Research article

Type and frequency of genetic variants for cardiovascular risk in patients with type 2 diabetes mellitus

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ABSTRACT

Introduction and Aim: Type 2 diabetes mellitus (T2DM) is associated with a wide range of cardiovascular diseases that comprise the largest cause of both morbidity and mortality for the diabetic patients. The aim is to study the allelic and genotypic frequencies of genetic variants associated with cardiovascular disease (CVD) in T2DM and to assess their contribution to the risk of cardiovascular complications in the patients.

Materials and Methods: The genotyping was performed by using Cardiovascular disease StripAssay kit (Vienna Lab) based on polymerase chain reaction and reverse hybridization. The following mutations were studied: FV G1691A (Leiden), FV H1299R (R2), Prothrombin G20210A, Factor XIII V34L, β-Fibrinogen − 455 G/A, PAI-1 4G/5G, GPIIIa L33P (HPA-1), MTHFR C677T, MTHFR A1298C, ACE I/D, Apo B R3500Q, Apo E2/E3/E4. Diabetic patients were divided in 2 groups: 1) patients with cardiovascular complications (N=20) and 2) patients without cardiovascular complications (N=16).

Results: In all diabetic patients, we found higher than population frequency for FV Leiden allele (5.5%), FVR2 allele (9.7%), PAI-1 4G allele (58.9%), β-Fibrinogen genotype -455G/A (38.9%) and ACE D/D genotype (36.1%). Statistically higher frequency was established for β-Fibrinogen −455 G/A genotype in the patients with cardiovascular complications compared to non-cardiovascular patients (55% vs. 18.7%).

Conclusion: We detected significantly higher frequency of β-Fibrinogen −455 G/A genotype in diabetic patients, especially in these with cardiovascular disease. Based on its pro-inflammatory role and its connection to possible thrombotic events, we assumed that patients would benefit from anti-inflammatory treatment.

Keywords: Type 2 diabetes mellitus; genetic predisposition; cardiovascular risk; fibrinogen mutation.

INTRODUCTION

Diabetes mellitus (DM) is a very serious health condition that has reached extremely high frequency worldwide nowadays. Almost half a billion people are living with the disease. Data from the current 9th edition of the International Diabetes Federation (IDF) atlas showed that DM is one of the diseases that growing very fast all around the world. It is found that in 2019, 463 million people have diabetes and this number is expected to reach 578 million by 2030, and 700 million by 2045. Type 2 DM accounts for the vast majority - around 90% - of the disease worldwide (1). It is well known that the long-term complications of diabetes can be present at diagnosis in people with type 2 diabetes. The disease is associated with a wide range of cardiovascular disease (CVD) that comprise the largest cause of both morbidity and mortality for the patients (2). The prevalence of coronary artery disease (CAD) is found to be around 21% and that of any CVD is around 32% in adults with diabetes (3). The morbidity from CVD in diabetic patients is 2 to 4-fold higher in comparison to people without diabetes. Patients with DM without myocardial infarction (MI) have exactly the same risk for CAD as people that have already have MI (4). The most common types of CVD that are found in patients with diabetes are arterial hypertension, coronary heart disease, cerebrovascular disease, peripheral artery disease as well as congestive heart failure. As a whole, CVD contribute for between one-third and one-half of all deaths.

As it is known, atherosclerosis is a chronic inflammatory and lipid-depository disease that can eventually lead to different cardiovascular events. Subclinical inflammation is observed in type 2 DM, obesity, insulin resistance. It is characterized by overexpression of cytokines which are produced by adipocytes, activated macrophages and other cells. Inflammatory mediators like plasminogen activator inhibitor – 1 (PAI-1), C-reactive protein (CRP), fibrinogen and others take part in signal pathways, in insulin action and in amplifying the inflammatory response (5). Increased plasma levels of fibrinogen have been found to be associated with the presence of -455G>A common mutation in the gene promoter region. In the study of Carter et al. both mutation and

plasma fibrinogen levels were associated with cardiovascular complications of non-insulin dependent diabetes mellitus. The results showed that fibrinogen levels were significantly higher in the patients with CAD than those without. The data suggested a relationship between the -455 G/A beta-fibrinogen gene polymorphism and the development of CAD in DM (6). In another study of Lam et al. the association between this gene mutation, fibrinogen plasma levels and the development of CAD have been investigated in patients with type 2 diabetes mellitus as well. They concluded that the -455G/A polymorphism of the b-fibrinogen gene is a genetic determinant of plasma fibrinogen concentrations and CAD in their cohort (7).

