Screening for Genetic Variants Associated with Cardiovascular Diseases in Patients with Type 2 Diabetes Mellitus

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Research article

Keywords: diabetes mellitus, cardiovascular disease, β-fibrinogen genotype -455G/A

Posted Date: December 28th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-51215/v2

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, GPIIIa 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.

Introduction

Diabetes mellitus (DM) is a very serious health issue that has reached extremely high levels worldwide nowadays. 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 million people have diabetes and this number is expected to reach 578 million by 2030, and 700 million by 2045. Unfortunately, three out of four patients are of working age and over 4 million people of the age 20–79 years are about to die from diabetes-

related causes in 2019. There is a 15% increase in the people with diabetes in Europe – they are 59 million in 2019 and their number is expected to rise to 66 million in 2030 and to 68 million in 2040, respectively. 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.

Metabolic syndrome is a specific set of symptoms, which plays an important role in cardiovascular morbidity and mortality. It is a progressive phenotype which is characterized by insulin resistance, abdominal obesity, hypertension, dyslipidemia or type 2 DM. 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. These cytokines are connected also with chronic inflammatory processes which cause lipid accumulation and development of atherosclerosis and CVD. Atherosclerosis is a complex multifactorial disease and the accelerating of the atherosclerotic process in DM may be explained by hyperglycemia, oxidative stress, accumulation of advanced glycation end products (AGEs), dyslipidemia, hyperinsulinemia, overexpression of inflammatory markers and genetic variabilities (5).

Methylenetetrahydrofolate reductase (MTHFR) catalyzes the conversion of 5,10-methylenetetrahydrofolate in 5-methyltetrahydrofolate. MTHFR plays role in the metabolism of folate and in the homeostasis of homocysteine. Frequent C677T polymorphism in MTHFR is connected with high risk of development of CVD, DM. The mutation leads to hyperhomocysteinemia – a risk factor for atherosclerosis (6). 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, which can be genetic or acquired. A study determines the prevalence of FVL, MTHFR C677T and MTHFR A1298C gene polymorphisms in patients with the disease. The results show that MTHFR A1298C gene was found in 77% among cases, followed by MTHFR C677T (67%) and FVL (17%) (7).

Renin–angiotensin–aldosterone system (RAAS) regulates the blood volume and pressure. It also has a role in the pathogenesis of atherosclerosis and can take part in the development of arterial hypertension, insulin resistance, DM, obesity, vascular and systemic inflammation. Angiotensin II activates intracellular signal pathways which cause atherothrombosis through inflammation, endothelial dysfunction, impaired fibrinolysis. Genetic polymorphism of RAAS genes including of angiotensin converting enzyme (ACE), angiotensin II type I receptor, angiotensinogen take part in atherosclerosis pathogenesis (8). 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 hypertonia and CAD occurrence (9, 10).

Plasma lipoproteins are made of hydrophobic core that consists of triglycerides and cholesterol esters, and of superficial monolayer of phospholipids, unesterified cholesterol and apolipoprotens. Increased levels of apolipoprotein B (apoB) – containing lipoproteins like LDL and chylomicron remnants cause atherosclerosis. Chylomicrons which contain apoB 48 are secreted in guts after meal while VLDL which have apoB 100 come from the liver. The metabolism of chylomicron remnants and VLDL in liver is facilitated by apolipoprotein E (apoE). ApoB 100 is responsible for LDL uptake in liver (11). 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 apoE accumulate lipoprotein remnants. Lipoprotein remnants with apoE stimulate accumulation of cholesterol esters in macrophages. In lesions most of apoE molecules are synthesized locally by macrophages (12).

Different studies evaluate hemostatic 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 (13). Factor V (FV) Leiden is a procoagulant mutation that is associated with venous and arterial thrombosis as well as pregnancy complications. The relationship between the factor V Leiden mutation and atherosclerosis is a matter of debate due to conflicting data. A study found a relevant increase in the prevalence of diabetes between patients with venous thromboembolism carriers of FVL compared to non-carriers of FVL although this was not statistically significant (14). Persistent hyperglycaemia in diabetes mellitus causes coagulopathies due to glycation of haemoglobin, prothrombin, fibrinogen and other proteins that are involved in the clotting 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 (15). Plasminogen activator inhibitor-1 (PAI-1) also known as endothelial plasminogen activator inhibitor or serpin E1 is a serine protease inhibitor (serpin) that functions as the main inhibitor of tissue plasminogen activator (tPA) and urokinase (uPA), the activators of plasminogen and the process of fibrinolysis. Elevated PAI-1 is an important risk factor for thrombosis and atherosclerosis (16). Circulating PAI-1 levels are found to be elevated in patients with CAD. There are studies that show that insulin resistance may be a regulator of PAI-1 expression. The production of PAI-1 by adipose could be an important contributor to the elevated plasma PAI-1 levels that are seen in patients with insulin resistance (17). Patients with metabolic syndrome typically present with significantly higher levels of PAI-1 (18). Prospective studies of patients with MI or CAD have showed the association between increased plasma PAI-1 levels and the risk of coronary events (17). A recent metaanalysis has also

proved that PAI-1 polymorphism (4G/5G) is associated with MI (19). PAI -1 is linked to RAAS too, which is an important contributor to vascular disease initiation and progression (20). Small drug molecules have been developed for PAI-inhibition. Tiplaxtinin, (PAI-039), and piperazine-chemotype molecules have been studied (21). Small molecules anti-PAI-1 that are orally bioavailable as TM5001, TM5007, TM5275, were tested in animal models, with some in vitro good results, but unfortunately they did not achieve enough data to be used (22). Factor XIII or fibrin stabilizing factor that is activated by thrombin to factor XIIIa. XIIIa is an enzyme of the blood coagulation system that crosslinks fibrin. Deficiency of XIII increases bleeding tendency (23). Human platelet antigens (HPA) are polymorphisms in platelet antigens. Platelets play a very important role in acute arterial occlusion and platelet hyperactivity may contribute to an increased risk for CVD. Platelets attach to subendothelial structures by specific receptors such as the collagen receptor glycoprotein (GP) Ia/IIa, or the primary von Willebrand factor (vWF) receptor GPIb/IX. After that they become activated and aggregate by cross-linking via the fibrinogen receptor GPIIb/IIIa (24). There is data that GPIIIa (HPA-1) may play a protective role in CVD. Iniesta et al. found that the platelet GP IIIa Polymorphism HPA-1 protects against subarachnoid hemorrhage and that the suggested platelet hyper-reactivity that is determined by this allele could reduce the risk to suffer that disease (25).

