Research paper

Factor XIII-A Val34Leu and Tyr204Phe variants influence clot kinetics in a cohort of South African type 2 diabetes mellitus patients

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Highlights

- A significant association between the Val34Leu polymorphism and T2DM was found.
- The 204Phe variant was more prevalent in controls compared to T2DM.
- A hypo-coagulable clot profile but stronger clot of platelet poor plasma was observed in T2DM.
- Higher clot strength and resistance was observed in the presence of the 34Leu variant.
- The presence of the 204Phe variant in T2DM influenced clot kinetics.

Abstract

Factor XIII, a transglutaminase that plays a crucial role in clot formation, consists of subunits A and B. Single nucleotide polymorphisms in Factor XIII-A have been linked to thrombotic risk. In Type 2 Diabetes mellitus (T2DM), a hypercoagulable state is thought to contribute to the high mortality rate associated with thrombotic diseases. Due to the lack of prevalence data of FXIII-A single nucleotide polymorphisms (SNPs) in T2DM in a South African cohort, this study assessed the prevalence FXIII-A Val34Leu (rs5985) and Tyr204Phe (rs3024477) SNP's and the effect on clot kinetics in T2DM.

Materials and methods: A cohort of T2DM patients (n = 100) and race, age and gender matched healthy controls (n = 101) were recruited following ethical approval. Thromboelastography® (TEG®) was used to assess the viscoelastic properties in platelet poor plasma (PPP) in controls (n = 91) and T2DM patients (n = 91) younger than 50 years old. Genomic DNA was isolated from whole blood using the Quick-DNA™ Miniprep Plus Kit and PCR-RFLP was used to genotype each sample for FXIII-A rs5985 and rs3024477 SNPs.

Results: TEG® analyses indicated a longer R-time ($p < 0.0001$) and higher TMRTG ($p < 0.0001$) in PPP of T2DM patients. Control and T2DM genotype distribution conformed to Hardy-Weinberg equilibrium ($p > 0.05$). There was a higher prevalence of the wildtype genotype of FXIII-A Tyr204Phe (rs3024477) SNP in T2DM (OR = 0.23, 95% CI = 0.12–

0.42, $p < 0.0001$). The 204Phe variant was more frequent in the Caucasians (OR = 0.39, 95% CI = 0.05–0.33, $p < 0.0001$). The presence of the 204Phe variant in T2DM affected TMRTG ($p = 0.0207$). The variant affected R time ($p = 0.0432$) and TMRTG ($p = 0.0209$ and $p = 0.0207$) in controls and T2DM, respectively.

Conclusion: An inverse association with T2DM and FXIII-A Tyr204Phe was found. A hypo coagulable PPP clot profile was observed in T2DM. A shorter reaction time was observed and but faster rate at which the clot reached maximum strength in both controls and T2DM in the presence of the 204Phe variant.

Keywords: Factor XIII; Type 2 Diabetes Mellitus; Polymorphism; Coagulation

Abbreviations

Factor XIII, Factor thirteen; Factor XIII-A, Factor thirteen subunit A; T2DM, Type 2 Diabetes mellitus; Val, Valine; Leu, Leucine; VT, Venous thrombosis; SNP, Single nucleotide polymorphism; RPL, recurring pregnancy loss; Kcat/Km, catalytic efficiency; Pro, proline; Tyr, tyrosine; Phe, phenylalanine; gDNA, genomic deoxyribose nucleic acid; TEG, thromboelastography; WB, Whole blood; R, reaction time; K, clot kinetics; α-angle, alpha angle; MA, maximum amplitude; CI, coagulation index; Lys 30, lysis of clot within 30 min; MRTG, maximum rate of thrombus generation; TMRTG, time to maximum rate of thrombus generation; TTG, total thrombus generation; PPP, platelet poor plasma; PCR, polymerase chain reaction; RFLP, Restriction Fragment Length Polymorphism; dNTPs, deoxynucleotide triphosphates; bp, base pairs; RE, restriction enzymes; OR, odds ratio; HWE, Hardy-Weinberg equilibrium; NAFLD, non-alcoholic fatty liver disease; CVD, cardiovascular disease; PAI, plasminogen activator inhibitor; tPA, tissue plasminogen activator.

1. Introduction

Fibrin and activated Factor XIII play an important role in clot formation. Any alteration in these two coagulation factors influences the coagulable state (Ariëns et al., 2002). The hypercoagulable state is usually observed in T2DM is attributed to chronic hyperglycaemia. The hypercoagulability is thought to contributes to the high mortality rate caused by thrombotic disease as a complication in T2DM patients (Holman et al., 2008). Factor XIII is a transglutaminase, which is composed of two A-subunits and two carrier B-subunits (FXIII-A2B2). During FXIII activation, it is cleaved by thrombin, to separate the A and B-subunits (Ariëns et al., 2002). It plays a crucial role in the development of cardiovascular diseases and studies have demonstrated that factor XIII is involved in deep vein thrombosis due to its high expression levels (Kool, 2017). Factor XIII-A mRNA found in platelets and monocytes indicating potential for *de novo* synthesis of FXIII-A in these cell for fibrin cross-linking and protein in the α-granules of platelets, and it is also found in megakaryocytes and tissue macrophages (Pitkänen et al., 2017).

