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Research Article

Association of PAI-1 4G/5G polymorphism with thrombotic risk in Sudanese patients with type 2 diabetes

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Introduction: The symptoms of hypercoagulability and hypofibrinolysis, which are both fatal and insignificant causes of death in this population, diabetes type 2-related mortality is linked to thrombotic complications, particularly cardiovascular ones. These complications can cause excessive fibrin deposition and accumulation within vessels, as well as the onset of thrombosis. The frequency of the 4G genotypes of the 4G/5G polymorphism of the PAI-1 gene in diabetic patients from Sudan remained unknown. The purpose of this study was to ascertain the genotype frequency of the PAI-1 gene's 4G/5G polymorphism and the relationship between this allele and thrombotic complications in individuals with type 2 diabetes from Sudan.

Methods: A case-control study with 50 healthy individuals serving as the control group and 70 diabetic patients. The polymorphism 4G/5G was genotyped using ASP-PCR (allele specific PCR), and the Hardy-Weinberg rule was used to determine the allelic frequency. For the 4G allele as a risk factor of thrombosis in diabetic patients, the allelic frequencies were determined using gene counting using the SNP-STAT program, and their connection with thrombotic complications was assessed using the X2 test and the odd ratio with (confidence intervals 95% and OR ≥1).

Results: The frequency of the 4G allele was significantly associated with the risk of thrombosis in diabetic patients, one-fold higher than that of the 5G allele (P value 0.027, CI=95%, OR =1), and there was no statistically significant difference in the frequency of the 4G allele among Sudanese diabetic patients compared to the control group (P value = 0.998).

Conclusion: Patients with type 2 diabetes who contain one 4G allele are at a high risk of thrombosis. Therefore, diabetic patients should be prescribed anticoagulant medications, namely type 2, for at least the short term to prevent thrombus formation, particularly cardiovascular events.

Keywords: *4G/5G polymorphism, Diabetic mellitus type 2, PAI-1 gene, mortality, Sudan*

Introduction

The PAI-1 gene, which spans 12.3 kb and has eight introns and nine exons, is found on human chromosome 7q21.3-22 (Eriksson et al., 1995; Ibrahim et al., 2022). It is the primary physiological inhibitor of tissue-type plasminogen activator (tPA) in the fibrinolytic system, which converts plasminogen into active plasmin, which cleaves fibrin. It is a member of the serine protease inhibitor (serpin) family. Patients with thrombotic illness frequently exhibit impaired fibrinolytic activity due to elevated PAI-1 expression (Chevilley et al., 2015; Mohamedahmed & Ibrahim, 2022). Several PAI-1 polymorphisms have been previously reported by different researchers to cause an increase in PAI-1 levels. The PAI-1 (rs1799889) -675 4G/5G insertion/deletion polymorphism at -675 in the promoter region has been described the most frequently to date (Parpugga et al., 2015). Two alleles containing four or five consecutive guanosines (4G and 5G) are produced by this polymorphism, and they differ in how they regulate the concentration of PAl-1 (Nordt et al., 2001). The plasma PAI-1 concentrations of subjects homozygous for the 4G allele are around 25% higher than those of subjects homozygous for the 5G allele (4G allele transcribes PAI-1 six times more than 5G allele). Young diabetic patients may be at higher risk for intravascular thrombosis and recurrent myocardial infarction (MI) due to the interaction of a particular PAI-1 genotype with Metabolic Syndrome factors, including plasma triglycerides, high density lipoprotein, plasma insulin, and visceral fat; circumference width, and lastly body mass index (Aburto-Mejía et al., 2017; Khalaf et al., 2019). The incidence of the 4G/5G polymorphism and its correlation with complications from type 2 diabetes are unknown in the Sudan, and the genetic expression and polymorphisms of PAI-1 remain poorly studied (Khalaf et al., 2019). The development of venous and arterial thrombosis may be linked to impaired fibrinolysis or hypofibrinolysis, which can be brought on by environmental or genetic factors. It has also been linked to atherosclerosis, obesity, diabetes, and hyperlipidemia (Almakey et al., 2021; Ibrahim et al., 2021).