Methylenetetrahydrofolate reductase (MTHFR) is an enzyme whose function is to convert the 5,10-methylene tetrahydrofolate in 5-methyl tetrahydrofolate. Thus, the enzyme regulates the folate metabolism and maintains the homocysteine levels in physiological range. The MTHFR gene mutation leads to hyper homocysteinemia – a risk factor for atherosclerosis (8). On the other hand, it is found that a number of common polymorphisms and mutations in the genes coding for Factor V Leiden (FVL) and MTHFR can contribute to deep vein thrombosis – a condition that can be associated with hypercoagulability, either genetic or acquired. In patients with deep vein thrombosis, the incidence of common genetic mutations FVL, MTHFR C677T and MTHFR A1298C was determined in a study of Ehsani M. et al. as follows: 77% for MTHFR A1298C, 67% for MTHFR C677T and 17% for FVL (9).

Renin–angiotensin–aldosterone system (RAAS) regulates the blood volume and pressure and also has a role in the pathogenesis of atherosclerosis and in the development of arterial hypertension, insulin resistance, DM, obesity, vascular and systemic inflammation. Genetic polymorphism of RAAS genes including gene for angiotensin converting enzyme (ACE), take part in atherosclerosis pathogenesis (10). The DD genotype of ACE is known to be connected to higher serum activity of ACE as well as the risk of left ventricular hypertrophy, arterial hypertension and CAD occurrence (11, 12). The high levels of apolipoprotein B (apoB), which contains low-density lipoproteins (LDL) and chylomicron, have been reported as well established risk factors for atherosclerosis (13). Genetic defect of apoB100 causes increased level of LDL which accumulates in plasma and leads to hypercholesterolemia and premature atherosclerosis. On the other hand, patients lacking apoE accumulate lipoprotein remnants (14).

The aim of our study was to investigate the allelic and genotypic frequencies of genetic variants associated with CVD in patients affected by type 2 DM with and without cardiovascular complications in order to estimate the additional contribution of the genetic variations in determining the risk of such complications.

MATERIALS AND METHODS

We collected 36 samples of peripheral venous blood – 19 men and 17 women, middle age 49,4±9,8 (from 30 to 77 years). They were divided in two groups according to their cardiovascular status: 20 with type 2 DM and CVD, middle age 56,3±10,8, and 16 with type 2 DM without CVD, middle age 42,5±10,8. Their general characteristics are shown on table 1. The collection of patients' samples was approved by the institutional ethical committee (Medical University, Sofia) with the approval No1209/2018. Each patient signed a written informed consent.

Table 1: General characteristics of participants in the subgroups

Parameters	Type 2 DM with CVD	Type 2 DM without CVD
Number	20	16
Age (years)	56.3±10.8	42.5±10.8
BMI (kg/m^2)	34.8±8.4	38.1±13.7
HbA1c (%)	8.8±1.8	7.9±2.3
MetS (%)	77.8	66.7

The statistical analysis of the data was performed through SPSS v.20.0 (SPSS, Chicago, USA). The data are expressed as middle value ± standard deviation (SD). Student's T-test and one-way analysis of variance (One-way ANOVA) are used for comparison of continuous variables and Pearson's chi-squared test for proportional comparisons. P-value less than 0,05 was considered statistically significant.

Our data do not show statistically significant difference in sex, middle age, BMI, HbA1c, the presence of metabolic syndrome (MeS) so these variables cannot influence the results from the DNA analysis.

We have used CVD StripAssay kit (Vienna Lab) based on polymerase chain reaction (PCR) and reverse hybridization. The procedure included three steps: 1. DNA isolation; 2. PCA amplification with biotinized primers; 3. Hybridization of amplificated products on test strip containing specific for the allele oligonucleotide probe immobilized on a massive of parallel bands (figure 1). The bound biotinized sequence are found with the help of streptavidin - alkaline phosphatase and color substrates.

The following mutations were studied: *FV* G1691A (Leiden), *FV* H1299R (R2), *Prothrombin* G20210A, *Factor XIII* V34L, *β-Fibrinogen* -455 G/A, *PAI-1* 4G/5G, *GPIIIa* L33P (*HPA-1*), *MTHFR* C677T, *MTHFR* A1298C, *ACE* I/D, *Apo B* R3500Q, *Apo* E2/E3/E4.

