population [42]. A previous study conducted in a south Indian population has reported that the MTHFR A1298C gene polymorphism might lead to an increased risk for the occurrence of acute myocardial infarction [43]. In contrary, the MTHFR A1298C variant was not associated with the development of type 2 diabetic nephropathy in Chinese population [44] and in Caucasians [45]. The occurrence of the mutant allele frequency varies in different population. This might be due to ethnic variations, geographical background, and interindividual differences of the studied population. The prevalence of the MTHFR A1298C gene polymorphism in healthy controls.

Conclusions and future direction

The MTHFR A1298C gene polymorphism is considered as a risk factor for the development of diabetes and its complications among Egyptians and may be an increased risk for the development of chronic complications. Future researchers may focus on the investigation of gene-nutrient interactions and epigenetic interactions for better understanding of the role of folate metabolism genes in the risk of diabetes and its complications among the Egyptian population . We recommend further researches to evaluate MTHFR as new target to decrease the incidence of diabetes mellitus.

References:

1. Cho, N.H., Shaw, J.E., Karuranga, S., Huang, Y., da Rocha Fernandes, J.D., Ohlrogge, A.W. and Malanda, B. (2018) IDF Diabetes Atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045. Diabetes Res ClinPract .138:271-81.
2. Factors Associated with Glycated Hemoglobin Levels > 6.5% among Diabetic Patients Attending Kenyatta National Hospital, Kenya Maina Charity Muringo1, Joseph Mutai2, John Gachohi (2021 Journal of Diabetes Mellitus, , 11, 10-25
3. Saeedi P, Petersohn I, Salpea P, Malanda B, Karuranga S, Unwin N, Colagiuri S, Guariguata L, Motala AA, Ogurtsova K, Shaw JE, Bright D, Williams R; IDF Diabetes Atlas Committee. Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9th edition. Diabetes Res Clin Pract. 2019 Nov;157:107843.
4. Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9th edition

5. Hegazi, R., El-Gamal, M. , Abdel-Hady, N. and Hamdy, O.(2015) Epidemiology of and Risk Factors for Type 2 Diabetes in Egypt . Annals of Global Health, 81(6)814-820.
6. Kanwal and Dsouza .(2019) Sirtuins and diabetes: optimizing the sweetness in the blood. Translational medicine communications 4:3.
7. Barbosa PR, Stabler SP, Machado AL, Braga RC, Hirata RD, Hirata MH, et al. Association between decreased vitamin levels and MTHFR, MTR and MTRR gene polymorphisms as determinants for elevated total homocysteine concentrations in pregnant women. Eur J Clin Nutr 2008;62:1010-21.
8. Sukla KK, Raman R. Association of MTHFR and RFC1 gene polymorphism with hyperhomocysteinemia and its modulation by vitamin B12 and folic acid in an Indian population. Eur J Clin Nutr 2012;66:111-8.
9. Yakub M, Moti N, Parveen S, Chaudhry B, Azam I, Iqbal MP, et al. Polymorphisms in MTHFR, MS and CBS genes and homocysteine levels in a Pakistani population. PLoS One 2012;7:e33222.
10. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, et al. A candidate genetic risk factor for vascular disease: A common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10:111-3.
11. Weisberg I, Tran P, Christensen B, Sibani S, Rozen R. A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. Mol Genet Metab 1998;64:169-72.
12. Friso S, Choi SW, Girelli D, Mason JB, Dolnikowski GG, Bagley PJ, et al. A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. Proc Natl Acad Sci U S A 2002;99:5606-11.
13. Finkelstein JD. Pathways and regulation of homocysteine metabolism in mammals. Semin Thromb Hemost 2000;26:219-25.
14. Yi P, Melnyk S, Pogribna M, Pogribny IP, Hine RJ, James SJ, et al. Increase in plasma homocysteine associated with parallel increases in plasma S-adenosylhomocysteine and lymphocyte DNA hypomethylation. J Biol Chem 2000;275:29318-23.
15. Crott J, Fenech M. Preliminary study of the genotoxic potential of homocysteine in human lymphocytes in vitro. Mutagenesis 2001;16:213-7.
16. Dong C, Yoon W, Goldschmidt-Clermont PJ. DNA methylation and atherosclerosis. J Nutr 2002;132:2406S-9S.
17. American Diabetes Association, (2017) Classification and Diagnosis of Diabetes. Diabetes Care . 40(Suppl. 1):S11–S24.
18. Enyioma, O., Michael, P.T., Abd-Ishakur, A., Mustapha, S. and Mona, A.(2002) Leptin, lipid and lipid metabolism related hormones in chronic renal failure in Arabia. Nephrology, 7:115-119.