Fibrinogen (factor I) is a glycoprotein complex that is 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 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 when euglycemia and euaminoacidemia are maintained in type 2 diabetic individuals but not in people without the disease. Enhanced fibrinogen production by insulin is supposed to be a main alteration leading to hyperfibrinogenemia and to cardiovascular risk in type 2 DM (29). Fibrinogen expression and deposition is also increased in obese people. The increase in fibrinogen expression and fibrin deposition leads to increased adipocyte inflammation and macrophage infiltration which suppresses glucose uptake and may lead to adipose tissue fibrosis. However, relationship between fibrinogen and insulin resistance is controversial. Free fatty acids may explain the relationship between fibrinogen and insulin resistance because a simultaneous increase in free fatty acids and fibrinogen is seen in variety of clinical and experimental condition. This relationship might also result from an inflammatory reaction that accompanies atherosclerosis (30). A common mutation -455 G/A in the promoter region of the beta-fibrinogen gene has been associated with elevated fibrinogen in plasma. Carter et al. studied the association of G/A polymorphism at position -455 of the beta-fibrinogen gene and fibrinogen levels in the development of CAD in people with non-insulin dependent DM. 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 (31). Lam et al. investigated the relation between the G/A-b-fibrinogen gene polymorphism and plasma fibrinogen concentration and its role in CAD in patients with type 2 DM and in non-diabetic control subjects. They concluded that the G/A455 polymorphism of the b-fibrinogen gene is a genetic determinant of plasma fibrinogen concentrations and CAD in their cohort (32).

Aim of the study

To investigate the allelic and genotypic frequencies of genetic variants 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.

Materials And Methods

We collected probes from peripheral venous blood of patients with type 2 diabetes mellitus. They were divided in two groups according to their cardiovascular status: i) with type 2 DM and CVD, middle age 56,3±10,8, and ii) with type 2 DM without CVD, middle age 42,5±10,8. Their general characteristics are shown on table 1.

Parameters	Type 2 DM withCVD	Type 2 DM without CVD
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

Table 1 - General characteristics of participants in the subgroups

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 is 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 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 D/D, Apo B R3500Q, Apo E2/E3/E4.

Principle of the test:

In vitro amplification (PCR; 2 separate reactions of a probe)

The amplificated products are stored in ice or at 2-8^{o} C for later use.

Test with electrophoresis

Analysis of the products of amplification with gel electrophoresis (2,8% agar gel).

Hybridization (45^{o} C; water bath system)

Result interpretation

The genotype of the probe is determined with the help of Collector TM sheet.

The processed Test strip is put in one of the fields, it is leveled up to the schematic draught with the help of the red marker line (top) and the green one (bottom) and is fixed with glue.

A positive reaction of the top control line shows the right function of Conjugate Solution and Color Developer. That line must always be positively coloured.

For each polymorphic position one of the following colour models must be present and the intensity of the positive lines may vary (figure 2).

For the three Apo E isoforms E2, E3 and E4 the following colour models can be seen:

The six possible homozygotic and heterozygotic Apo E genotypes (E2/2, E3/3, E4/4, E2/3, E2/4, E3/4) correspond to the shown bands.

The allelic 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.

In our cohort the number of patients studied is 36, that are 72 alleles – these are 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.

Results

Figure 4 and 5 show the results from the genotyping of 12 genetic variants in risk genes in diabetic patients with and without CVD.

1.1. Results from the genotyping of Factor V Leiden and HR2

Altogether for all patients a frequency of 5,5% is found – more than two-fold increase than the world population frequency of 1,9% and 2,9% in Europe. According to 1000 Genomes database the frequency of the heterozygotes is 2% and in our cohort we found it 11%. No connection between the mutation of FV Leiden and cardiovascular complications has been established, even in the group of the patients without CVD a higher frequency of the mutation is found – 9,4%. We found also a higher than population frequency for FV H1299R (R2) – 9,7% in comparison to world frequency 5,7% and 6% in Europe.

Allele/Genotype	DM with CVD	DM without CVD	All
FV (Leiden)	2.5%	9.4%	5.5%
FV G/A	5%	18.8%	11%
FV A/A	0	0	0
FV (R2)	8.8%	10.7%	9.7%
FV H/R	17.6%	21.4%	19.4%
FV R/R	0	0	0

Table 2 – Allelic and genotypic frequencies of FV G1691A (Leiden) and FV H1299R (R2)

1.2. Results from the genotyping of Prothrombin

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%.

Allele/Genotype	DM with CVD	DM without CVD	All
Prothrombin 20210A	2.5%	0	1.4%
Prothrombin G/A	5%	0	2.8%
Prothrombin A/A	0	0	0

Table 3 - Allelic and genotypic frequencies of Prothrombin G20210A

1.3. Results from the genotyping of PAI – 1

We found higher frequency of the pathogenic allele – 58,6% compared to 26,9% world population frequency according to Ensambl and 38,9% in Europe. The frequency of the homozygotes is 31,4% in comparison to 20,9% in the world and 29,4% in Europe.

Allele/Genotype	DM with CVD	DM without CVD	All
PAI-1 4G	65%	50%	58.6%
PAI-1 4G/5G	60%	46.7%	54.3%
PAI-1 4G/4G	35%	26.7%	31.4%

Table 4 - Allelic and genotypic frequencies of PAI-1 4G/5G

1.4. Results from the genotyping of Factor XIII

In our cohort was found lower frequency of the minor allele of 11,1% 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 that genetic variant.

Allele/Genotype	DM with CVD	DM without CVD	All
Factor XIII 34L	7.5%	15.6%	11.1%
Factor XIII V/L	15%	31.2%	22.2%
Factor XIII L/L	0	0	0

Table 5 - Allelic and genotypic frequencies of Factor XIII V34L

1.5. Results from the genotyping of β-Fibrinogen

The allelic frequency in our group is 22,2% which is higher than the world population frequency – 16,9%, and that in Europe – 20,3%. According to 1000 Genomes database the population frequency of the heterozygotes is 22% and we found it 38,9%. It increases statistically significant in the group with CVD compared to the one without CVD – 55% versus 18,7% - figure 11.