The allelic and genotypes frequencies of each of the investigated genetic variants were determined and were compared to the population frequencies from genomic databases – The Genome Aggregation

Database (gnomAD), 1000 Genomes Project phase 3 database, Ensembl Genome Browser.

RESULTS

In our cohort the number of patients studied was 36 (meaning 72 alleles for each gene) – there were 20 patients from the first group (40 alleles) and 16 from the second one (32 alleles). For some genetic variants the number is less due to unsuccessful analysis. Figure 1 and 2 show the results from the genotyping of 12 genetic variants at risk genes in diabetic patients with and without CVD.

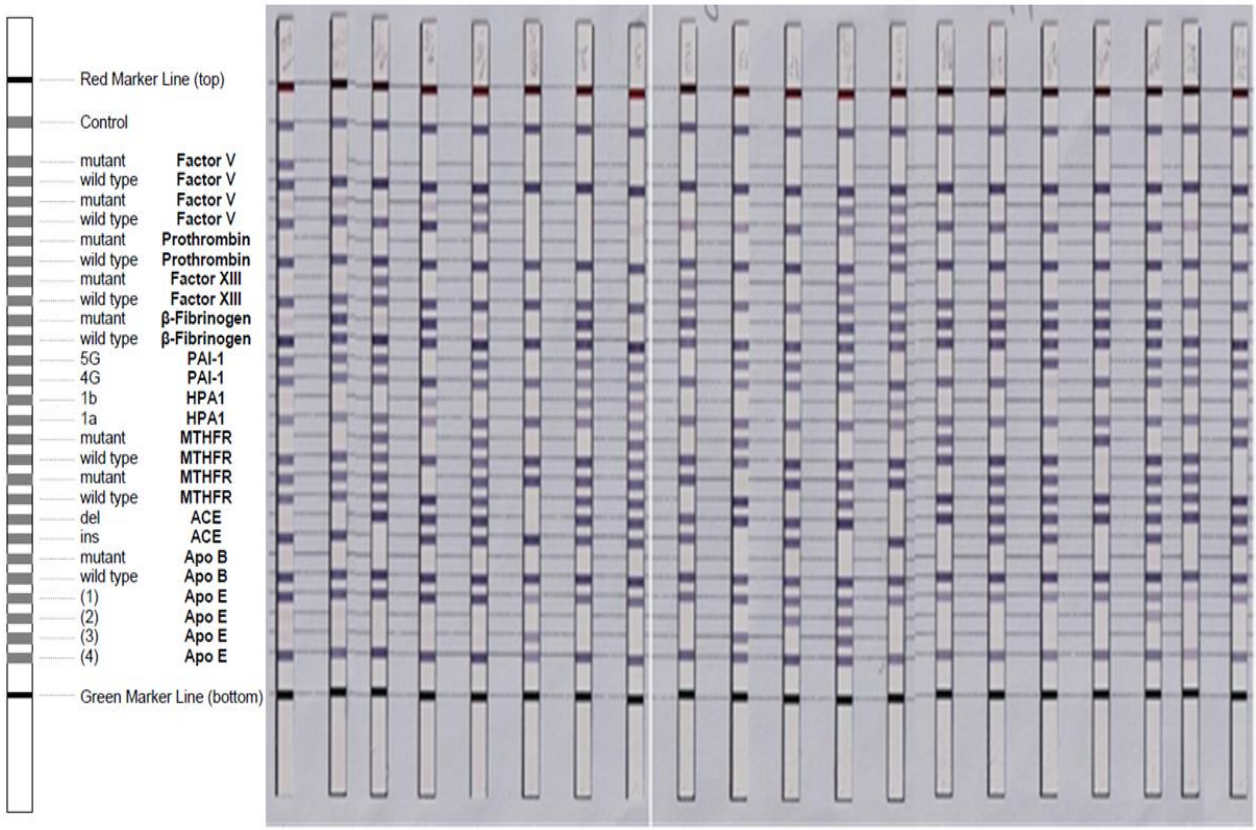

Fig. 1: Results from the genotyping of 12 genetic variants at risk genes in diabetic patients with CVD