Protein function such as catalytic activity, ligand binding or DNA binding properties maybe impaired if there is a single nucleotide polymorphism in the coding region of a gene. Genetic variations in genes that encode coagulation factors play a significant role in the

pathophysiology of diseases. The most commonly found and studied include: thrombophilic gene polymorphisms include factor V Leiden (rs6025), prothrombin gene G20210A (rs1799963), fibrinogen gamma 10034 > T (rs2066865) and FXIII-A Val34Leu located on exon 2c.103 G > T (rs5985) (Tinholt et al., 2016). These variants increases or decreases the risk of a venous thrombosis (VT) (Tinholt et al., 2016). The prothrombin G20210A and fibrinogen gamma increases the risk of developing VT, while FXIII Val34Leu has been shown to reduce the risk of developing VT (Uitte de Willige, 2005, Van Hylckama Vlieg et al., 2002).

There have been five common SNPs found for FXIII-A where there is an amino acid exchange and Val34Leu is commonly found in high frequencies in the Caucasian population (Mezei, 2017). The presence of this SNP increases the rate at which factor XIII is activated, and affects how the fibrin network forms (Wartiovaara, 2000).

The effect of FXIII Val34Leu has been investigated in recurring pregnancy loss (RPL), with no link found in European and South American populations (Jung, 2017). However, there was an association found in the Asian population (Jung, 2017). FXIII deficiency has been associated with severe bleeding disorders and the risk of having miscarriage in female individuals (Weger et al., 2001). Several studies have shown that there is an association between the FXIII Val34Leu variant and the reduced risk of developing strokes, myocardial infarctions and deep vein thrombosis, however it is also associated with the risk of having intracerebral haemorrhage (Weger et al., 2001, Catto, 1999, Fransco, 1999). One study demonstrated that there is a protective effect of the FXIII Val34Leu variant against the development of arterial thrombosis (Weger et al., 2001).

A study conducted by de Lange et al. (de Lange et al., 2006) demonstrated that the FXIII-A Val34Leu variant plays an important role in the activation of FXIII, by assessing the 6 different variants that can be found in the FXIII A-subunit and their effect to FXIII activation (de Lange et al., 2006). Studies have suggested that the FXIII-A Val34Leu variant has an effect on the activation of thrombin, as it reduces the stability of the fibrin and the cross linking of other coagulation factors (Hancer et al., 2006, Ariëns et al., 2000). Ariens *et al.* (2000) not only showed microscopic changes in clot structure but also that FXIII 34Leu was cleaved by thrombin more rapidly and by lower doses than 34Val and kinetic analysis of activation peptide release showed that the catalytic efficiency (kcat /Km) of thrombin was reduced by 0.3 $(\mathrm{mmol/L})^{-1} \times sec^{-1}$ for FXIII 34Leu than for 34Val (Ariëns et al., 2000). Thus, factors such as gene-gene interaction, heterogeneous genetics and environmental backgrounds need to be taken into consideration as they play a key role in the presence of a SNP variant and a disease (Hancer et al., 2006).

Other FXIII subunit-A SNP variants are Tyr204Phe located on exon 5 (rs3024477) and Pro564Leu (rs5982) located on exon 8 (Naderi, 2016). These variants are associated with a decrease in the plasma levels of FXIII (Reiner, 2001). Furthermore, it is suggested that Pro564Leu is involved in the reduced specific activity of FXIII (Reiner, 2001). However, in other studies this Pro564Leu SNP variant is also shown to increase the activity of FXIII (Anwar, 1999). Reiner *et al.* suggested that the Tyr204 and 564Leu variants can be used as genetic marker of haemorrhagic stroke in women (Reiner, 2001); and Pruissen *et al.* found a strong association between the Tyr204Phe variant and the risk of young women having ischaemic strokes (Pruissen, 2008), and it increases the risk by 9-fold (Naderi, 2016).

It is estimated that there are 425 million type 2 diabetes mellitus (T2DM) individuals worldwide (Ogurtsova et al., 2017). >16 million are individuals from Africa, which is estimated to increase to 41 million by the year 2045 (Ogurtsova et al., 2017). In South Africa there were 1 826 100 T2DM cases in 2017 (Ogurtsova et al., 2017). T2DM is characterized by the combination of insulin resistance (due to the insufficient insulin production response), hyperglycaemia, inflammation, obesity, central adiposity, hypertension, hypertriglyceridemia and hypoalphalipoproteinemia (Hancer, 2002, Patani et al., 2018).

Considering the lack of scientific evidence on the presence of FXIII-A SNP's rs5985 and rs3024477 in T2DM in a South African cohort, the prevalence and effect of two SNP variants Val34Leu (rs5985) and Tyr204Phe (rs3024477) on clot kinetics was assessed in this study.