Allele/Genotype	DM with CVD	DM without CVD	All
β-Fibrinogen - 455 A	27.5%	15.6%	22.2%
β-Fibrinogen - 455 G/A	**55%** **p<0.03**	18.7%	38.9%
β-Fibrinogen -455A/A	0	6.2%	2.7%

Table 6 - Allelic and genotypic frequencies of β-Fibrinogen -455 G/A

1.6. Results from the genotyping of HPA1

The found from us allelic frequency is 12,5% and is comparable to the world population frequency – 12,1%; in Europe – 15,2%.

Allele/Genotype	DM with CVD	DM without CVD	All
HPA-1b	7.5%	18.7%	12.5%
HPA-1a/1b	15%	37.4%	25%
HPA-1b/1b	0	0	0

Table 7 - Allelic and genotypic frequencies of GPIIIa L33P (HPA-1)

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. The highest frequency is that of the PAI-1 variant in patients with DM. The frequency of Factor XIII polymorphism is lower than that in world population frequency 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 13.

1.7. Results from the genotyping of MTHFR

The allelic frequency of MTHFR 677T we found is 25% and is a little lower than that of world population – 31%, and in Europe – 32%. The allelic frequency of MTHFR 1298C in our study is 38,9% and is higher than that in world – 29%, and in Europe – 32%.

Allele/Genotype	DM with CVD	DM without CVD	All
MTHFR 677T	22.5%	28.1%	25%
MTHFR C/T	25%	31.2%	27.8%
MTHFR T/T	10%	12.5%	11.1%
MTHFR 1298C	47.5%	28.1%	38.9%
MTHFR A/C	55%	31.2%	44.4%
MTHFR C/C	20%	12.5%	16.7%

Table 8 - Allelic and genotypic frequencies of MTHFR C677T и MTHFR A1298C

1.8. Results from the genotyping of ACE

We found a frequency of the homozygotes of the pathologic allele that is 36,1% and is higher than population frequency in Europe – 25%.

Allele/Genotype	DM with CVD	DM without CVD	All
ACE Del	55%	65.6%	59.7%
ACE I/D	50%	43.7%	47.2%
ACE D/D	30%	43.7%	36.1%

Table 9 - Allelic and genotypic frequencies of ACE I/D

1.9. Results from the genotyping of ApoB

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

1.10. Results from the genotyping of ApoE

The frequency of the risk allele E4 we found is 13,9% and is comparable to the world population frequency – 13,8%q and that in Europe – 16,1%

Allele/Genotype	DM with CVD	DM without CVD	All
Apo E3/E4	10%	18.7%	13.9%
Apo E2/E4	5%	0	2.8%
Apo E3/E3	85%	81.3%	83.3%

Table 10 - Allelic and genotypic frequencies of Apo E2/E3/E4

Discussion

The investigation of the allelic frequency of 12 genetic variants connected to cardiovascular risk in Bulgarian patients with type 2 DM showed more than two-fold increase in comparison to population frequency for the following alleles:

- 5,5% for FV (Leiden) mutation compared to 1,9% in world population and 2,9% in Europe population (no connection between FV (Leiden) mutation and cardiovascular complications has been established - in the group of patients without CVD the frequency of the mutation is higher – 9,4% compared to 2,5% in the group of patients with CVD)

- 58,6% for PAI-1 4G in comparison to 26,9% in world population

Increased frequency in comparison to world population for the following genotypes has been found:

- 38,9% for β-fibrinogen 455 G/A compared to 22% in world population

- 36,1% for ACE D/D compared to 25% in world population

Lower frequency for Factor XIII 34L and MTHFR C677T in the investigated groups in comparison to world population has been found. It is suggested that Factor XIII 34L may have protective role in CVD development.

No statistically significant difference between the investigated groups with and without CVD in the allelic and genotypic frequency in 11 out of 12 studied genetic variants has been found.

A statistically significant higher frequency in heterozygotes for β-fibrinogen 455 G/A in the group of patients with DM and CVD has been found – 55% in comparison to 18,7% in the group without CVD. It is 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.

Conclusion

In our study we aimed at investigating the allelic and genotypic frequencies of genetic variants that have are supposed to have strong association with CVD in patients with type 2 DM with and without

cardiovascular complications in order to try to estimate the additional contribution of the genetic variations in determining the risk of such complications. We found a statistically significant higher frequency in heterozygotes for β-fibrinogen 455 G/A in the group of patients with DM and CVD which is also seen in other studies. 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

Funding:

The study was supported by Grant of Bulgarian National Scientific Foundation with No КП-06-Н 33/10, 2019