Researchers have examined global assays for fibrinolysis capacity biomarkers that would suggest decreased fibrinolysis, such as changes in active t-PA levels or elevated plasminogen activator inhibitor-1 (PAI-1), a significant hypofibrinolytic marker, alpha-2-antiplasmin (Plasmin Inhibitor), and thrombin activatable fibrinolysis inhibitor (TAFI) (Longstaff, 2018). While high amounts of PAI-1 hinder the fibrinolytic process, high levels of fibrinogen are linked to a more compact clot formation. It has been demonstrated that elevated serum glucose causes fibrinogen to become more glycated, and glycated fibrinogen clots have a more compact shape and are more resistant to lysis. Recent research has demonstrated that the fibrinolytic process is hampered by post-translational alteration in fibrinogen caused by glycoaldehyde, a byproduct of protein glycation (Alzahrani & Ajjan, 2010). Young people (less than 45 years old) who have survived myocardial infarction and those who have experienced recurrent MI have higher levels of PAI-1 protein, which is linked to an increased risk of CVD in diabetes. PAI-1, which is generated by adipose tissue and endothelial cells, has long been thought to be the primary inhibitor of fibrinolysis in diabetes. Elevated levels of PAI-1 in this cohort appear to be related to hormonal (hyperinsulinemia) and metabolic (hyperglycemia and hypertriglyceridemia) abnormalities, which are commonly observed in T2DM patients (Schneider & Sobel, 2012). Prior research showed that hyperglycemia promotes coagulation while hyperinsulinemia suppresses fibrinolysis in healthy individuals, mainly by increasing PAI-1 production. This helps to explain why obesity and type 2 diabetes together raise PAI-1 considerably more than either condition alone (Kearney et al., 2017). Patients who are at high risk of occlusive vascular events have benefited considerably from antiplatelet therapy with aspirin since the 1970s.

The antiplatelet medications that are currently on the market, however, vary in their efficacy. According to clinical data, aspirin resistance was considerably more common in patients who had previously experienced an ischemic attack or stroke, and those who did not respond to aspirin had a ten-fold higher risk of repeat vascular events than those who were aspirin-sensitive. Finding viable strategies for improving antiplatelet treatments that prevent platelet-mediated atherothrombosis would be crucial from a clinical standpoint. In order to better identify patients and choose antiplatelet drugs that may be able to lower plasma PAI-1 levels for improved stroke prevention, plasma PAI-1 levels and activity may therefore be useful biomarkers (Tjärnlund-Wolf et al., 2012).

Materials and methods

Research design: To ascertain the 4G allele frequency of the PAI-1 gene in individuals with diabetes, an analytical case control study was conducted. Between June 2020 and January 2021, 70 patients in Wad Medani, Gezira state in central Sudan, visited the *Aldaraja* Health Center's Diabetic Clinic and were diagnosed with diabetes based on their HbA1c and Glucose Tolerance Test (GTT). Both men and women with type 2 diabetes who were under control and those who were not were chosen. They ranged in age from 28 to 86. A thorough questionnaire that included clinical data and patient

demographics was used to gather their medical history and personal information. As controls, 50 non-diabetic individuals who were matched with cases and were seen as outpatients at the *Aldaraja* Health Center's diabetic clinic served as controls.

Ethical consideration: The Diabetic Clinic at *Aldaraja* Health Center provided ethical permission, and the Ministry of Health in Gezira State's local Research Medical Ethics Committee (REC) granted ethical approval.

Study population: Patients with diabetes of different kinds and those under the age of eighteen make up the study population. Additionally excluded were patients who were pregnant, taking contraceptive pills, had neuropathy or nephropathy, smoked, were on standard anticoagulant medication at the time of admission (either heparins or coumarin derivatives), had liver disease, or had hypertension prior to diabetes mellitus.

Sample collection and preparation: Following a 12-hour fast, 6 ml of venous blood was drawn from each participant using an aseptic technique. To estimate HbA1c, 3 ml of blood was submerged in 0.75 ml of EDTA tube. The fine care rapid quantitative test was performed using the principal fluorescence immunoassay technology, and the sample was stored at -20 °C for DNA extraction. After centrifuging the sample at 2000 rpm for five minutes, the blood sample was submerged in a lithium heparin tube for the estimation of FBG using the spectrophotometric technique and SPINREACT reagent (Bain et al., 2016).

DNA extraction: The G-DEX™IIb Genomic DNA Extraction Kit [for Blood] from Intron was used to separate genomic DNA from whole blood leucocytes. Using a GenQant photometer, DNA was quantified by diluting the DNA (10 µl DNA to 90 µl nuclease-free water), vortexing it for 15 seconds, and letting it sit at room temperature for 10 minutes to homogenize it. The diluted DAN was then read at 260 nm, the protein at 280 nm, and the DNA yield was automatically calculated; the samples' mean ratio was 1.58.