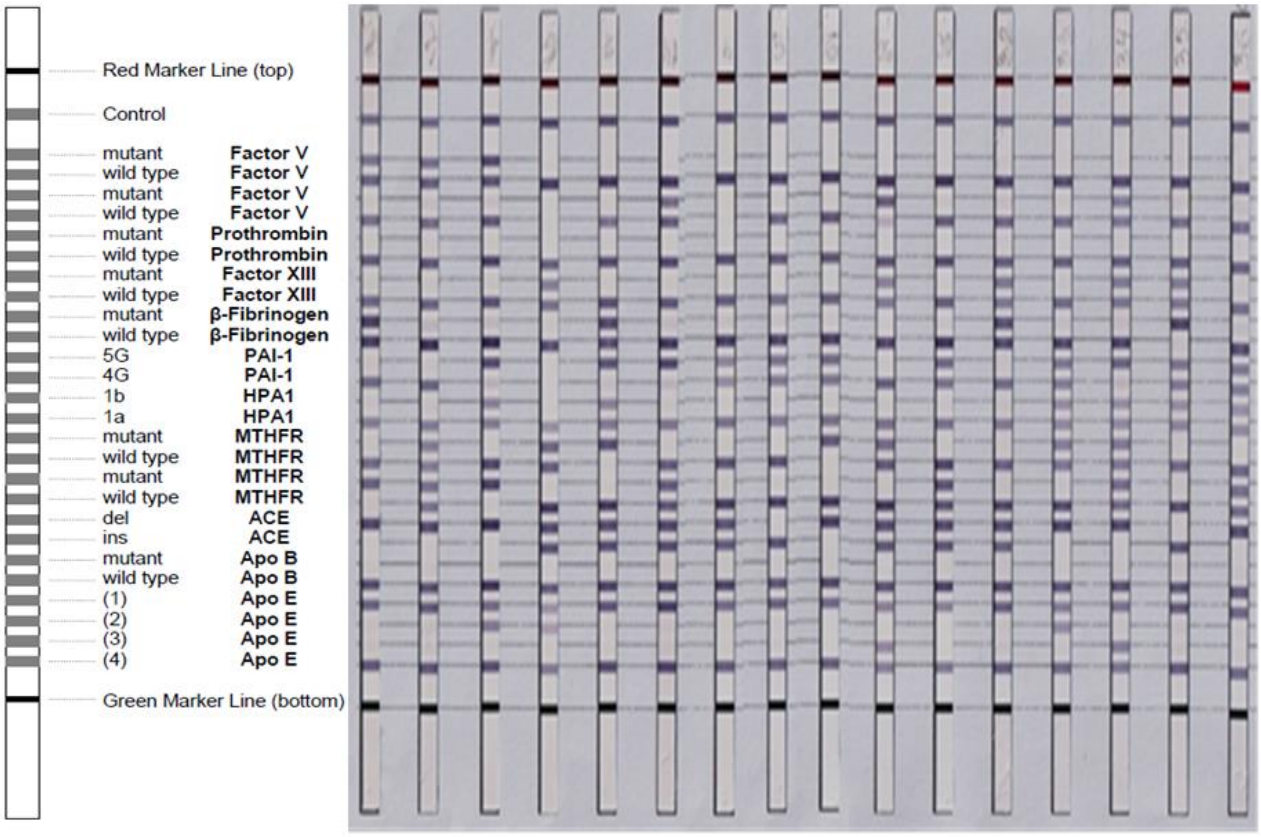

Fig. 2: Results from the genotyping of 12 genetic variants in risk genes in diabetic patients without CVD

Results from the genotyping of factors for congenital thrombophilia (Table 2)

Results from the genotyping of Factor V Leiden and HR2

Altogether for all patients a frequency of 5.5% was found for *Factor V* Leiden mutation (Table 2) – it is about two-fold increase than the population frequency in the world (1.9%) and in Europe (2.9%). According to 1000 Genomes database the frequency of the heterozygotes is 2% and, in our cohort, it was 11%. No connection between the mutation of FV Leiden and cardiovascular complications has been established. We found also a higher than population frequency for *FV* H1299R (R2) – 9.7% in comparison to frequency of 5.7% in the world and 6% in Europe.

Results from the genotyping of Prothrombin G20210A

We found a frequency of 1.4% and it is comparable to the world population frequency of 0.8% and to that in Europe – 1.1% (Table 2). The mutation was found only in DM patients with CVD not reaching statistical significance.

