Screening for Genetic Variants Suggests \( \beta \)-fibrinogen -455 G/A Genotype as a Contributor to Cardiovascular Complications in Type 2 Diabetes Mellitus

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Research article

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Abstract

Background

Diabetes mellitus is associated with a wide range of cardiovascular diseases that comprise the largest cause of both morbidity and mortality for the diabetic patients. Our objective was to study the allelic and genotypic frequencies of genetic variants that have shown a strong association with cardiovascular disease in diabetic patients with and without cardiovascular complications and to assess the additional contribution of genetic variation in determining the risk for such complications.

Methods

We have used 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, GPIIIα L33P (HPA-1), MTHFR C677T, MTHFR A1298C, ACE I/D, Apo B R3500Q, Apo E2/E3/E4. 36 diabetic patients divided in 2 groups were analyzed: 1) 20 diabetic patients with cardiovascular disease and 2) 16 diabetic patients without cardiovascular disease.

Results

We found higher than population frequency for the following alleles/genotypes – 5.5% for FV Leiden allele, 9.7% for FVR2 allele, 38.9% for β-Fibrinogen genotype − 455G/A, 58.9% for PAI-1 4G allele, 36.1% for ACE D/D genotype. Statistically higher frequency was established for β-Fibrinogen − 455 G-A in the patients with cardiovascular disease compared to non-cardiovascular disease (55% vs. 18.7%).

Conclusions

We detected high 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, patients would benefit from anti-inflammatory treatment.

Background

Diabetes mellitus (DM) is a very serious health issue that has reached extremely high incidence worldwide nowadays (1). Almost half a billion people are living with the disease. Findings of the current 9th edition of the International Diabetes Federation (IDF) atlas state that DM is one of the diseases that grows very fast all around the world. It is found that in 2019 463 millions people have diabetes and this number is expected to reach 578 millions by 2030, and 700 millions by 2045. It is well known that the long-term complications of diabetes can be present at the time of diagnosis in people with type 2
diabetes. DM is associated with a wide range of cardiovascular disease (CVD) comprising 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% in adults with diabetes, the percentage increasing to 32% when consider any CVD (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 the people already affected by MI (4). The most common types of CVD 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 to mortality of one-third to one-half of DM patients.

As it is known, atherosclerosis is a lipid-depository condition leading to different cardiovascular diseases and is associated with chronic inflammation. Subclinical inflammation is observed in type 2 DM, obesity, and metabolic syndrome with insulin resistance as well. It is characterized by overexpression of cytokines 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 signaling pathways, connected to insulin action and inflammatory response (5). A common mutation – 455 G/A in the promoter region of the beta-fibrinogen gene has been associated with elevated fibrinogen levels in plasma. Carter et al. studied the association of -455G/A gene polymorphism and fibrinogen levels for the development of CAD in people with non-insulin dependent DM. The results showed significantly higher fibrinogen levels in the patients with CAD than in those without CAD. The data suggested a relationship between the – 455 G/A beta-fibrinogen gene polymorphism and the development of CAD in DM (6). Lam et al. investigated the relation between the – 455G/A-b-fibrinogen gene polymorphism and plasma fibrinogen concentration, elucidating its role for CAD in patients with type 2 DM and in non-diabetic control subjects. They concluded that the – 455G/A polymorphism in the b-fibrinogen gene is a genetic determinant of plasma fibrinogen concentrations and risk factor for CAD in their cohort (7).

Methylenetetrahydrofolate reductase (MTHFR) catalyzes the conversion of 5,10-methylenetetrahydrofolate in 5-methyltetrahydrofolate. It plays role in the metabolism of folate and in the regulation of homocysteine levels. Frequent C677T polymorphism in MTHFR is associated with high risk of CVD development. The mutation leads to hyperhomocysteinaemia – a risk factor for atherosclerosis (8). On the other hand, it was found that common polymorphisms and mutations in the genes encoding Factor V Leiden (FVL) and MTHFR can contribute to deep vein thrombosis – a condition caused by hypercoagulability, which can be genetic or acquired. A study determined the incidence of FVL, MTHFR C677T and MTHFR A1298C gene polymorphisms in patients with the abovementioned disease. The results detected MTHFR A1298C polymorphism in 77% of cases, followed by polymorphisms MTHFR C677T (67%) and FVL (17%) (9).