19. Abraham, E.C., Nathan, D.M., Derger, U.(1978) The clinical information value of glycosylated haemoglobin assay. Diabetes,27:931 – 938.
20. Trinder, P.(1969) Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. J Ann ClinBiochem, 6: 24-25.
21. Bachorik, P.S., Walker, R.E. and Kwiterovich,(1982) Determination of high density lipoprotein-cholesterol in human plasma stored at -7OOC. Journal of Lipid Research.23.
22. Rifai, N. and Warnick, R. (2006) Lipids, lipoproteins, apolipoproteins and other cardiovascular risk factors. In: Tietz Textbook of Clinical Chemistry and Molecular Diagnosis. Carl, A. B, Edward, R. A and David, E. B [edrs.]. Saunders. [4th edition]; Ch 26. PP. 918-922.
23. Fossati, P. and Prenciphe, L.(1982) Determination of serum triglyceride. ClinChem , 28: 207-210.
24. Friedewald, W., Levy, R. and Fredrickson, D.(1972)Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. ClinChem, 18:499- 502.
25. Fawcett, J.K. and Scott, J.E. (1960) A Rapid and Precise Method for the Determination of Urea. Journal of Clinical Pathology, 13, 156-159.
26. Bartels, H., Bohmer, M. and Heierli, C. (1972) Serum creatinine determination without protein precipitation. Clinica Chimica Acta, 37, 193-197.
27. Kalin A, Alatas O, Colak O. Relation of plasma homocysteine levels to atherosclerotic vascular disease and inflammation markers in type 2 diabetic patients. Eur J Endocrinol 2008;158:47–52.
28. Frosst P, Blom HJ, Milos R, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10:111–3
29. Van der Put NMJ, Gabreels F, Stevens EMB. A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? Am J Hum Genet 1998;62:1044–51.
30. Hussain M, Naqvi SB, Khan MA, Rizvi M, Alam S, Abbas A, et al. Direct cost of treatment of diabetes mellitus Type 2 in Pakistan. Int J Pharm Pharm Sci 2014;6:261-4.
31. Andayani TM, Ibrahim MI, Asdie AH. The association of diabetesrelated factor and quality of life in Type 2 diabetes mellitus. Int J Pharm Pharm Sci 2010;2:139-45
32. Human methylenetetrahydrofolate reductase: isolation of cDNA, mapping and mutation identification. Goyette P, Sumner JS, Milos R, Duncan AM, Rosenblatt DS, Matthews RG, Rozen R Nat Genet. 1994 Jun; 7(2):195-200
33. Kluijtmans, L.A., van den Heuvel, L.P., Boers, G.H., Frosst, P., Stevens, E. M., van Oost, B. A., den Heijer, M., Trijbels, F. J., Rozen, R., and Blom, H. J. (1996). Molecular genetic analysis in mild hyperhomocysteinemia: a common mutation in the methylene tetra hydrofolate reductase gene is a genetic risk factor for cardiovascular disease. Am J Hum Genet 58(1), 35-41