2. Materials and methods

2.1. Materials

Cups and pins (catalogue number (cat. no.: 6211)) and 0.2 M Calcium Chloride (cat. no.: 7003) for thromboelastography were purchased from Barker Medical (Johannesburg, South Africa). Consumables were purchased from Starlab (Milton Keynes, UK) and reagents for buffers were purchased from Sigma-Aldrich (St. Louis, Missouri, United States); restriction enzymes (RsaI: NEB R0167S, DdeI: NEB R0175S) from New England BioLabs (Ipswich, Massachusetts, USA), GoTaq Polymerase for PCR from Promega (Madison, Wisconsin, USA), Genomic-DNA (gDNA) isolation kits from Zymo Research (Irvine, California, USA) and nucleotides from Bio-Line (London, UK).

2.2. Patient recruitment and sample collection

Following statistical, MSc committee and ethical approval (Ethical approval no.: 269/2017, 100 Type 2 diabetes mellitus patients were recruited from the Steve Biko Academic Hospital, Diabetic Clinic, Pretoria, South Africa and 100 healthy controls were recruited. Following completion of informed consent, 5 ml of blood was collected in citrate tubes via venipuncture. The inclusion criteria for T2DM patients included: patients must have been diagnosed with T2DM for at least 3 months. The exclusion criteria for healthy controls included: they should not be smokers, on any chronic medication or oral contraceptives.

2.3. Thromboelastography

Thromboelastography (TEG) was used to measure the quality of coagulation in WB (Thakur, 2012). The viscoelasticity parameters of clot formation assessed include reaction time (R; sec)-the time elapsed until the first measurable clot forms, clot kinetics (K; sec)- the time elapsed until the clot reaches a fixed strength (20 mm), α-angle (°)- reflects the speed of fibrin accumulation, maximum amplitude (MA; mm)- reflects the strength of the clot, coagulation index (CI; dynes/sec)- provides a representation of a patients coagulable state, Lys 30 (% of the lysed clot)- lysis of a clot within 30 min, clot firmness (G; dynes/cm^2)- the shear elastic modulus strength, maximum rate of thrombus generation (MRTG; Dyn cm^{-2} s^{-1})- the maximum speed of the clot growth, time to maximum rate of thrombus generation (TMRTG; min)- the time period before the maximum speed of the clot growth and total thrombus generation (TTG; Dyn.cm^{-2})- the total clot resistance.

Whole blood was centrifuged (2000 xg, 10 min) to get platelet rich plasma (PRP), then the PRP was centrifuged (2000 xg, 20 min) to get platelet poor plasma (PPP) which was used to asses clotting parameters. A cup was mounted into the TEG instrument (TEG®5000 Haemonetics, Switzerland). The maximum amount that the cup held was 360 µl; where 20 µl was calcium chloride which served as the coagulation activator and 340 µl PPP from each sample. The machine has a thin wire, which measured the coagulation process as the sample in the cup was gently vortexed. For the analysis of the coagulation process, a specialized TEG programme was used to monitor coagulation and clotting kinetics, using specific pre-selected parameters in Table 1. A thromboelastograph was generated from which the clotting kinetics was obtained for each sample.

Table 1. TEG parameters normal value ranges.

Parameters	**Normal ranges** (Pretorius et al., 2017)
Reaction time (R time)	9–27 min
Clotting time (K time)	2–9 min
Alpha angle (α angle)	22–58°
Maximum amplitude (MA)	44–64 mm
Maximum rate of thrombus generation (MRTG)	0–10 Dyn cm − 2 s − 1
Time to maximum rate of thrombus generation (TMRTG)	5–23 min
Total thrombus generation (TTG)	251–1014 Dyn.cm − 2

2.4. Deoxyribonucleic acid isolation

Genomic deoxyribonucleic acid (gDNA) was isolated from 150 µl of WB using the Quick-DNA™ Miniprep Plus Kit (Zymo Research). An aliquot of 200 µl cell lysis buffer and 20 µl of Proteinase K was added to each 150 µl WB sample in 1.5 ml microcentrifuge tubes. The tubes were mixed thoroughly for 10–15 s (s) and incubated [55 °C, 10 min (min)]. Equivolume (370 µl) genomic binding buffer was added to the sample and vortexed (10–15 s). The homogenized mixture was then transferred into a Zymo-Spin™ IIC-XL column in a collection tube centrifuged (12 000 xg, 1 min) and the flow through discarded while the column was placed into a fresh collection tube. 400 µl of DNA pre-wash buffer was added to the spin column in a fresh collection tube and centrifuged (12 000 xg, 1 min). The collection tube was emptied and 700 µl g-DNA wash buffer was added to the spin column and it was then centrifuged (12 000 xg, 1 min). The eluate collection tube was decanted and 200 µl g-DNA wash buffer was added to the spin columns and it was centrifuged (12 000 xg, 1 min). Thereafter, the collection tube with the flow through was discarded. The spin columns were transferred into a clean 1.5 ml microcentrifuge tube, and 75 µl DNA elution buffer was added directly on to the matrix and incubated [5 min, room temperature (RT)]. The tubes were then centrifuged (12 000 xg, 1 min) to elute the DNA. The eluted DNA was then stored at −20 °C, quantified using spectrophometrically (Nanodrop 2000c, Thermo-Fischer, South Africa), standardised to 50 ng/µl and used for polymerase chain reaction (PCR).

