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ASSESSING GENE EXPRESSION OF COAGULATION FACTORS AND FACTOR XIII-A POLYMORPHISMS IN TYPE 2 DIABETES MELLITUS PATIENTS

BY

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DECLARATION

I, Marry-Ann Ntanyane Phasha, declare that:

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DEDICATION

“A lot of people have gone further than they thought they could because someone else thought they could”

-Zig Ziglar

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PUBLICATIONS

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PRESENTATIONS

The paper titled: Assessing gene expression of coagulation factors and factor XIII-A polymorphisms in type 2 diabetes mellitus patients. Phasha, M. N, Soma P, Pretorius E, Bester J, Phulukdaree A. was accepted for presentation at the following institutional, national and international conferences (Appendix 7):

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ABSTRACT

Background: Type 2 diabetes mellitus (T2DM) is characterized by chronic hyperglycaemia, inflammation and coagulopathies due to cytokine activation of IL-1β and IL-6. The aim of the study was to assess the mRNA levels of coagulation factors and the identification of two FXIII SNPs (Val34Leu and Tyr204Phe) in T2DM patients (n=100) compared to controls (n=100).

Methodology: Thromboelastography (TEG) was used to assess the viscoelastic properties of pre-selected clot parameters. Quantitative PCR (qPCR) was used to assess mRNA levels of key coagulation factors (factor XIII-A, factor XII and tissue factor). PCR-Restriction fragment length polymorphism (PCR-RFLP) was used to identify the presence or absence of Val34Leu and Tyr204Phe using specific restriction enzymes.

Results: From the pre-selected viscoelastic properties, TEG showed that the R time and TMRTG were prolonged by 17.67 mins (26.15±2.56 min vs. 8.48±0,92 min) and 12.76 mins (24.85±2.41 min vs. 12.09±1.15 min) ($p<0.0001$) respectively. There was a reduction in MA by 2.55 $dyn.cm^{-2}s^{-1}$ (3.94±0.61 dyn.cm−2 s−1 vs. 5.73±0.54 dyn.cm−2 s−1, p=0.0074) and TTG by 121 $dyn.cm^{-2}$ (213.8±26.29 dyn cm−2 vs. 335.5 ± 32.33 dyn cm−2, p= 0.0385) in T2DM patients compared to controls. A 4.38-fold decrease in mRNA levels of FXIII-A in T2DM (delta ct: 10.94±0.44) was observed, compared to controls (delta ct: 13.07±0.48), p=0.0033. The genotype distribution in both T2DM and control individuals conformed to the Hardy-Weinberg Equilibrium (HWE) ($p>0.05$). An association of the Val34Leu SNP and T2DM was found, (OR) = 1.670, 95% CI=0.97-2.89, p=0.0747) . An even stronger association was found with the Tyr204Phe SNP and T2DM (OR=3.57, 95% CI=2.37-5.45, $p<0.0001$). The levels of FXIII-A mRNA was significantly altered in T2DM (delta ct: 14.46±0.82 vs. 12.03±0.49, p=0.0105) in the presence of the wild type (Tyr/Tyr) compared to the variant (Tyr/Phe and Phe/Phe), respectively. The polymorphism had an effect on the viscoelastic properties as showed by measurements obtained in the TEG variables in T2DM patients, the 34Leu variant increased the MRTG by 1.35 Dyn.cm-2.s-1 (4.92±0.84 Dyn.cm-2s-1 vs. 3.57±0.78 Dyn.cm-2s-1, p=0.0457) and TTG by 120 Dyn.cm-2 (301.3±38.22 Dyn.cm-2 vs. 181.3±31.68 Dyn.cm-2, p=0.0367). The 204Phe variant reduced the R time by 3.79 mins (6.68± 0.73min vs. 10.47± 1.63min, p=0.033),

the TMRTG by 13.1 min (14.49±2.59 min vs. Tyr/Tyr 27.52±3.01 min, p=0.0017) and increased the MRTG by 3.95 Dyn.cm-2.s-1 (6.74±1.17 Dyn.cm-2s-1 vs. Tyr/Tyr 2.79±0.47 Dyn.cm-2s-1, p=0.0020). In controls the 34Leu variant had no effect on the viscoelastic properties and the 204Phe variant reduced the R time by 3.88 min (6.59±0.73 min vs. 10.47±1.63 min, p=0.0334).

Conclusion: This study has showed that T2DM patients have a hypocoagulable (PPP) clot profile while the formed clot has an increased clot strength. An association of the Val34Leu SNP as well as the Tyr204Phe SNP with T2DM was observed, with an indication that the polymorphic variant, 204Phe, offering a protective effect being more prevalent in controls.

LIST OF ABBREVIATIONS

A	Adenine
AA	Amino acid
ABCC8	ATP Binding Cassette Subfamily C Member 8
AGEs	Advanced glycation end-products
BI	Brain infarction
Bp	Base pairs
C	Cytosine
CALPN10	Calpin 10 (CALPN10)
Cat no	Catalogue number
CD4+T	Cluster of differentiation 4 T lymphocytes
cDNA	Complimentary deoxyribose nucleic acid
CHD	Coronary heart disease
CRP	C-reactive protein
CVD	Cerebrovascular disease
DNA	Deoxyribose nucleic acid
dNTPs	Deoxynucleotide triphosphates
DVT	Deep venous thrombosis
EIF2AK3	Eukaryotic translation initiation factor 2-alpha kinase 3
ER	Endoplasmic reticulum
FIX	Factor nine
FV	Factor seven
FVII	Factor eight
FX	Factor ten