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

Acknowledgements -

The study was supported by Grant of BNSF with No КП-06-ПН 33/10, 2019

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Figures

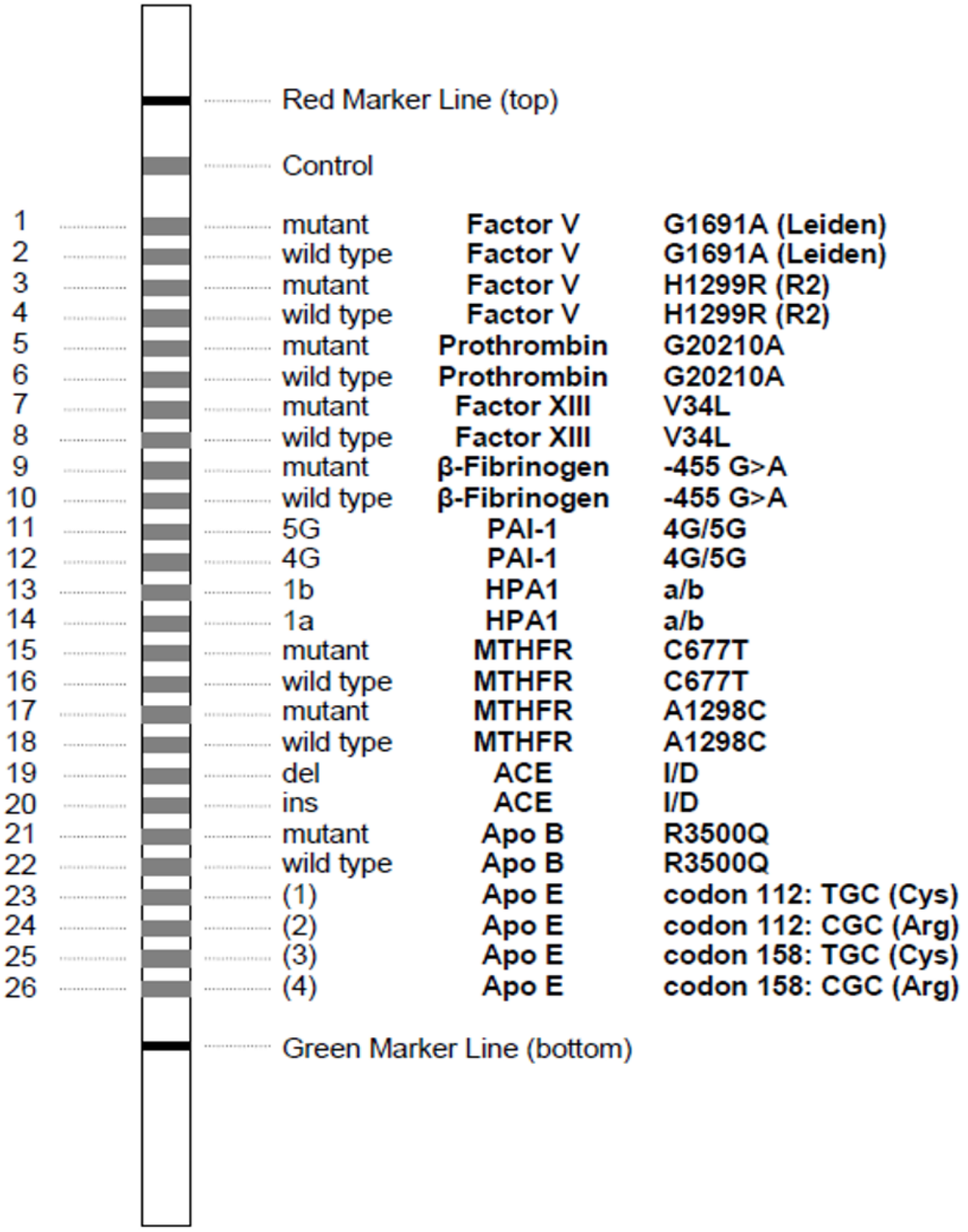


Figure 1

Test strip for detection of allele – specific hybridization

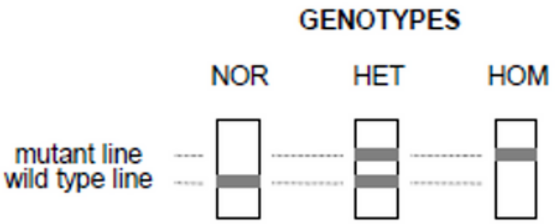


Fig. 2a

	wild type line	mutant line	genotype
NOR	**positive**	negative	normal
HET	**positive**	**positive**	heterozygous
HOM	negative	**positive**	homozygous mutant

Figure 2

Possible results of a test strip

E2 (112: Cys, 158: Cys) lines (1) + (3)
E3 (112: Cys, 158: Arg) lines (1) + (4)
E4 (112: Arg, 158: Arg) lines (2) + (4)