2.5. Polymerase chain reaction and Restriction Fragment Length polymorphism

Primer concentrations for each FXIII-A SNP (Val34Leu and Tyr204Phe) were optimized. Go Taq® Hot start green master mix was used to amplify the FXIII-A gene sequences, the master mix contained GoTaq® Hot start polymerase, dNTPs, $MgCl_2$ and reaction buffers and primers (400 nM). Val34Leu [192 base pairs (bp)] Forward: 5′-CATGCCTTTTCTGTTGTCTTC-3′, Reverse: 5′-TACCTTGCAGGTTGACGCCCCGGGGCACTA-3′and Tyr204Phe (113 bp) Forward: 5′-

GGAAACAGTCTGGTTTGGTAA-3′, Reverse: 5′- ACCCCGATGTCATTCAGGACG-3′ were amplified in a 25 µl reaction. The primers were subjected to PCR with an initial denaturation at 95 °C for 2 min, denaturation at 95 °C for 30 s, annealing for 30 s (Val34Leu: 48.2 °C and Tyr204Phe: 48.9 °C), extension at 74 °C for 30 s and final extension at 74 °C for 5 min.

The restrictions were set up in a 25 µl reaction (3 µl PCR product, 5 µl 1X NE buffer and 1 µl RE (1 000U) (New England BioLabs) as outlined in Table 2.1. The restriction fragments were then analysed using gel electrophoresis on a 3% agarose gel (GR Green stained) for 30 min, visualised and analysed.

Table 2. Restriction enzymes and conditions.

	Val34Leu	**Tyr204Phe**
Restriction enzyme	*Dde*I	*Rsa*I
Restriction cut site	5′…C↓TNAG…3′ 3′…GANT↑C…5′	5′…GT↓AC…3′ 3′…CA↑TG…5′
Restricted fragments sizes	161 and 31 bp	91 and 22 bp
Incubation temperature	37 °C and inactivation at 65 °C for 20 min	37 °C and inactivation at 65 °C for 20 min

2.6. Statistical analysis

Differences between T2DM clots and control clots were determined by using a two-tailed non-parametric *t*-test (Mann Whitney test) using Graphpad Prism software. The differences were considered to be significant when $p < 0.05$. The Hardy-Weinburg equilibrium was calculated using the Court-lab calculator, and the association analysis was conducted using the recommended Fischers Exact Test.

3. Results

3.1. Cohort demographics

The mean age of the controls was 43.52 (22–66) years old and the T2DM group was 59.64 (32–56) years old. The ratio of males to females in the control group was 1:3.69, and in the T2DM group was 1:0.69. Race matched controls and T2DM comprised 33.7% Black South African and 66.3% Caucasian South African. Clinical parameters were verified using patient hospital records. Majority (74.1%) of the T2DM patients were co-morbid with hypertension and minority (37%) had chronic diseases such as hypercholesterolemia, kidney failure, hypothyroidism and hyperthyroidism. Controls were found from students and staff populations on a randomized basis provided they met the inclusion criteria. In order to eliminate gender and ethnicity bias, the samples selected to assess clot kinetics and, all samples genotyped were also evaluated as a total cohort and stratified according to ethnicity to calculate an association with T2DM [odds ratio (OR)].

3.2. Clot properties

Thromboelastography analyses indicated altered clot kinetics in T2DM compared to controls. The time taken for the first measurable clot to form (R-time) was significantly longer in T2DM patients compared to controls (Table 3, $p < 0.0001$). The clot strength (MA) and the

time it took for the clot to reach a fixed clot (K time) were slightly higher in T2DM compared to controls, however no significant difference was observed. The time period before the TMRG was higher (Table 3, $p < 0.0001$) in T2DM.

Table 3. Coagulation profile of healthy controls compared to type 2 diabetes mellitus.

TEG Parameter	T2DM	Control	P value
Reaction time (min)	23.85 ± 2.36	8.45 ± 1.01	<0.0001 ***
Maximum amplitude (mm)	38.91 ± 2.16	36.28 ± 2.17	n.s.
K value (min)	3.58 ± 0.43	2.87 ± 0.28	n.s.
Alpha angle (degrees)	59.24 ± 2.54	63.91 ± 1.92	n.s.
MRTG (Dyn.cm-2 s-1)	4.13 ± 0.66	5.29 ± 0.56	n.s.
TMRTG (min)	25.04 ± 2.28	12.38 ± 1.30	<0.0001***
TTG (Dyn.cm-2)	229.9 ± 28	341.3 ± 36.43	n.s.

Data is represented as the mean and standard error of the mean. Where $^*p < 0.05$ derived from the Mann-Whitney *U* test is considered significant. TEG: thromboelastT2DM: type 2 diabetes mellitus, K value: clotting time, MRTG: Maximum rate of thrombus generation, TMRTG: Time to maximum rate of thrombus generation and TTG: Total thrombus generation.