Results from the genotyping of PAI –1 4G/5G

We found higher frequency of the pathogenic allele 4G – 58.6% (Table 2) compared to 26.9% world population frequency according to Ensemble genome database. The frequency of the homozygotes 4G/4G was 31.4% in comparison to 20.9% in the world and 29.4% in Europe.

Results from the genotyping of Factor XIII V34L

In our cohort we found lower frequency of 11.1% for the minor allele (Table 2) compared to 21.9% world population frequency and 25.2% in Europe. It is important to note that in the group with CVD the frequency is even lower – 7.5%, which suggests a protective role of this genetic variant.

Results from the genotyping of β-Fibrinogen -455 G/A

The overall allelic frequency of the pathological allele in our group was 22,2% which is higher than the world population frequency – 16,9%, and close to that in Europe – 20,3%. According to 1000 Genomes database the population frequency of the heterozygotes is 22%, but it was 38,9% in our cohort (Table 2). It increases significantly in the group with CVD compared to the group without CVD – 55% versus 18,7% - fig. 3.

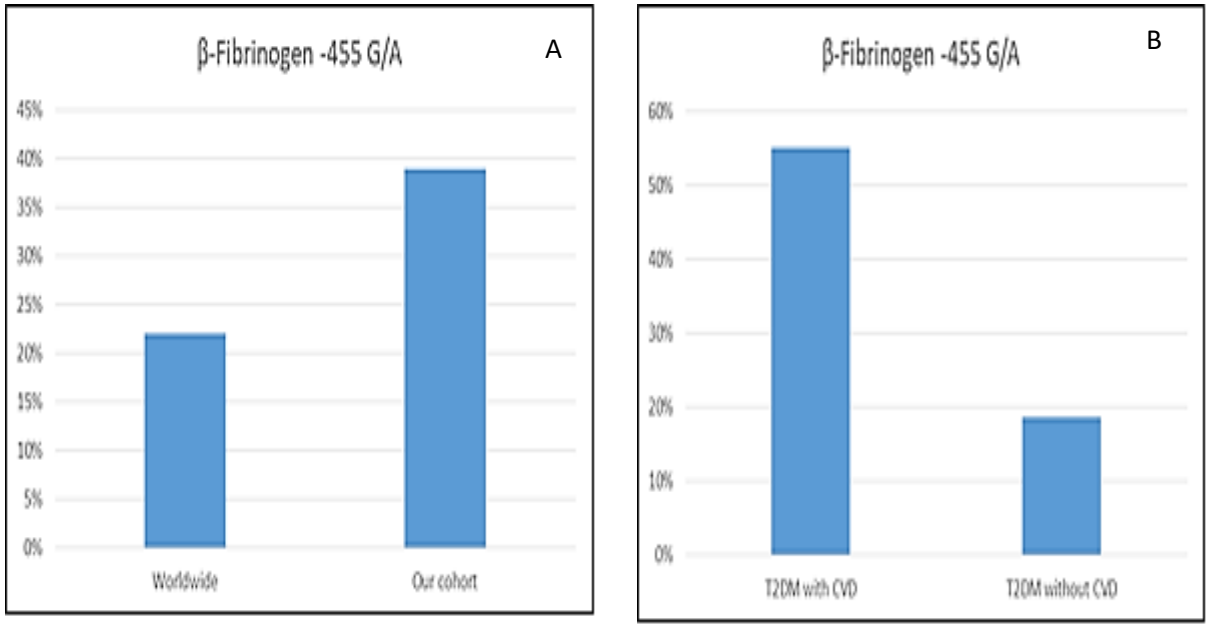

Fig. 3: Frequency of the heterozygotes -455 G/A for β-Fibrinogen in our cohort compared to worldwide (A) and in both DM patient's groups (B)

Results from the genotyping of HPA1 (GPIIIa L33P)

We found allelic frequency of 12.5% (Table 2) which is comparable to the world population frequency – 12.1%; and that in Europe – 1.2%.

To conclude about the factors contributing to congenital thrombophilia we found higher frequencies for most of them than in the world population frequency but not reaching statistical significance (Fig. 4A). The highest frequency is that of the *PAI-1* 4G variant in patients with DM. The frequency of *Factor XIII* polymorphism was lower than that in the world which is in accordance to the suggested protective role of this polymorphism. When comparing the frequencies in the groups with and without CVD only the variants of PAI-1 and Fibrinogen showed higher frequency in the group with CVD – fig. 4B.