Renin–angiotensin–aldosterone system (RAAS) regulates the blood volume and pressure and take part in the development of arterial hypertension. It also has a role in the pathogenesis of atherosclerosis, vascular and systemic inflammation, as well as in insulin resistance, DM and obesity. Genetic
polymorphism of the gene for angiotensin converting enzyme (ACE), belonging to RAAS, has a role in atherosclerosis pathogenesis (10). The DD genotype of ACE is known to be connected to higher serum activity of ACE as well as to high risk of left ventricular hypertrophy, arterial hypertension and CAD (11, 12). Increased levels of apolipoprotein B (apoB) – containing lipoproteins like LDL and chylomicron remnants cause atherosclerosis as well (13). Genetic defect of apoB 100 causes increased level of LDL which accumulates in plasma and leads to hypercholesterolemia and premature atherosclerosis. On the other hand, patients which lack apolipoprotein E (apoE) accumulate lipoprotein remnants, whereas these with apoE stimulate accumulation of cholesterol esters in macrophages (14).

The aim of our study was to investigate the allelic and genotypic frequencies of variants in the genes that have shown strong association with CVD in patients with type 2 DM and the presence or absence of cardiovascular complications in order to estimate the additional contribution of the genetic variations in determining the risk of such complications.

**Methods**

**Patients’ selection**

We conducted a cross-sectional study with patients from the Department of Endocrinology, Clinics of Diabetology in Medical University of Sofia. The patients were given an informed consent which they signed and were acquainted with the aims, conditions and risks from their participation in the study according to the Declaration of Helsinki and the rules for Good medical practice. The study has been approved by the Scientific Research Ethics Committee of Medical University of Sofia. All participants were interviewed for the presence of diabetes mellitus (diagnosed by the World Health Organization criteria for diabetes) and cardiovascular disease (arterial hypertension, coronary artery disease, stroke – diagnosed by the cardiologic guidelines for these diseases) and their duration. Their weight (in kilograms, kg) and height (in meters, m) were measured and the Body Mass Index (BMI) calculated using the formula kg/m².

In all patients we measured HbA1c (NGSP certified) in whole blood probes with immunoturbidimetric method (Roche Diagnostics) as well as fasting lipid profile – total cholesterol, HDL and LDL cholesterol, triglycerides using enzyme colorimetric method (Roche Diagnostics). LDL is calculated by the Friedwald equation - [LDL-cholesterol] = [Total cholesterol] - [HDL-cholesterol] - ([Triglycerides]/2.2) where all concentrations are given in mmol/L, the calculation is not valid when the Triglyceride level is ≥ 4.5 mmol/L. (15). Metabolic syndrome (MeS) was diagnosed by the IDF criteria.

We collected 36 probes of peripheral venous blood – 19 men and 17 women, middle age 49,4 ± 9,8 (from 30 to 77 years). The patients 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.
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/m²)  | 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 mean value ± standard deviation (SD). The results were tested for normality of distribution and the parametric T-test has been applied. Pearson's chi-squared test is used for proportional comparisons. P-value less than 0.05 is statistically significant.

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

**Molecular-genetic 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. PCR amplification with biotinized primers; 3. Hybridization of amplified products on test strip containing specific for the allele oligonucleotide probe immobilized on a strip of parallel bands (Fig. 1). The bound biotinized sequence are detected by streptavidin - alkaline phosphatase and colour 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 genotypic frequencies for 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 is 36, corresponding to 72 alleles for each gene – these are distributed in 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, respectively.
1.1 Results from the genotyping of factors for congenital thrombophilia – Table 2

Results from the genotyping of Factor V Leiden and R2

Altogether for all patients a frequency of 5,5% was found for Factor V Leiden mutation (Table 2) – it is 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%. 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. No connection between both FV mutations and cardiovascular complications has been established.