3.3. Frequency of Factor XIII-A Val34Leu and Tyr204Phe polymorphisms

Confirmation of the presence of SNPs was done using PCR and RFLP. Control and T2DM genotype distribution conformed to Hardy-Weinberg equilibrium (HWE) ($p > 0.05$). There was no significance for the wildtype Val34Val (G/G) (Odds ratio (OR) = 0.79, 95% confidence interval (CI) = 0.41–1.55, p = 0.4947) genotype assessed in T2DM, and there was statistical significance for the Tyr204Tyr (A/A) (OR = 0.22, 95% CI = 0.12–0.42, $p < 0.0001$) genotype assessed in T2DM patients. Table 4 illustrates the Val34Leu and Tyr204Phe genotype distribution. The presence of each polymorphism in each ethnic group was also assessed, and the 34Leu variant was rare in both ethnicities, Blacks (OR = 1.06, 95% CI = 0.19–5.98, p = 0.9484) and Caucasians (OR = 0.89, 95% CI = 0.35–2.26, p = 0.8042). The 204Phe variant was more frequent in the Caucasian population (OR = 0.39, 95% CI = 0.05–0.33, $p < 0.0001$) compared to the Black population (OR = 0.33, 95% CI = 0.09–1.22, p = 0.0885). Table 5 shows that there was significance in the allele frequencies for the Tyr204Phe polymorphism, (RR = 0.5314, 95% CI = 0.39–0.72, OR = 0.2895, 95% CI = 0.17–0.51, Chi-square df = 19.55, 1, $p < 0.0001$***, fisher's exact $p < 0.0001$***).

Table 4. Genotype frequencies in control and T2DM.

	Genotype					
	Val/Val G/G	**Val/Leu G/T**	**Leu/Leu T/T**	**Tyr/Tyr A/A**	**Tyr/Phe A/T**	**Phe/Phe T/T**
Controls ***(n = 101)***	76	21	4	19	13	69
T2DM ***(n = 100)***	80	17	3	57	5	41
Total Caucasians ***(n = 101)***	75	24	2	30	12	69
Total Blacks ***(n = 54)***	46	5	3	26	2	26
Black Controls ***(n = 14)***	12	1	1	4	0	10
Black with T2DM ***(n = 40)***	34	4	2	22	2	16
Caucasian Controls ***(n = 64)***	47	16	1	9	10	45
Caucasian with T2DM ***(n = 37)***	28	8	1	21	2	14

Stratification by race resulted in the exclusion of coloured and/or Indian subjects.

Table 5. Allele frequencies for Val34Leu and Tyr204Phe polymorphisms.

	Control	**T2DM**	**Total**	Empty Cell	**Control**	**T2DM**	**Total**
Val	97	97	194	**Tyr**	32	62	94
Leu	25	20	45	**Phe**	82	46	128
Total	122	117	239	**Total**	114	108	222

3.4. The effect of the presence of Val34Leu and Tyr204Phe variants on clot kinetics

The Val34Leu polymorphism had no effect on clot properties in control subjects. The 204Phe variant only affected the reaction time in control subjects with a much lower time in the presence of the variant (Table 6, 6.59 ± 0 73 vs. 10.89 ± 2.05, $p = 0.0432$*). The presence of the polymorphism also affected TMRTG (Table 6, 9.98 ± 0.92 vs. 14.79 ± 2.28, $p = 0.0209$).

The presence of the 204Phe variant in T2DM affected the TMRTG (18.80 ± 3.60 vs. 27.83 ± 2.76, $p = 0.0207$*), with a decrease in the amount of time taken for the clot to form, but faster rate at which the clot reached maximum strength. The maximum rate of thrombus generation was substantially higher but the time taken before the maximum speed of the clot growth could be reached was reduced (Table 6).

Table 6. Val34Leu and Tyr204Phe variants and clot kinetics.

TEG Parameter	Control			T2DM		
	Val/Val	**Val/Leu + Leu/Leu**	**P value**	**Val/Val**	**Val/Leu + Leu/Leu**	**P value**
Reaction time (min)	8.90 ± 1.42	7.30 ± 0.74	n.s.	25.07 ± 2.86	20.40 ± 4.05	n.s.
Maximum amplitude (mm)	35.55 ± 2.53	37.96 ± 4.37	n.s.	40.07 ± 2.58	36.59 ± 3.98	n.s.
MRTG (Dyn.cm-2 s-1)	4.76 ± 0.65	6.28 ± 1.02	n.s.	4.02 ± 0.85	4.42 ± 0.8	n.s.
TMRTG (min)	13.56 ± 1.88	10.16 ± 0.98	n.s.	25.78 ± 2.88	22.93 ± 3.27	n.s.
TTG (Dyn.cm-2)	335.60 ± 43.15	351.90 ± 70.17	n.s.	204.61 ± 33.54	301.2 ± 45.52	n.s.
	Tyr/Tyr	**Tyr/Phe + Phe/Phe**	**P value**	**Tyr/Tyr**	**Tyr/Phe + Phe/Phe**	**P value**
Reaction time (min)	10.89 ± 2.05	6.59 ± 0.73	0.0432*	22.52 ± 2.45	27.22 ± 5.5	n.s.
Maximum amplitude (mm)	38.70 ± 3.62	34.5 ± 3.84	n.s.	38.99 ± 2.46	38.70 ± 4.63	n.s.
MRTG (Dyn.cm-2 s-1)	4.74 ± 0.88	5.84 ± 0.69	n.s.	3.07 ± 0.51	6.48 ± 1.67	n.s.
TMRTG (min)	14.79 ± 2.28	9.98 ± 0.92	0.0209*	27.83 ± 2.76	18.80 ± 3.60	0.0207*
TTG (Dyn.cm-2)	362.40 ± 54.63	320.10 ± 49.70	n.s.	208.7 ± 30.51	277.1 ± 59.50	n.s.