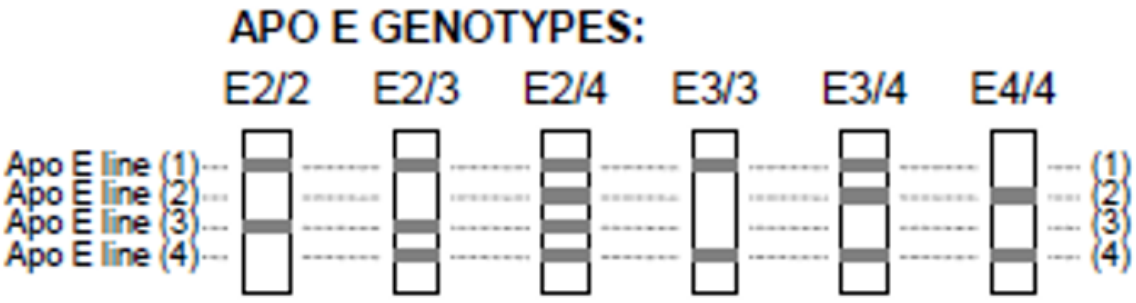


Figure 3

Possible results of a test strip for Apo E

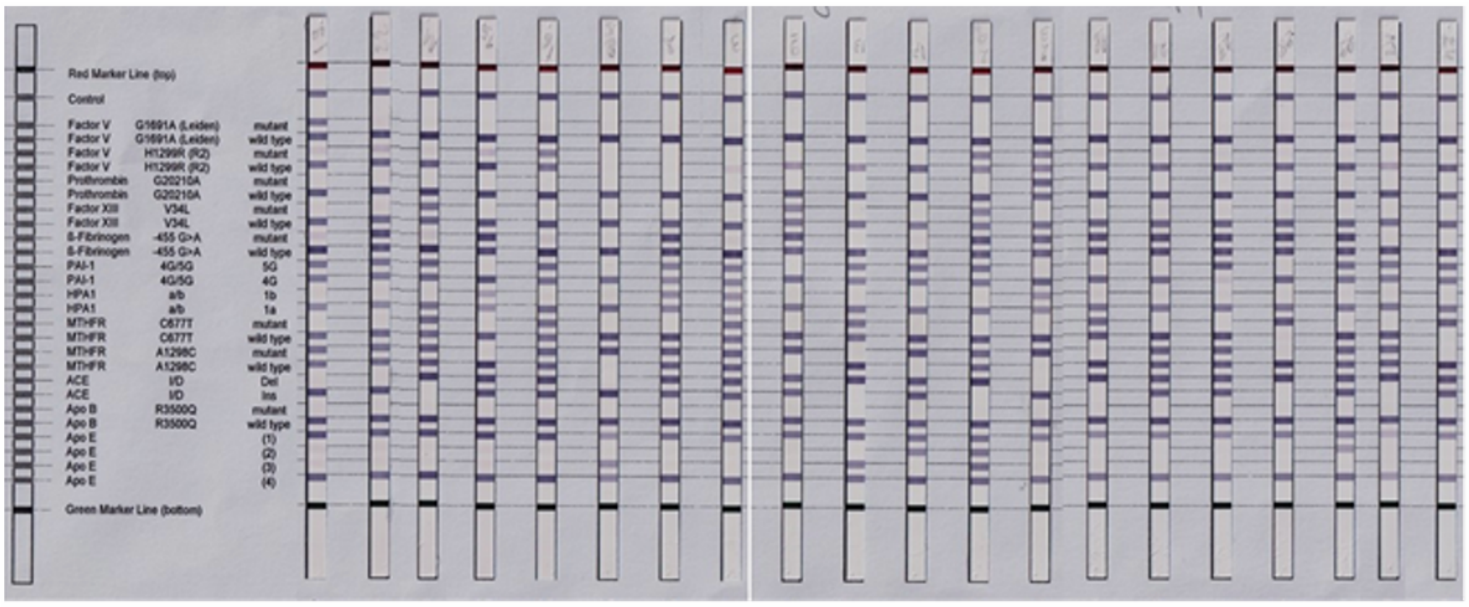


Figure 4

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

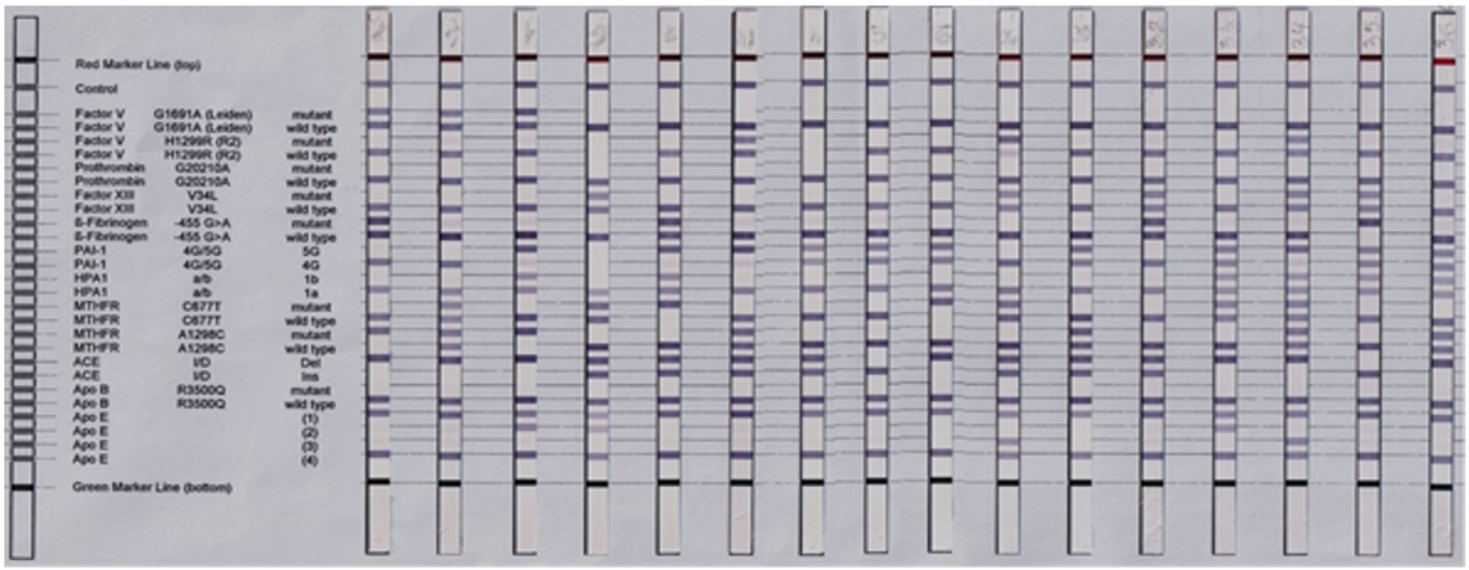


Figure 5

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

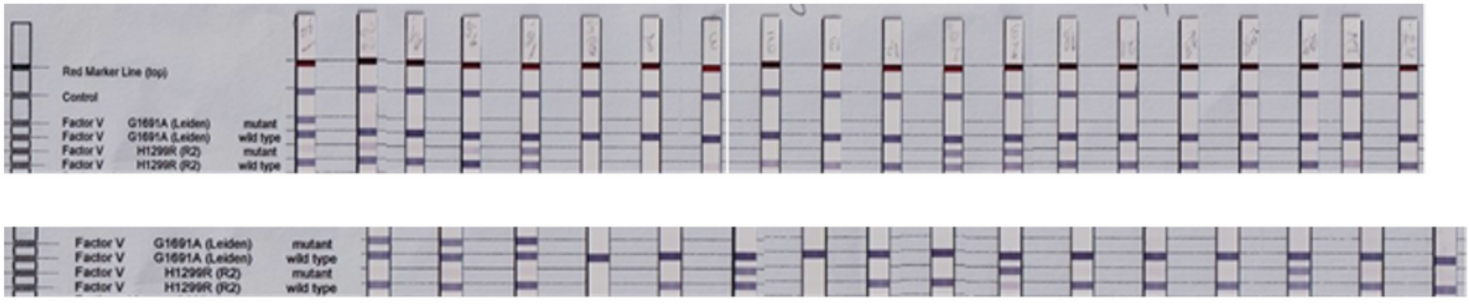


Figure 6

Results from the genotyping of FV G1691A (Leiden) and FV H1299R (R2) in patients with DM with CVD (top) and without CVD (bottom)

Figure 7

Results from the genotyping of Prothrombin G20210A in patients with DM with CVD (top) and without CVD (bottom)