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%)   | 19/35 (54.3%)|
| PAI-1 4G/4G         | 7/20 (35%)  | 4/15 (26.7%)   | 11/35 (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%), | 3/16 (18.7%) | 14/36 (38.9%)|
| β-Fibrinogen -455 A/A| p<0.03     | 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         |

Results from the genotyping of ProthrombinG20210A

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-1G4/5G
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% and we found it 38.9% (Table 2). It increases significantly in the group with CVD compared to the one without CVD – 55% versus 18.7% - figure 3

**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 – 15.2%.

In order 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 (Figure 4A). The highest frequency was found for PAI-1 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 the polymorphism. When comparing the frequencies in the groups with and without CVD only the variants of PAI-1 and Fibrinogen show higher frequency in the group with CVD – figure 4B.

**1.2 Results from the genotyping of MTHFR C677T and A1298C**

The allelic frequency of MTHFR 677T we found was 25% (Table 3) and is a little 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 Frequencies

| Allele/Genotype | DM with CVD  | DM without CVD | All     |
|----------------|-------------|----------------|---------|
| MTHFR 677T     | 9/40 (22.5%)| 9/32 (28.1%)   | 18/72 (25%) |
| MTHFRC/T       | 5/20 (25%)  | 5/16 (31.2%)   | 10/36 (27.8%) |
| MTHFRT/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%) |
| MTHFRA/C       | 11/20 (55%) | 5/16 (31.2%)   | 16/36 (44.4%) |
| MTHFRC/C       | 4/20 (20%)  | 2/16 (12.5%)   | 6/36 (16.7%)  |

### 1.3 Results from the genotyping of ACE I/D

We found a frequency of the homozygotes for the pathologic allele of 36.1% (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%) |

### 1.4 Results from the genotyping of ApoB R3500Q and ApoE

The ApoB mutation was not found in any of the patients and its world population frequency is 1:5000.

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

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

| Allele/Genotype | DM with CVD  | DM without CVD | All     |
|----------------|-------------|----------------|---------|
| ApoE3/E4       | 2/20 (10%)  | 3/16 (18.7%)   | 5/36 (13.9%) |
| ApoE2/E4       | 1/20 (5%)   | 0              | 1/36 (2.8%)  |
| ApoE3/E3       | 17/20 (85%) | 13/16 (81.3%)  | 30/36 (83.3%) |

### Discussion
Different studies evaluate thrombophilia’s gene variants and atherothrombotic and cardiovascular complications. Diabetic patients are affected by abnormalities of the coagulation cascade and are predisposed to thrombotic events because of metabolic changes and acquired or inherited coagulation defects (16). FVL is a procoagulant mutation associated with venous and arterial thrombosis as well as with pregnancy complications. Persistent hyperglycaemia in diabetes mellitus causes coagulopathies due to haemoglobin glycation, prothrombin, fibrinogen and other proteins involved in the coagulation pathway. Shortened activated partial thromboplastin time (aPTT) and prothrombin time (PT) reflect hypercoagulable state, which is associated with an increased thrombotic risk and different CVD (17). The relationship between the factor V Leiden mutation and atherosclerosis is a matter of debate due to conflicting data. We found higher frequency of 5.5% for FVL in all studied diabetic patients, without correlation to cardiovascular complications. A study found a relevant increase in the prevalence of diabetes among patients with venous thromboembolism carriers of FVL compared to non-carriers of FVL although this was not statistically significant (18).