Genotyping of 4G/5G polymorphism of *PAI-1* gene: Study participants and controls were screened for 4G/5G polymorphisms (rs179989 insertion/deletion) in the -675 promoter rejoin using Allele Specific Primer PCR. Two primers for internal control and three primers for the ASP-PCR genotyping technique of 4G/5G polymorphisms (rs 179989 insertion/deletion). Sequence of primers Internal controls for HB1 and HB2 are 5' CAACTTCATCCACGTTCACC '3 and 5'GAAGAGCCAAGGACAGGTAC '3, respectively. Primers downstream for 4G/5G polymorphism genotyping 4G specific primer 5'TGCAGCCAGCCACGTGATTGTCTAG'3 The particular primers 5'GTCTGGACACGTGGGGA'3 and 5G Fifth, 'GTCTGGACACGTGGGGG'3 (Falk et al., 1995).

Master mix preparation: Add Taq PCR Master Mix to the master mix that was utilized. (2x conc.) 5 U/µl of Taq DNA Polymerase, 1.0 mL of APSTP1100A, 20 mM of Tris-HCl (pH8.8) PCR, 100 mM of KCl, 0.2% of Triton® X-100, 4 mM of MgCl2, 0.5 mM of dNTP, and 1x protein stabilizer sediment loading dye (Falk et al., 1995).

PCR temperature profile for 4G allele: initial denaturation at 94ºC for 5 minutes, denaturation at 94ºC for 1 minute, annealing at 58ºC for 45 seconds, and extension at 72ºC for 45 seconds for 35 cycles. Hold at 4ºC∞ for detection of 138 bp after final extension at 72ºC/5 min for 1 cycle (Falk et al., 1995).

PCR temperature profile for 5G allele: 94ºC for 5 minutes of initial denaturation, 94ºC for 1 minute of denaturation, 58ºC for 45 seconds of annealing, and 72ºC for 45 seconds of extension for 40 cycles Final Extension: Hold at 4ºC∞ for 1 cycle at 72ºC/5 min (Falk et al., 1995).

PCR Methods: Each sample underwent two PCR reactions, one for each allele. Both the upstream (HB1) and downstream (HB2) primers produced a 264-bp control band. 138-bp bands were amplified by each allele-specific primer and downstream primer. For the 4G allele-specific reactions, 4G/4G homozygotes produced 268- and 138-bp bands, while for the 5G allele-specific reaction, they produced a 268-bp band. 5G/5G homozygotes showed 268-bp bands for the 4G allele-specific reaction and 268- and 138-bp bands for the 5G allele-specific reaction, while 4G/5G heterozygotes had 268- and 138-bp bands for both reactions (Falk et al., 1995).

Agarose gel electrophoresis: 1.5 µl of ethedium bromide was added to 10 ml of agarose gel after 2.5% of the gel had been dissolved in 100 ml of 1x Tris- borate EDTA buffer. Gel wells were filled with 15 µl of PCR product and 5 µl of 100 bp DNA ladder, which included bromophenol blue dye and was used as a visual aid to track the migration process during

agarose gel electrophoresis. The gel was then run at 120 V for 25 minutes. The product was then visualized using a transilluminator under UV light, and the soft wear program took a picture of it and saved it.

Data analysis: Version 21 of the Statistical Package for Social Sciences (SPSS) was used to do the statistical analysis. The ≃2analysis was used to examine the significance of the variations in genotypes, alleles, and their relationship to thrombotic complications between the diabetes patient and control groups. Gene counts using the SNP-STAT tool were used to determine the allelic frequencies, and genotypes were assessed. All PAI-1 genotype's observed numbers were compared to what would be predicted for a population in Hardy-Weinberg equilibrium $(p+q)^2 = p^2+2pq+q^2 = 1$, where p is the frequency of allele 4G and q is the frequency of allele 5G. Additionally, the 4G allele's odds ratio (OR ≥1 and 95% CI) was employed as a risk factor for thrombosis in diabetes patients.

Results

A total of 120 samples were taken, 70 for diabetic patient type 2 and 50 healthy individuals as control group (Table 1).

Table 1. Demographical data of the study groups case and control

Demographics data	Diabetic patients	Control subjects
Number (n) **%**	70 58.3%	50 41.7%
Mean Age / years	65.2	33.7
Gender **Male** **Female**	 41.4% 58.6%	 32% 68%
Mean BMI	28.1	26.3

The genotype distribution of 4G/5G polymorphism of PAI-1 gene in the diabetic patients type 2 group and control subjects were 4G/4G, 6% (n = 4 and 3), 4G/5G, 44% (n = 31 and 22), and 5G/5G, 50% (n = 35 and 25) respectively (Table 2).