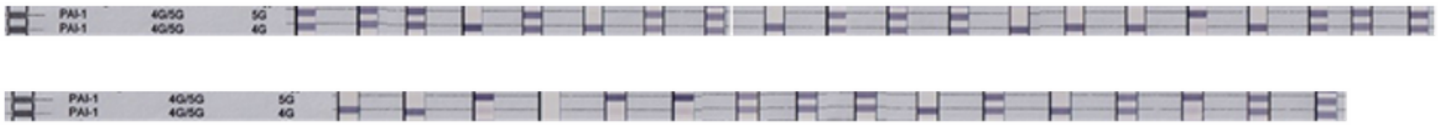


Figure 8

Results from the genotyping of PAI-1 4G/5G in patients with DM with CVD (top) and without CVD (bottom)

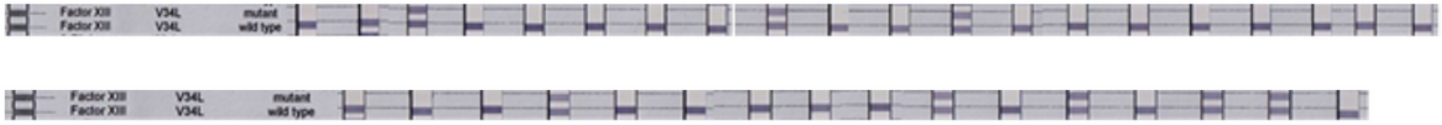


Figure 9

Results from the genotyping of Factor XIII V34L in patients with DM with CVD (top) and without CVD (bottom)

Figure 10

Results from the genotyping of β-Fibrinogen -455 G/A in patients with DM with CVD (top) and without CVD (bottom)

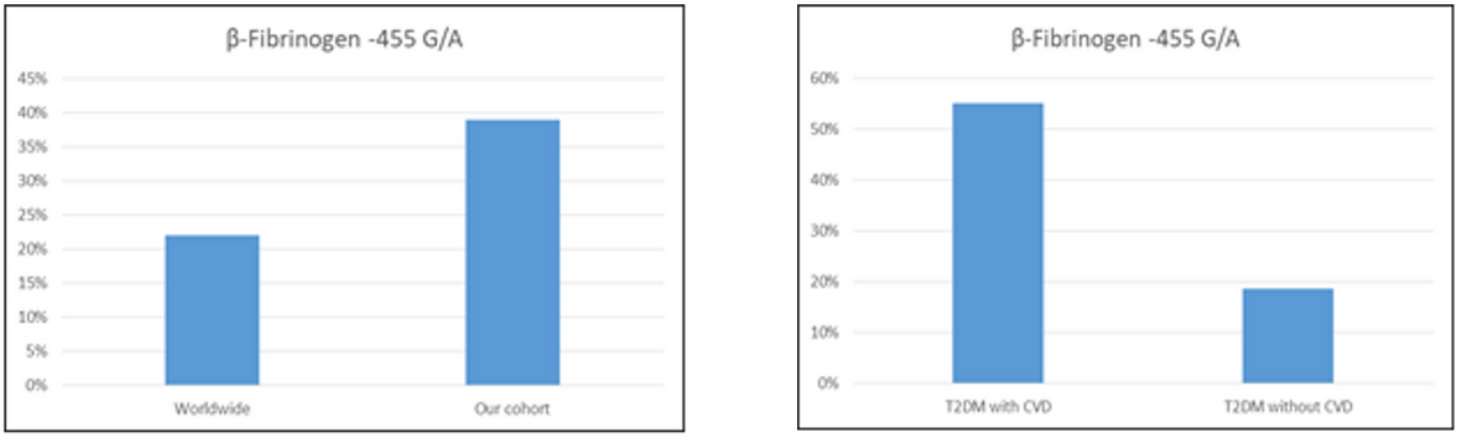
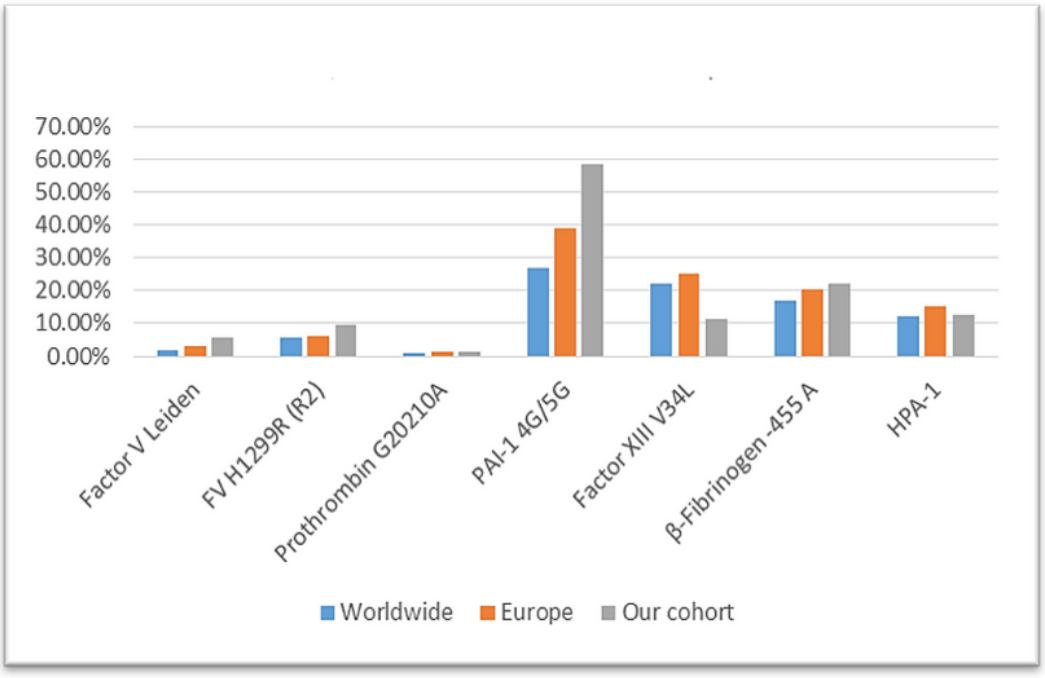


Figure 11

Frequency of the heterozygotes of the mutation for fibrinogen

Figure 12

Results from the genotyping of GPIIIa L33P (HPA-1) in patients with DM with CVD (top) and without CVD (bottom)