34. Fletcher O, Kessling AM. MTHFR association with arteriosclerotic vascular disease? Hum Genet 1998;103:11–21
35. Chang YH, Fu WM, Wu YH, Yeh CJ, Huang CN, Shiau MY. Prevalence of methylenetetrahydrofolate reductase C677T andv A1298C polymorphisms in Taiwanese patients with Type 2 diabetic mellitus. Clin Biochem 2011;44(17–18):1370–4.
36. Benrahma H, Abidi O, Melouk L, Ajjemami M, Rouba H, Chadli A, et al. Association of the C677T Polymorphism in the Human Methylenetetrahydrofolate Reductase (MTHFR) Gene with the Genetic Predisposition for Type 2 Diabetes Mellitus in a Moroccan Population. Genet Test Mol Biomarkers 2012;16(5):383–7
37. El-Baz R, Settin A, Ismaeel A, Khaleel AA, Abbas T, Tolba W, Abd Allah W, Sobh MA. MTHFR C677T, A1298C and ACE I/D polymorphisms as risk factors for diabetic nephropathy among type 2 diabetic patients. J Renin Angiotensin Aldosterone Syst. 2012 Dec;13(4):472-7. doi: 10.1177/1470320312444651. Epub 2012 May 3. PMID: 22554825.
38. Sarah W. El Hajj Chehadeh, Herbert F. Jelinek, Wael A. Al Mahmeed, Guan K. Tay, Unini O. Odama, Gehad E.B. Elghazali, Habiba S. Al Safar, Relationship between MTHFR C677T and A1298C gene polymorphisms and complications of type 2 diabetes mellitus in an Emirati population, Meta Gene, Volume 9, 2016, Pages 70-75, ISSN 2214-5400, https://doi.org/10.1016/j.mgene.2016.04.002. (https://www.sciencedirect.com/science/article/pii/S221454001630010X)
39. Ay A, Alkanli N, Sipahi T, Gulyasar T, Ustundag S, Guldiken S, et al. Investigation of the relationship between MTHFR, IRS and CALCA gene polymorphisms and development of diabetic nephropathy in patients with type 2 diabetes mellitus. Biotechnol Biotechnol Equip. 2018;32:1257-65
40. Sun J, Xu Y, Xue J, Zhu Y, Lu H. Methylenetetrahydrofolate reductase polymorphism associated with susceptibility to coronary heart disease in Chinese Type 2 diabetic patients. Mol Cell Endocrinol 2005;229:95-101.
41. Fekih-Mrissa N, Mrad M, Klai S, Mansour M, Nsiri B, Gritli N, et al. Methylenetetrahydrofolate reductase (C677T and A1298C) polymorphisms, hyperhomocysteinemia, and ischemic stroke in Tunisian patients. J Stroke Cerebrovasc Dis 2013;22:465-9.
42. Settin A, El-Baz R, Ismaeel A, Tolba W, Allah WA. Association of ACE and MTHFR genetic polymorphisms with Type 2 diabetes mellitus: Susceptibility and complications. J Renin Angiotensin Aldosterone Sys 2015;16:838-43.
43. Angeline T, Jeyaraj N, Tsongalis GJ. MTHFR gene polymorphisms, B-vitamins and hyperhomocystinemia in young and middle-aged acute myocardial infarction patients. Exp Mol Pathol 2007;82:227-33.
44. Wang D, Bai L, Zhai Q, Li Y, Cao M, Hai J, et al. Association of MTHFR C677T and A1298C polymorphisms with the development of Type 2 diabetic nephropathy and their interaction with environmental factors. Int J Clin Exp Pathol 2017;10:3778-85.

45. Moczulski D, Fojcik H, Zukowska-Szczechowska E, Szydlowska I, Grzeszczak W. Effects of the C677T and A1298C polymorphisms of the MTHFR gene on the genetic predisposition for diabetic nephropathy. Nephrol Dial Transplant 2003;18:1535-40.