Table 2. Distribution of genotyping of 4G/5G polymorphism of PAI-1 gene among study population

Genotype frequencies (n=120)						
	All subjects		Control		Diabetic patients	
Genotype	**Count**	**Proportion**	**Count**	**Proportion**	**Count**	**Proportion**
4G/4G	7	0.06	3	0.06	4	0.06
5G/4G	53	0.44	22	0.44	31	0.44
5G/5G	60	0.5	25	0.5	35	0.5

There were no statistical differences in genotyping distribution when compared the diabetic patients with control group for 4G/5G polymorphism of PAI-1 gene (P value = 0.998) (Table 3).

Table 3. Cross-tabulation for genotyping 4G/5G polymorphism of PAI-1 gene in case and control

Model	Genotype	Control	Diabetic patients	P value
Co-dominant	**5G/5G**	25 (50%)	35 (50%)	0.998
	4G/5G	22 (44%)	31 (44.3%)	
	4G/4G	3 (6%)	4 (5.7%)	

Distribution of 4G/5G polymorphism of PAI-1 gene according to thrombotic complication in diabetic patient type 2, diabetic patient without thrombosis and control group, there were no diabetic patient with thrombosis had 5G/5G genotype, and the most of them had 4G/5G genotype (Figure 1).

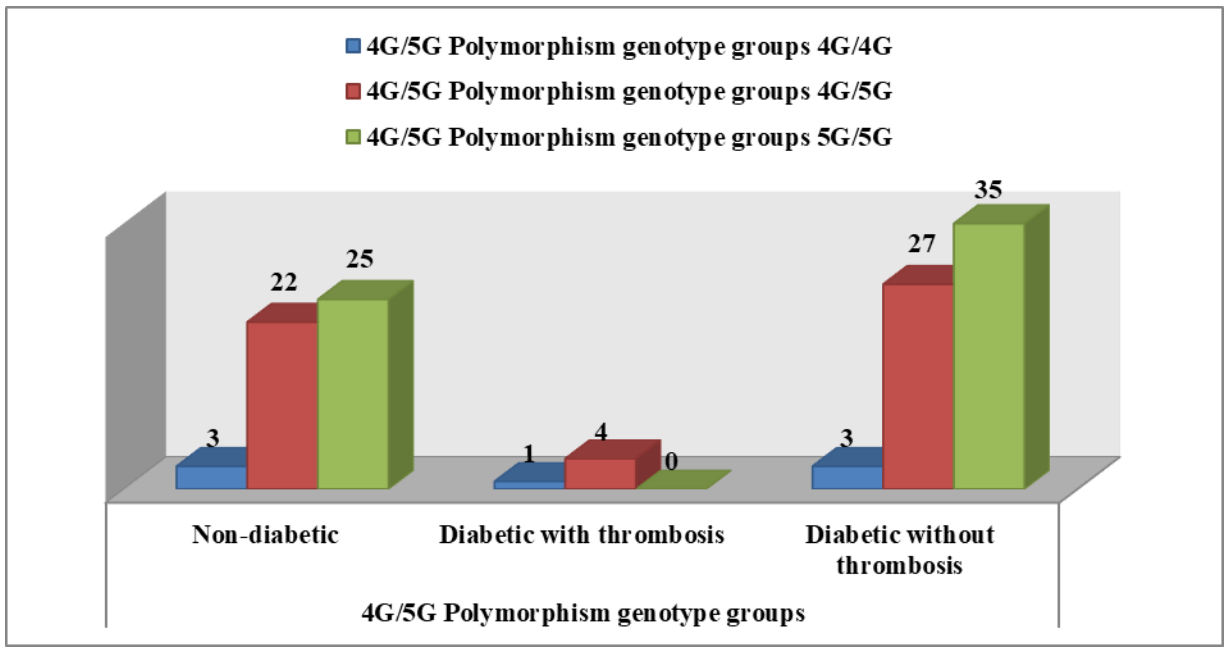

Figure 1. Distribution of 4G/5G polymorphism among diabetes with and without thrombotic complication and control

Distribution the alleles of 4G/5G polymorphism according to thrombotic complication in diabetic patient type 2, diabetic patient without thrombosis and control group; the highest 4G allele was found in the diabetic patients with thrombosis (60%) (Figure 2).