Our study revealed high frequency of 58.6% for the pathologic allele in the gene for Plasminogen activator inhibitor-1 (PAI-1) also known as endothelial plasminogen activator inhibitor or serpin E1 - a serine protease inhibitor (serpin) that functions as the main inhibitor of tissue plasminogen activator (tPA) and urokinase, the activators of plasminogen-related process of fibrinolysis. Elevated PAI-1 is an important risk factor for thrombosis and atherosclerosis (19). Circulating PAI-1 levels are found to be elevated in patients with CAD. Couple of studies showed that insulin resistance may be a regulator of PAI-1 expression. The production of PAI-1 by adipose tissue could be an important contributor to the elevated plasma PAI-1 levels that are seen in patients with insulin resistance (20). Patients with metabolic syndrome typically present with significantly higher levels of PAI-1 (21). Prospective studies of patients with MI or CAD have showed the association between increased plasma PAI-1 levels and the risk of coronary disease (20). A recent meta-analysis has also proved that PAI-1 polymorphism (4G/5G) is associated with MI (22). PAI − 1 is linked to RAAS too, which is an important contributor to vascular disease initiation and progression (23). Taken this data together, we assumed that diabetic carriers of PAI-1 4G polymorphism are highly predisposed to its adverse effects and targeted treatment is worthy to be investigated. Small drug molecules have been developed for PAI-inhibition - Tiplaxtinin, (PAI-039), and piperazine-chemotype molecules have been studied (24). Small anti-PAI-1 molecules have been tested in animal models, with some good results in vitro, but unfortunately they did not achieve enough data to be used (25).

One important finding from our study is the significantly higher frequency of β-Fibrinogen − 455 G/A heterozygotes in diabetic patients with CVD compared to non-CVD patients. Fibrinogen (factor I) is a glycoprotein produced by the liver. In case of tissue and vascular injury it is converted by thrombin to fibrin and then to a fibrin-based blood clot which acts to occlude blood vessels and stop bleeding. Fibrinogen is a "positive" acute-phase protein and its blood levels rise in response to certain conditions like systemic inflammation or tissue injury (26). 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 by increasing platelet-platelet interaction (27). Fibrinogen is considered as
being involved in thrombotic occlusion and in the final stage of atherothrombosis. There are studies suggesting that fibrinogen may play a more active role in the development and progression of atherosclerotic plaque (28). On the other hand, fibrinogen production and plasma concentration are increased in type 2 DM. It is not known whether altered response to insulin contributes to hyperfibrinogenemia in diabetic patients. Fibrinogen production is acutely increased by insulin even in individuals with controlled type 2 diabetes but not in people without the disease. Increased fibrinogen production by insulin is supposed to be a main cause for hyperfibrinogenemia and associated cardiovascular risk in type 2 DM (29). Fibrinogen production and deposition is also increased in obese people. The increased fibrinogen production and fibrin deposition lead to increased adipocyte inflammation and macrophage infiltration which suppresses glucose uptake. However, relationship between fibrinogen and insulin resistance is controversial. The possible explanation is the increase in free fatty acids which has been seen in variety of clinical and experimental condition of insulin resistance. This relationship might also result from an inflammatory reaction that accompanies atherosclerosis (30).

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.

**Conclusions**

In our study we aimed at investigating the allelic and genotypic frequencies of genetic variants that are supposed to have strong association with CVD, in patients with 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. We found a significantly higher frequency in heterozygotes for β-fibrinogen – 455 G/A polymorphism in the group of patients with T2DM and CVD. This comes to show that fibrinogen is really an important contributor to the pathogenesis of CVD, especially in patients with type 2 DM.

**Declarations**

**Ethics approval and consent to participate:**

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.

**Consent for publication:**

It is included in the text of the Informed consent signed by the patient. All participants in the study signed the Informed consent.

**Availability of data and materials:**

All data and material are available in the Molecular Medicine Centre, Medical University Sofia
Competing interests:

No

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Authors’ contributions:

All authors contributed to the study conception and design. Methodology: IM, MM, MH, VP, ID; Formal analysis and investigation: IM, PG, RN, TM, ID; Writing – original draft preparation: IM, ID; Writing - review and editing: PG, RN, TM; Funding acquisition: ID; Resources: ID; Supervision: ID

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Figures

Figure 1

Results from the genotyping of 12 genetic variants at risk genes in diabetic patients with CVD
Figure 2

Results from the genotyping of 12 genetic variants in risk genes in diabetic patients without CVD

A

B

Figure 3

Frequency of the heterozygotes -455 G/A for β-Fibrinogen in our cohort compared to worldwide (A) and in the both DM patients groups (B)
Figure 4

Frequencies of the studied variants for congenital thrombophilia in the different populations (A) and patients groups (B).