Table 2: Allelic and genotypic frequencies of factors for congenital thrombophilia

Allele/Genotype	DM with CVD	DM without CVD	All
FV (Leiden)	1/40 (2.5%)	3/32 (9.4%)	4/72 (5.5%)
FV G/A	1/20 (5%)	3/16 (18.8%)	4/36 (11%)
FV A/A	0	0	0
FV (R2)	3/34 (8.8%)	3/28 (10.7%)	6/62 (9.7%)
FV H/R	3/17 (17.6%)	3/14 (21.4%)	6/31 (19.4%)
FV R/R	0	0	0
Prothrombin 20210A	1/40 (2.5%)	0	1/72 (1.4%)
Prothrombin G/A	1/20 (5%)	0	1/36 (2.8%)
Prothrombin A/A	0	0	0
PAI-1 4G	26/40 (65%)	15/30 (50%)	41/70 (58.6%)
PAI-1 4G/5G	12/20 (60%)	7/15 (46.7%)	19/ A 54.3%)
PAI-1 4G/4G	7/20 (35%)	4/15 (26.7%)	11/ 31.4%)
Factor XIII 34L	3/40 (7.5%)	5/32 (15.6%)	8/72 (11.1%)
Factor XIII V/L	3/20 (15%)	5/16 (31.2%)	8/36 (22.2%)
Factor XIII L/L	0	0	0
β-Fibrinogen - 455 A	11/40 (27.5%)	5/32 (15.6%)	16/72 (22.2%)
β-Fibrinogen - 455 G/A	**11/20 (55%), p<0.03**	3/16 (18.7%)	14/36 (38.9%)
β-Fibrinogen - 455 A/A	0	1/16 (6.2%)	1/36 (2.7%)
HPA-1b	3/40 (7.5%)	6/32 (18.7%)	9/72 (12.5%)
HPA 1a/1b	3/20 (15%)	6/16 (37.4%)	9/36 (25%)
HPA 1b/1b	0	0	0