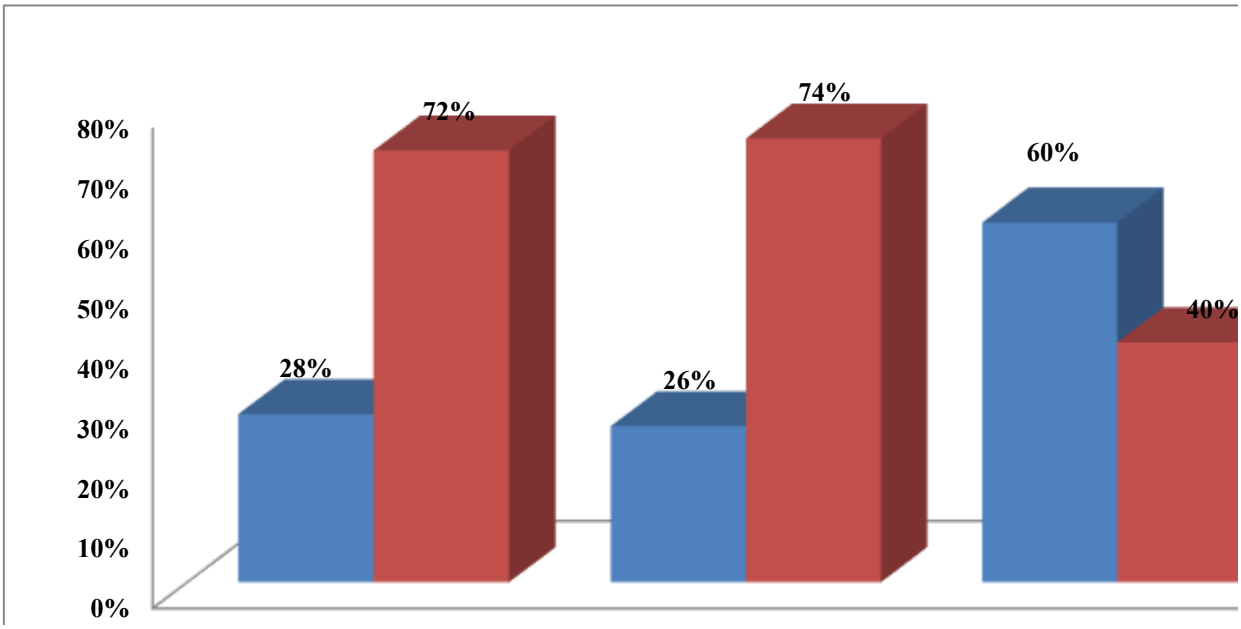

Figure 2. Distribution the allele of 4G/5G polymorphism among diabetes with and without thrombotic complication and control

In both diabetic with and without thrombosis the frequency of the 4G allele had significant association to the risk of thrombosis in diabetic patients one-fold as compare to 5G allele (P value = 0.027, OR = 1) as shown in (Table 4).

Table 4. Cross-tabulation for the presence of thrombosis and 4G allelic frequency

Present of thrombosis	4G/5G alleles		Total	P value	OR (CI 95%)
	4G allele	5G allele			
No					
Count	30	35	65		
% Within Present of thrombosis	46.2%	53.8%	100.0%		
% Within 4G and 5G	85.7%	100.0%	92.9%		
% Of Total	42.9%	50.0%	92.9%		
Yes					
Count	5	0	5		
% Within Present of thrombosis	100.0%	0%	100.0%	0.027	1
% Within 4G and 5G	14.3%	0%	7.1%		
% Of Total	7.1%	0%	7.1%		
Total Count	35	35	70		
% Within Present of thrombosis	50.0%	50.0%	100.0%		
% Within 4G and 5G	100.0%	100.0%	100.0%		
% of Total	50.0%	50.0%	100.0%		

In both diabetic groups with and without thrombosis, the 4G/5G genotype had significant association of thrombosis among the diabetic patients as compare to 5G/5G genotype (P value = 0.021), whereas patients with 4G/4G genotype hardly had significant association as compare to 5G/5G (P value = 0.046) (Table 5).