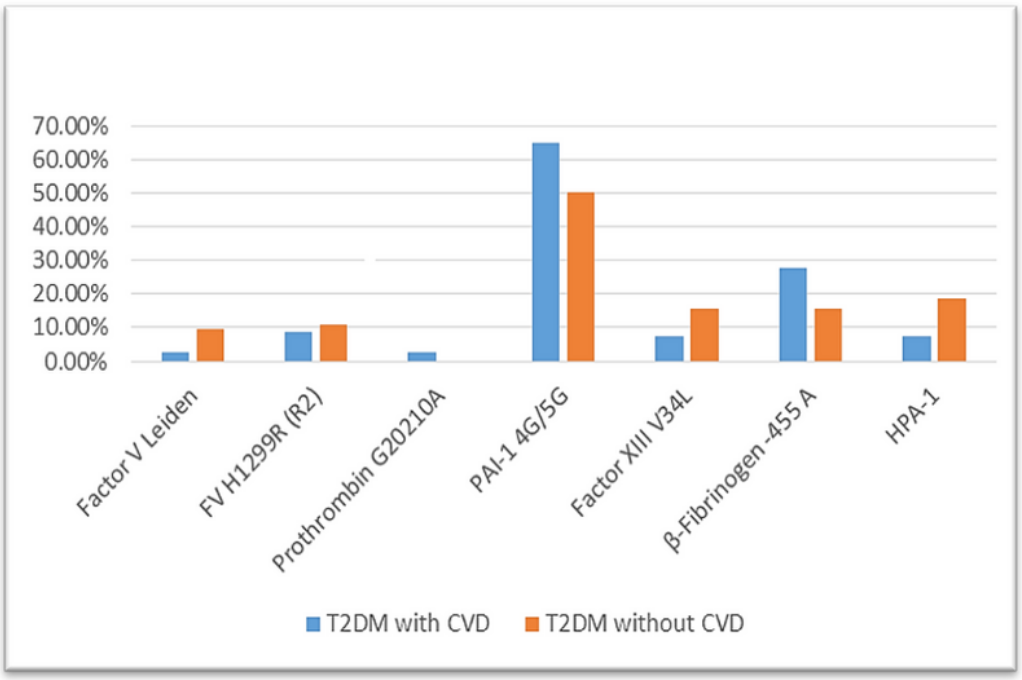


Figure 13

Frequencies of the studied variants of congenital thrombophilia in the different groups and populations.

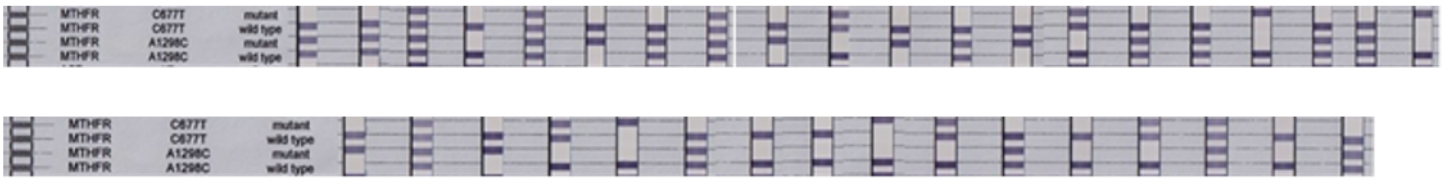


Figure 14

Results from the genotyping MTHFR C677T и MTHFR A1298C in patients with DM with CVD (top) and without CVD (bottom)

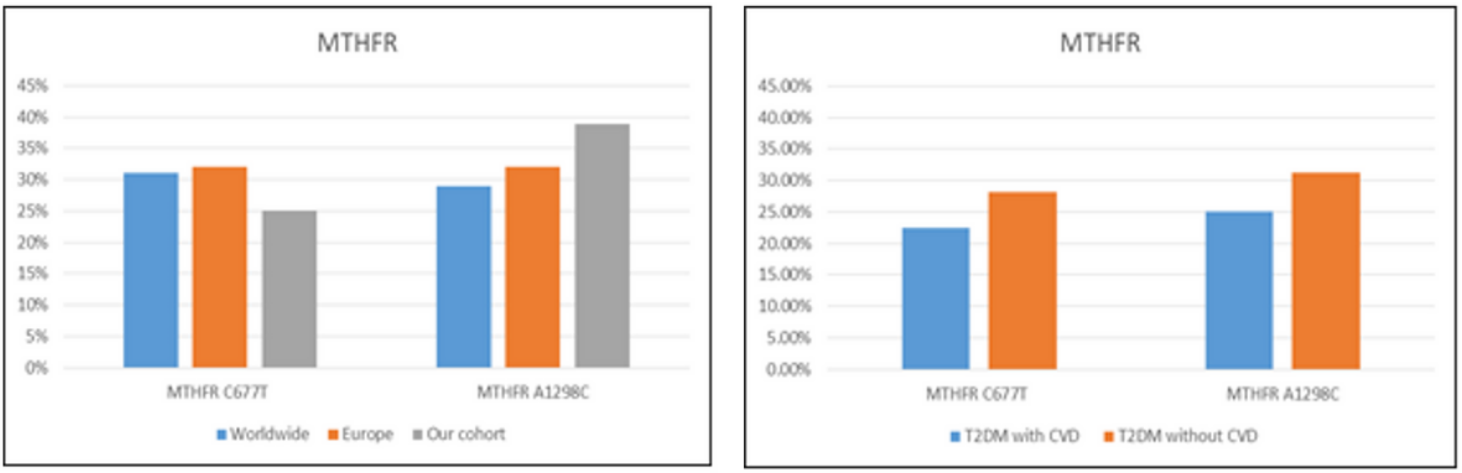


Figure 15

Allelic and genotypic frequencies of MTHFR C677T и MTHFR A1298C

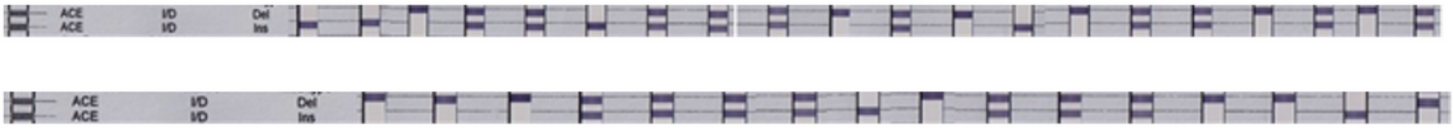


Figure 16

Results from the genotyping ACE I/D in patients with DM with CVD (top) and without CVD (bottom)