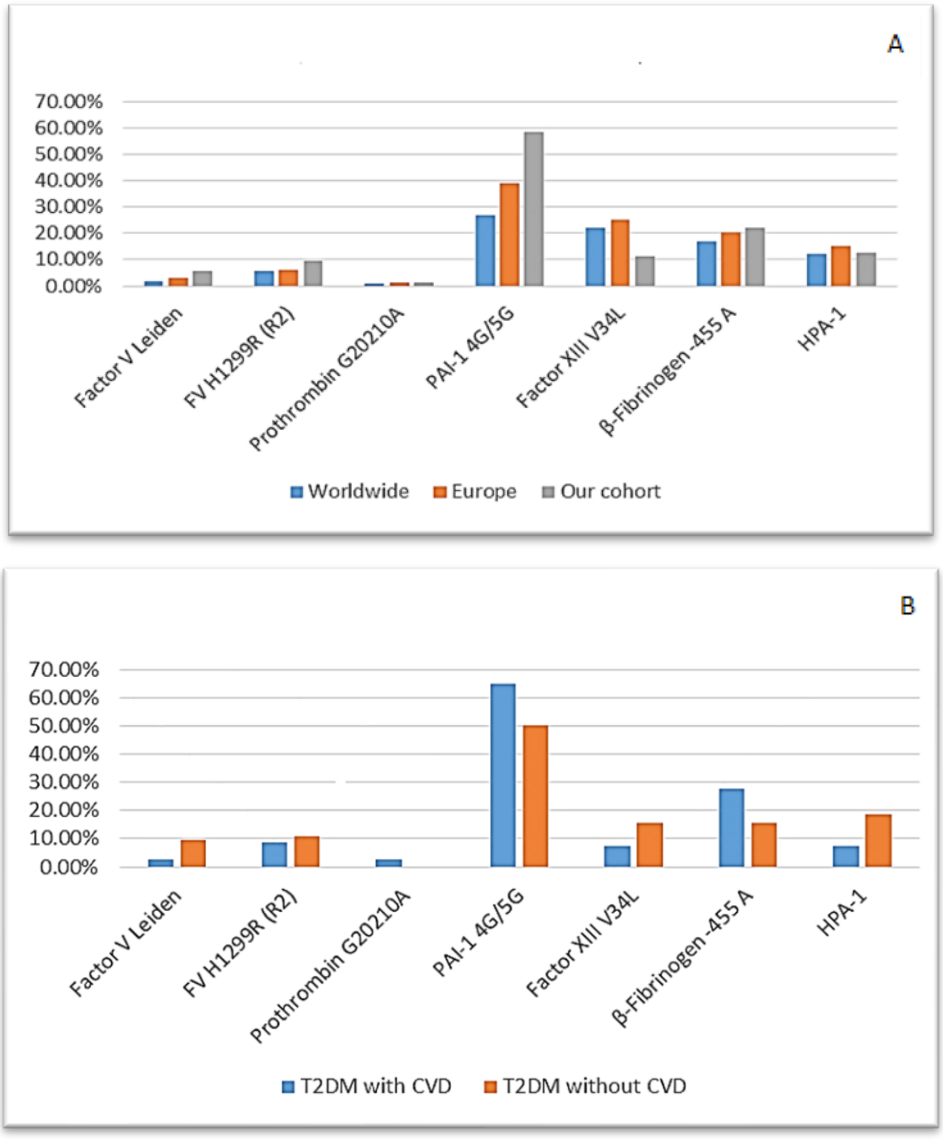

Fig. 4: Allele frequencies of the studied variants for congenital thrombophilia in the different populations (A) and patients' groups (B).

Results from the genotyping of MTHFR C677T and A1298C

The allelic frequency of MTHFR 677T in our cohort was 25% (Table 3) and it is a bit lower than that of world population – 31%, and that in Europe – 32%. The allelic frequency of MTHFR 1298C in our study was 38,9% and it is higher than that in world – 29%, and in Europe – 32%.

Table 3: Allelic and genotypic frequencies of MTHFR C677T и MTHFR A1298C

Allele/Genotype	DM with CVD	DM without CVD	All
MTHFR 677T	9/40 (22.5%)	9/32 (28.1%)	18/72 (25%)
MTHFR C/T	5/20 (25%)	5/16 (31.2%)	10/36 (27.8%)
MTHFR T/T	2/20 (10%)	2/16 (12.5%)	4/36 (11.1%)
MTHFR 1298C	19/40 (47.5%)	9/32 (28.1%)	28/72 (38.9%)
MTHFR A/C	11/20 (55%)	5/16 (31.2%)	16/36 (44.4%)
MTHFR C/C	4/20 (20%)	2/16 (12.5%)	6/36 (16.7%)

Results from the genotyping of ACE I/D

We found a frequency of 36.1% for the homozygotes of the pathologic allele (Table 4) which is higher than population frequency in Europe – 25%.

Table 4: Allelic and genotypic frequencies of ACE I/D

Allele/Genotype	DM with CVD	DM without CVD	All
ACE Del	22/40 (55%)	21/32 (65.6%)	43/72 (59.7%)
ACE I/D	10/20 (50%)	7/16 (43.7%)	17/36 (47.2%)
ACE D/D	6/20 (30%)	7/16 (43.7%)	13/36 (36.1%)

Results from the genotyping of ApoB R3500Q and ApoE

The mutation *ApoB* R3500Q was not found in any of the patients and its world population frequency is 1:500.

The frequency of the risk allele E4 in *ApoE* was 13.9% (Table 5) and it is comparable to the world population frequency – 13.8% and that in Europe – 16.1%

DISCUSSION

Different studies evaluate hemostasis gene variants and atherothrombotic and cardiovascular complications. Patients with diabetes are known to have coagulation cascade abnormalities and are prone to thrombotic events due to metabolic changes and acquired or hereditary coagulation defects (15). The glycation of hemoglobin, prothrombin, fibrinogen, and other proteins involved in the clotting cascade, caused by persistent hyperglycemia in diabetic patients, leads to coagulopathies. There is also decreased activated partial thromboplastin time (aPTT) and prothrombin time (PT) which are hallmarks of hypercoagulable state carrying an increased thrombotic risk for these patients and cause CVD as well (16). Factor V Leiden (FVL) is a mutation associated with congenital thrombophilia, increasing the risk for venous and arterial thrombosis, as well as implicated in complications of pregnancy. The link between the factor V Leiden mutation and atherosclerosis is not clearly understood. Our results showed a high incidence of 5.5% for FVL in all studied patients with diabetes, without showing an association with cardiovascular complications. One study found an increase in the prevalence of diabetes among patients with venous thromboembolism who are carriers of FVL compared with non-carriers of FVL, without reaching statistical significance (17).