4. Discussion

Type 2 diabetes mellitus is a multifactorial disease and the complications associated with the disease are continuously under investigation. Two pathways, inflammation and coagulation play a central role in the progression and development of coagulopathies in T2DM patients (Levi and van der Poll, 2005). The main coagulation factors production site is found in the hepatocytes and the sinusoidal endothelial cells in the liver (Lallukka et al., 2017). Low-grade inflammation, hyperglycaemia, insulin resistance and obesity are characteristics of metabolic non-alcoholic fatty liver disease (NAFLD) and these factors make individuals to be susceptible to T2DM, venous thrombosis and cardiovascular disease (CVD) (Lallukka et al., 2017). (Lallukka et al., 2017) demonstrated that insulin-resistant individuals have increased clotting factor activity (Lallukka et al., 2017). Their results demonstrated an increase in FXIII activity and no changes in the expression level of FXIII-A in the liver in insulin resistant individuals (Lallukka et al., 2017).

Insulin resistance has been demonstrated to increased levels of plasminogen activator inhibitor (PAI-1) and tissue plasminogen activator (tPA), thus enhancing the risk of a thrombotic event due to hypofibrinolysis in T2DM individuals (Kearney et al., 2017).

Premature atherosclerosis in T2DM patients plays an important role in increasing the risk of cardiovascular complications in these patients (Kearney et al., 2017). In T2DM, hypofibrinolysis and prothrombic characteristics are associated with increased clot time (Kearney et al., 2017).

The coagulation factor XIII which is required for the last step during the coagulation cascade is a transglutaminase which is composed of two subunits (the A subunit which is the catalytic one and the B which is composed of two carrier proteins) (Elbaz et al., 2000). During FXIII activation by thrombin, the transglutaminase forms covalent e-(g-glutamyl)-lysyl bonds between the fibrin monomers and this results in a stable fibrin clot which is resistant to fibrinolysis (Elbaz et al., 2000). One study showed that FXIII cross-linking decreased the fibrin fibre diameter, which increases resistance to fibrinolysis (Hethershaw et al., 2014). They also demonstrated that there is an additional function of FXIII during clot formation, which "influences fibrin clot formation", which then results in the production of a clot that consists of thinner fibrin fibres (Hethershaw et al., 2014).

Each of the TEG parameters are influenced by coagulation factors. Both fibrinogen and FXIII influence clot strength, as their interaction results in a cross-linked fibrin structure (Maatman et al., 2018). Deficiency of coagulation factors FXII, FXI,FVIII,FII (thrombin) and FI (fibrinogen) affects the reaction time (R time) and clot strength (MA) (Nielsen, 2005). Absence of FXII results in "no clot formation", while the absence of FXI results in an increased R-time and decreased α-angle (Nielsen, 2005). Interestingly, the absence of FIX decreases the R time, whereas FVIII absence was increased and the α-angle was decreased (Nielsen, 2005). On the other hand, absence of FII (thrombin) results in no clot production, whereas the absence of FXIII results in a longer R-time, and lower α-angle (Nielsen, 2005). The clot strength and fibrin crosslinking is mainly affected by FXIII and FI (fibrinogen), in addition to FII, FVII, FX and FXII (Nielsen, 2005). Factor I also plays an important role in the speed of fibrin accumulation (α-angle) (Nielsen, 2005).

In a study by Viswanathan et al. (Viswanathan et al., 2014) they showed that the time it takes for the first measurable clot to form (R time) was prolonged in T2DM patients (Viswanathan

et al., 2014). They also found a decrease in the clot strength (Viswanathan et al., 2014). In another study done by Maatman et al. (Maatman et al., 2018) they found that there is an increase in the clot strength in T2DM patients compared to controls (37.0 ± 8 vs 34.1 ± 8 mm; p < 0 001) which is associated with an increased risk of T2DM patients developing myocardial infarction (Maatman et al., 2018). They also found no significant difference for the R time and the K time in T2DM patients (Maatman et al., 2018).

The combination of TEG parameters, can be used to determine if an individual has a hyper- or hypo-coagulable clot profile. According to Pretorius et al. (Pretorius et al., 2017), a hypercoagulable clot profile is characterised by a reduction in the reaction time (R time), clot kinetics (K time), an increase in the speed of fibrin accumulation (α-angle), clot strength (MA), maximum speed of clot growth (MRTG), a reduction in the time from clot initiation till the maximum clot formation (TMRTG) and an increase in the total clot resistance (TTG) (Pretorius et al., 2017). While a hypo-coagulable clot profile is characterized by increased in the reaction time, clot kinetics and time period before the maximum speed of clot growth and an decrease in the speed of fibrin accumulation, clot strength, the maximum speed of clot growth and total clot resistance (Pretorius et al., 2017). From the results, of this study, there was increased reaction time and time period before the maximum speed of clot growth, the reduction of maximum speed of clot growth and total resistance which demonstrated a hypocoagulable clot profile for T2DM patients.