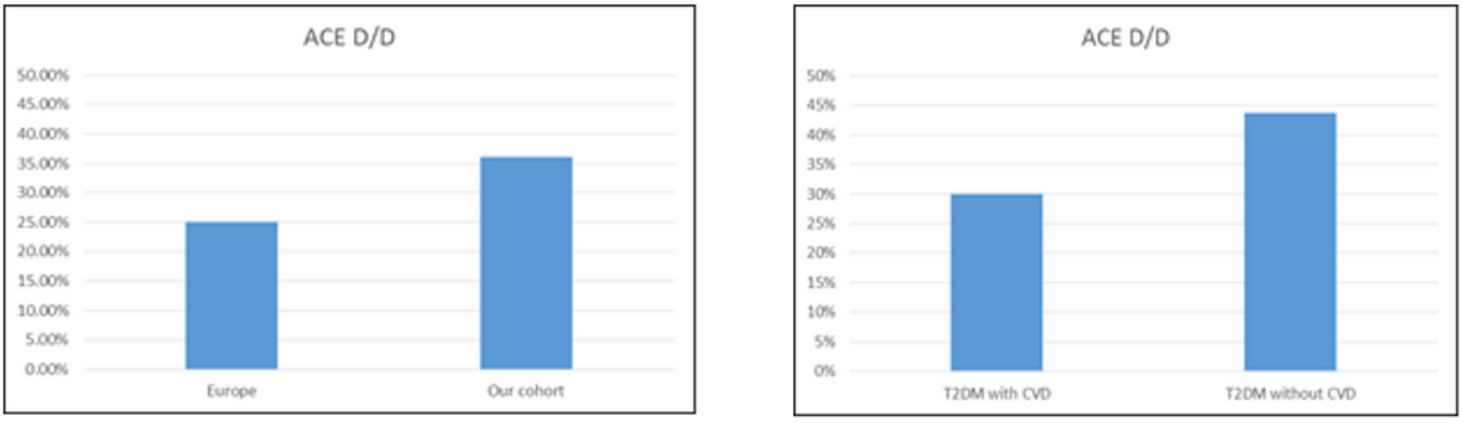


Figure 17

Allelic and genotypic frequencies of ACE I/D

Figure 18

Results from the genotyping Apo B R3500Q in patients with DM with CVD (top) and without CVD (bottom)

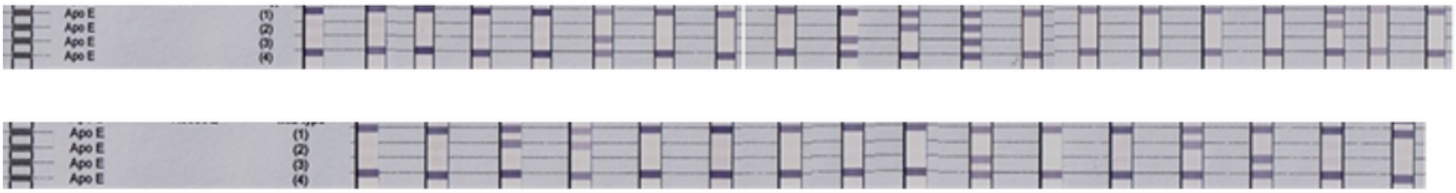
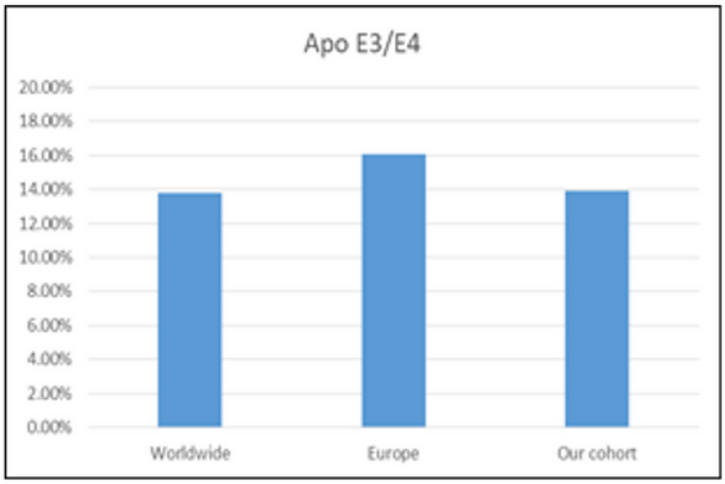


Figure 19

Results from the genotyping Apo E2/E3/E4 in patients with DM with CVD (top) and without CVD (bottom)



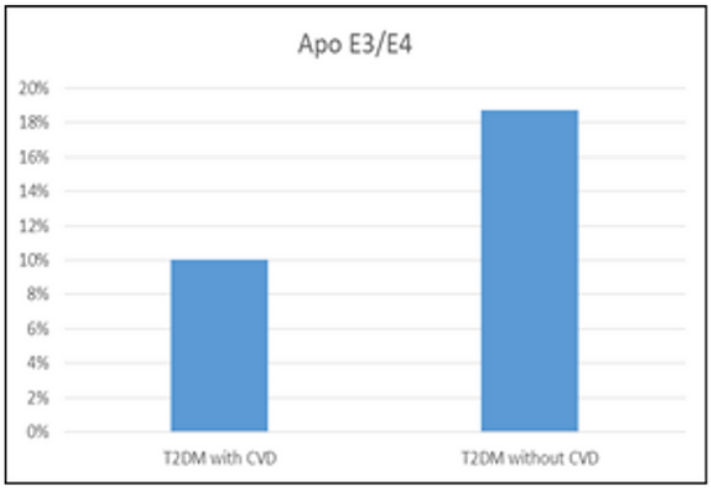


Figure 20

Allelic and genotypic frequencies of Apo E2/E3/E4