We detected high frequency of 58.6% for *PAI-1* 4G allele in the studied diabetic patients, pointing the role of this serine protease inhibitor, which inhibits the tissue plasminogen activator (tPA) and urokinase (uPA), both doing an activation of plasminogen-related process of fibrinolysis. Elevated PAI-1 is an important risk factor for thrombosis and atherosclerosis (18). Circulating PAI-1 levels are found to be elevated in patients with CAD. It was suggested that the increased plasma levels of PAI-1, seen in patients with insulin resistance, could arise by the production of PAI-1 in adipose tissue (19). Patients with metabolic syndrome typically present with significantly higher levels of PAI-1 (20). Prospective studies of patients with myocardial infarction (MI) or CAD have showed the association between increased plasma PAI-1 levels and the risk of coronary events (19). The association of PAI-1 polymorphism (4G/5G) with myocardial infarction has been revealed in a recent meta-analysis (21). The existing link between PAI-1 and renin-angiotensin-aldosterone system suggests its contribution to the development of vascular disease as well (22).

We suppose an increased predisposition to adverse cardiovascular effects in diabetic patients who are carriers of *PAI-1* 4G polymorphism. Since PAI-1 targeted treatment has been reported, it is worthy to be studied. There are small molecules developed to inhibit PAI-1 - Tiplaxtinin, (PAI-039), and piperazine-chemotype molecules (23). Orally administered molecules such as TM5001, TM5007, TM5275, were tested in animal models. So far, some in vitro good

results were achieved, but they are still not tested in clinical trials (24).

One important finding from the present study is the significantly higher frequency of β-Fibrinogen -455 G/A heterozygotes in diabetic patients with CVD compared to non-CVD patients. Fibrinogen is the Factor I in the coagulation cascade – it represents a complex of glycoproteins, produced by the liver. During tissue and vessel damage, it is converted to fibrin under the action of thrombin, then the fibrin is organized into clot and occlude blood vessel to stop the bleeding. It is now established that fibrinogen is an acute-phase protein which increases its levels during inflammatory events or tissue injury (25). Studies have shown that high levels of fibrinogen are associated with CAD and may contribute to vascular disease by increasing blood viscosity thus stimulating fibrin formation, or increasing platelet-platelet interaction (26). Fibrinogen is considered as being involved in thrombotic occlusion and in the final stage of atherothrombosis. On the other hand, fibrinogen production and plasma concentration are increased in type 2 DM. There is no clear evidence whether altered insulin signaling is connected to hyperfibrinogenemia in diabetic patients. In type 2 diabetic patients, fibrinogen levels acutely increased along with insulin even in normal levels of glucose and amino acids, but not in non-affected persons. A recent study suggested that fibrinogen levels were associated with glucose levels and HbA1c in patients with CAD. They showed associations of all these parameters with the major cardiovascular complications in patients with prediabetes and diabetes, thus pointing the essential role of fibrinogen in cardiovascular risk determination in these patients (27). The fibrinogen's increase could be also result of an inflammatory reaction accompanying the process of atherosclerosis (28). We supposed that the role of β-fibrinogen as pro-inflammatory protein along with its thrombotic effects may increase the risk for CVD in patients with DM.

Table 5: Allelic and genotypic frequencies of Apo E2/E3/E4

Allele / Genotype	DM with CVD	DM without CVD	All
Apo E3/E4	2/20 (10%)	3/16 (18.7%)	5/36 (13.9%)
Apo E2/E4	1/20 (5%)	0	1/36 (2.8%)
Apo E3/E3	17/20 (85%)	13/16 (81.3%)	30/36 (83.3%)

CONCLUSION

In our study we aimed at investigating the allele and genotype frequencies of genetic variants strongly associated with CVD in patients with type 2 DM with and without cardiovascular complications to estimate the additional contribution of genetic variations in determining the risk of such complications. We found a statistically significant higher frequency of heterozygotes for β-fibrinogen -455 G/A in the group of DM patients with CVD. This comes to show that fibrinogen is really an important contributor to the pathogenesis of CVD, especially in patients with type 2 DM.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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