The coding gene of the FXIII-A subunit is composed of 15 exons, mapped on the 6p24-25 chromosome, with a size of 160 kb, while the B-subunit is composed of 12 exons that are separated by 11 introns, mapped on the 1q31-32 chromosome, with a size of 28 kb (Naderi, 2016). The subunit contains 731 amino acids (aa) and 5 domains which are the activation peptide (1–37 aa), beta-sandwich (38–183 aa), catalytic core (184–515 aa), beta-barrel 1 (516–627 aa) and 2 (628–731 aa) (Naderi, 2016). There are different SNP target sides on subunit A (Ariëns et al., 2002).

The stability of a clot can be affected by factors such as the presence of polymorphisms on FXIII. Factor XIII-A Val34Leu variant is a nucleotide change from a G > T which results in a valine to leucine amino acid substitution and is located on exon 2 near the thrombin cleavage site (Elbaz et al., 2000, Attié-Castro et al., 2000). It has been suggested to result in the increased activity of the transglutaminase and increased fibrin cross-linking (Pruissen, 2008, Elbaz et al., 2000, Attié-Castro et al., 2000, Corral, 2000). Balogh et al. (Balogh et al., 2000) showed that at low thrombin concentration (0.5 U/ml), the rate at which the activated FXIII peptide is released was faster for the homozygous FXIII-A Leu/Leu protein variant compared to the wild-type FXIII-A Val/Val (Balogh et al., 2000). The heterozygous Val/Leu protein demonstrated an intermediate speed and they proposed that the rate of speed was influenced by the location of the variant near the thrombin activation site (Balogh et al., 2000). One study showed that FXIII-A activation is increased by 2–3 folds in individuals that carry the Val34Leu variant, however, this had a negative effect on the cross-linking structure, where the variant produced thin and less porous fibres (Jeddi-Tehrani et al., 2010).

Many FXIII-A Val34Leu studies have been done on conditions such as vascular thrombosis (Corral, 2000), myocardial infarction (Hancer et al., 2006, Elbaz et al., 2000, Reiner et al., 2002), deep venous thrombosis (Elbaz et al., 2000), ischaemic stroke (Pruissen, 2008), coronary artery disease (Naderi, 2013) and recurrent pregnancy loss (Jeddi-Tehrani et al., 2010). Many of these studies focused on the association of the variant with the investigated condition. The variant had a protective effect against myocardial infarction (Hancer et al.,

2006), deep venous thrombosis, brain infarction (Elbaz et al., 2000) and vascular thrombosis (Corral, 2000). However, the mechanism behind the protective effect is still not well understood and it has been hypothesized that the variant enhance the activation rate of FXIII that then leads to the inadequate fibrin cross-linking (Elbaz et al., 2000). Another proposed hypothesis is that there is linkage disequilibrium between FXIII-A Val34Leu and other functional FXIII-A variants (Elbaz et al., 2000).

The prevalence of the Val34Leu variant differs amongst different ethnic groups. Hancer et al. (Hancer et al., 2006) found that the variant is high in Western countries where it occurred at 48% and 51% in England (Catto, 1999, Kohler et al., 1998), 45.8% in the USA, 43% in Italy, 45.1% in Hungary, 50.2% in France and 28.9% in the Brazilian and African populations (Hancer et al., 2006). In addition, it is rarely found in the Asian population (Hancer et al., 2006). Another study done by Attie-Castro *et al.* (2000) found that the variant was more frequent in Brazilians (allele frequency = 0.0306) and Portuguese Caucasians (f = 0.204) (Attié-Castro et al., 2000). The study also found that the prevalence of the variant in the Black population was lower compared to other populations (Attié-Castro et al., 2000).

Ma et al. (Ma et al., 2015) found that the presence of the 34Leu variant (Val34Leu) increases the risk of developing intracerebral haemorrhage (OR = 1.23, 95% CI = 0.94–1.61, p = 0.13) (Ma et al., 2015). A study conducted by Yildirm et al. (Yildirm, 2017) demonstrated that the genotype distribution of Val34Leu was high for the homozygous wild type Val/Val (68.5%), heterozygous Val/Leu (29.2) and homozygous variant Leu/Leu (2.4%) (Yildirm, 2017). They stated that the results they obtained did not indicate if the polymorphism was able to enhance the predisposition of thrombosis or have any protective effect against thrombosis (Yildirm, 2017).

There are other polymorphisms located on the FXIII A subunit, for example, Tyr204Phe, which is located on exon 5 and Pro564Leu which is located on exon 2 (Jeddi-Tehrani et al., 2010). Both of these polymorphisms have been linked to the decrease in FXIII-A levels and activity. Furthermore, they also contribute to the early breakdown of FXIII before its activation (Jeddi-Tehrani et al., 2010).