Table 5. Cross-tabulation for the presence of thrombosis and 4G/5G genotype frequency of PAI-1 gene in diabetic patients

4G/5G Polymorphism genotype groups		Present of thrombosis		Total	P value
		No	Yes		
4G/4G	Count	3	1	4	0.046
	% Within 4G/5G Polymorphism genotype groups	75.0%	25.0%	100.0%	
	% Within Present of thrombosis	4.6%	20.0%	5.7%	
4G/5G	Count	27	4	31	0.021
	% Within 4G/5G Polymorphism genotype groups	87.1%	12.9%	100.0%	
	% Within Present of thrombosis	41.5%	80.0%	44.3%	
5G/5G	Count	35	0	35	Ref
	% Within 4G/5G Polymorphism genotype groups	100.0%	0%	100.0%	
	% Within Present of thrombosis	53.8%	0%	50.0%	
Total	Count	65	5	70	
	% Within 4G/5G Polymorphism genotype groups	92.9%	7.1%	100.0%	
	% Within Present of thrombosis	100.0%	100.0%	100.0%	

Discussion

New methods to lower cardiovascular morbidity and mortality in individuals with diabetes mellitus, particularly type 2, may be developed with a better understanding of the mechanisms causing vascular thrombosis rather than traditional considerations. This study's risk allele 4G among Sudanese type 2 diabetic patients did not differ statistically from the control group (P value = 0.998), and its prevalence was higher than that of Aburto-Mejía's et al. (2017) study of diabetes in Mexico, which included three different African ethnic groups. The frequency of the 4G allele was lower in Africans (13%), compared to Indian and white people (54% and 58%, respectively) (Aburto-Mejía et al., 2017). Around 15% of people have the 4G allele, in South Africa (de Lange et al., 2013). However, the frequency of the 4G allele was 54.7% in the diabetes patients in Egypt (Khalaf et al., 2019). According to Festa et al., 2003; Naran et al., 2008, the most common

genotypes of the 4G/5G polymorphism of PAI-1 in Sudanese diabetic patients and controls were 5G/5G 50%, 4G/5G 44%, and finally 4G/4G 6%. The latter genotype was higher than the African population by 2.6%. Due to ethnic differences, these studies confirmed that the prevalence of the 4G allele and homozygous 4G genotype was lower in African diabetic groups and higher in Sudanese communities, but not higher than in Egyptian diabetic populations. In accordance with Khalaf et al. (2019) and the study by Zhao & Huang, 2013 reported that the most common genotype among diabetic patients with thrombotic complications with statistical difference was 4G/5G (80%) (P value = 0.021), followed by 4G/4G (20%) (P value = 0.046) and 5G/5G 0%. These findings indicated that the PAI-1 4G/5G polymorphism was significantly associated with type 2 DM risk, and that circulating PAI-1 levels could predict the progression of type 2 DM to thrombosis (Khalaf et al., 2019; Zhao & Huang, 2013). Khalaf et al. (2019) also reported that the allelic frequencies of the 4G and 5G alleles were 60% and 40%, respectively, in diabetic patients with thrombosis; in diabetic patients with vascular complications, the allelic frequencies were 62.0% and 38%, respectively. The PAI-1 (4G/5G) polymorphism may not be a risk factor for diabetes mellitus, diabetic retinopathy, diabetic nephropathy, or diabetic coronary artery disease, according to a meta-analysis, which contradicts our findings (Xiao et al., 2011). Along with the traditional risk factors for thrombosis, this study discovered that patients with type 2 diabetes who carried the 4G allele were one-fold more likely to experience thrombosis than those who carried the 5G allele (P value = 0.027, OR = 1 and CI 95%). This finding is consistent with a study conducted by Khalaf et al., (2019) in diabetic patients in Egypt (P value = 0.007, OR = 3 and CI 95%).

Conclusion

Having one 4G allele increased the risk of thrombosis in diabetic patients with type 2 diabetes because the 4G/5G and 4G/4G genotypes are substantially linked risk factors for thrombosis. In Sudanese patients with type 2 diabetes, 4G/5G genotypes are more likely than 4G/4G genotypes to experience thrombotic complications. In order to bolster the results of this investigation, we suggested that additional case control studies involving a sizable sample of diabetic patients with thrombosis be conducted. The PAI-1 polymorphism may be helpful in future studies to develop fresh approaches to the early detection and management of these issues. Additionally, it might aid in the selection and dosage of patients receiving safe anticoagulant and fibrinolytic medication.

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Author contributions

Each author made a significant intellectual contribution, reviewed and approved the final manuscript version, and consented to take responsibility for all elements of the work.

Conflict of interest

The authors declare no conflict of interest.

Ethics approval

Ethical approved was obtained from the Research Ethics Committee, Faculty of Medical Laboratory Sciences, University of Gezira (15-2-2020).

Ethical concern and Informed consent

All participants provided written informed consent, and the study followed the Declaration of Helsinki ethical guidelines.

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