Common risk factors that contribute to the development of atherosclerosis are smoking, obesity, hypertension and diabetes, which are commonly found in patients with cardiovascular diseases and it has been shown that there is a 4-fold increase risk for developing ischaemic stroke associated with the Leu/Leu genotype and a 2-fold increase in the presence of the Tyr204Phe variant (Reiner et al., 2002). In a study done by Jeddi-Tehrani et al. (Jeddi-Tehrani et al., 2010), they suggested that the presence of the Tyr204Phe and Pro564Leu polymorphism is associated with an increased risk of recurring pregnancy loss (RPL) (Jeddi-Tehrani et al., 2010).

In another study done by Pruissen et al. (Pruissen, 2008), they showed that patients that had one allele copy of the 204Phe variant (Tyr/Phe and Phe/Phe) had a 9-fold increased risk for ischaemic stroke (Pruissen, 2008). The presence of both allele copies (Phe/Phe) increased the risk much higher by 77-fold compared to the wild-type (Tyr/Tyr) (Pruissen, 2008), while there was a 8-fold increase risk for the intermediate genotype (Tyr/Phe) (Pruissen, 2008). In the present study, there was a decrease in the reaction time of clot formation for patients with T2DM with the 204Phe variant. However, research suggests that the presence FXIII-A Val34Leu and Tyr204Phe polymorphisms did not change the risk of developing myocardial infarction in both the dominant and individual genotypes (Siegerink et al., 2009).

A separate FXIII-A polymorphism, found on exon 5, which is a A > 3 > T substitution, results in a tyrosine to a phenylalanine (Tyr204Phe). Barbosa et al. (Barbosa et al., 2004) found that the occurrence of the Tyr204Phe was not different between patients that have recurrent miscarriages and controls (Barbosa et al., 2004). They also showed that the Tyr204Phe variant is rare in the Brazilian population (Barbosa et al., 2004). In another study they found that there is no association of recurrent miscarriages and Val34Leu polymorphisms (López Ramírez et al., 2006).

In other results they found that individuals that carried 2 copies of the 564Leu allele had a 4-fold increased risk of developing haemorrhagic stroke (OR = 4.3) (Reiner, 2001). While carriers of either 1 or 2 copies of the 34Leu allele had a reduced risk of developing haemorrhagic stroke (OR = 0.7) (Reiner, 2001). They suggested that due to the fact that the presence of Tyr204Phe and Pro564Leu were at low frequency in Black individuals compared to Caucasians, other genetic and environmental factors were involved in the high prevalence of haemorrhagic strokes in Black individuals [90]. In the current study, the 34Leu and 204Phe variants were more prevalent in the Caucasian T2DM and control individuals compared to Black individuals.

The presence of the 34Leu and 204Phe variants depicted a hypercoagulable profile in T2DM patients. The 204Phe variant reduced the reaction time in T2DM patients and in controls. There was also a reduction in the time period before the maximum speed of clot growth and an increase in the maximum speed of clot growth. The 34Leu variant increased the maximum speed of clot growth and the total resistance in T2DM patients. In a study done by Schroeder et al. (Schroeder et al., 2001), they found that the presence of the 34Leu variant decreases the clot formation time (R time, p = 0.031) which also characterises a hypercoagulable clot profile (Schroeder et al., 2001).

This study has shown that T2DM patients have a hypo-coagulable (PPP) clot profile while the formed clot has an increased clot strength. Clot parameters including the reaction time, maximum speed of clot growth, the time period before maximum speed of clot growth and the total resistance were altered in T2D patients. This contradiction to the hypercoagulable state found in other studies may be due to the lack of cellular components such as platelets and erythrocytes which play a key role in the hypercoagulability. The exclusion of these components in the current study enabled the assessment of how factor XIII-A and fibrin/ogen influences clot kinetics.

In this study both Val34 and Tyr204Phe alleles were more prevalent in control subjects compared to T2DM patients. No association of the Val34Leu SNP and T2DM was found. An inverse association was found with the Tyr204Phe SNP and T2DM with an odds ratio of 0.22 with an indication that the polymorphic variant, 204Phe, may offer a protective effect being more prevalent in controls.

Stratification of the clot kinetics by Val34Leu genotypes showed only a slight increase in time for the clot to reach maximal strength and total clot resistance in the presence of the polymorphic variant.

Clot kinetics stratified by the Tyr204Phe genotypes showed that the presence of the 204Phe variant caused an altered coagulation profile, indicative of a possible protective effect, in concordance with literature, observed in the control group that also had the highest prevalence of this genotype.

5. Study limitations

This study has several limitations which were unavoidable due to the limited time available for the completion of the study.

Firstly, the study included samples from patients and controls of mixed race, both black and Caucasian. The black ethnic group was not stratified by clan lineage due to lack of such data.

The age range for the samples was wide, and inflammation is influenced by age, which influences coagulation. We therefore excluded samples from patients older than 50 in both controls (n = 9 excluded) and T2DM (10 excluded) groups for the TEG analysis.

We were unable to complete linkage disequilibrium using the NCBI website since there is a lack of a population code for blacks or Caucasians in South Africa.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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