FXI	Factor eleven
FXII	Factor twelve
FXIII	Factor thirteen factor
G	Guanine
GWAS	Genome-wide association studies
HDL	High density lipoproteins
HK	High molecular weight kininogen
ICH	Intracerebral haemorrhage
IL	Interleukin
IRS-1	Insulin receptor substrate-1
JNK	c-Jun N-terminal kinase
K	Clotting time
LPS	Lipopolysaccharide
MA	Maximum amplitude
$MgCl_2$	Magnesium chloride
MI	Myocardial infarction
Min	Minutes
ml	Millilitre
mRNA	Messenger ribonucleic acid
MRTG	Maximum rate of thrombus generation
MTNR1B	Melatonin receptor 1B
NAFLD	Non-alcoholic fatty liver disease
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor kappa-B

NO	Nitric oxide
OR	Odds ratio
PAI-1	Plasminogen activator inhibitor
PARs	Protease activated receptors
PCR	Polymerase chain reaction
PGL2	Prostacyclin
PK	Plasma kallikrein
PKC	Protein kinase C
PPARγ	Peroxisome proliferator-activated receptor gamma
PPK	Plasma pre-kallikrein
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PRR	Pattern recognition receptors
qPCR	Quantitative polymerase chain reaction
R	Reaction time
RE	Restriction endonuclease
RFLP	Restriction length polymorphism
ROS	Reactive oxygen species
RPL	Recurring pregnancy loss
RT	Room temperature
Sec	Seconds
SNPs	Single nucleotide polymorphisms
T	Thymine
T1DM	Type 1 diabetes mellitus

T2DM	Type 2 diabetes mellitus
TEG	Thromboelastography
TF	Tissue factor
Th	T-helper cells
TLRs	Toll-like receptors
TMRTG	Time to the maximum rate of thrombus generation
TNF-α	Tumour necrosis factor – alpha
tPA	Tissue-plasminogen activator
T-reg	T- regulatory cells
TTG	Total thrombus generation
VCAM	Vascular cell adhesion molecule
VT	Venous thrombosis
VTE	Venous thromboembolism
vWF	von Willibrand factor
WB	Whole blood
µL	Microlitre

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Chapter 1

Introduction

It is estimated that there are 425 million type 2 diabetes mellitus (T2DM) individuals worldwide [1]. More than 16 million are individuals from Africa, which is estimated to increase to 41 million by the year 2045[1]. In South Africa there were 1 826 100 T2DM cases in 2017 [1]. 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 [2-3].

There is a link between insulin resistance, inflammation and oxidative stress. Advanced glycation end-products (AGEs) plays a central role in this link as any increase in AGEs results in an increase in inflammation and oxidative stress [4]. It is found that the combination of oxidative stress, AGEs and hyperglycaemia can lead to the stimulation of inflammation and altered coagulation [5].

Hyperglycaemia plays a key role in determining the level of inflammation and hypercoagulation in T2DM patients. Elevated levels of chemokines, pro-inflammatory and coagulation factors can serve as predictors of diseases progression in T2DM and associated diseases such as coronary heart disease [6]. Pro-inflammatory and coagulation markers such as tumour necrosis factor – alpha (TNF-α), interleukin (IL) -β, IL-6, C-reactive protein (CRP), tissue factor, fibrinogen and plasminogen activator inhibitor-1 (PAI-1) are found to be elevated and are believed to play a major role in the pro-coagulant state [7-8].

The risk of developing coagulopathies is high in T2DM due to the imbalance between clot formation and lysis, resulting in hypercoagulation and hypofibrinolysis [9]. Increase in hypercoagulation and hypofibrinolysis activity can contribute to the formation of atherosclerosis in T2DM patients in that the antifibrinolytic factors such as TAFI are upregulated, which is one of the factors that contribute to hypofibrinolysis [10-14]. Platelet activation and endothelial dysfunction are key contributing factors to the development of coagulopathies observed. Platelets play a role in haemostasis, inflammation and immunity [15]. Platelets become resistant to the inhibitory effect of insulin and anti-aggregants such as nitric oxide (NO) and prostacyclin and this leads to the

hyperactivity of platelets observed in T2DM [16]. Damage to the endothelial wall creates a site for platelet adhesion, which is then followed by changes in platelet shape, degranulation and then platelet activation [17].

Platelet activation and vasoconstriction are the first mechanisms to be activated during haemostasis, this is followed by the activation of the coagulation cascade. During the coagulation cascade, the contact (intrinsic) and tissue factor (extrinsic) pathways come together to activate factor (F) IX. The contact pathway can also be activated during contact of blood with a negatively charged material or surface. This stimulates the activation and production of thrombin and fibrin fibre formation. The contact pathway consists of FXII, FXI, plasma pre-kallikrein (PKK), plasma kallikrein (PK) and high molecular weight kininogen (HK) [18-19]. Activation of FXII leads to the activation of FXI, which then furthers activates FIX. Factor IXa binds to FVIIIa on the surface of the activated platelet, and together activate FX [19].

Fibrin and activated FXIII play an important role in clot formation, any alteration in these two coagulation factors results in an altered coagulable state [20]. Due to hyperglycaemia in T2DM, a hypercoaguable state is usually observed, and this contributes to the high mortality rate caused by thrombotic disease as a complication in T2DM patients [21]. 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 [20]. 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 [22]. 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 [23].

In addition to hyperglycaemia, oxidative stress and inflammation, genetic variation also plays a role in the progression of the pathogenesis of T2DM. These genetic variations include inversions, substitutions, copy number variations and single nucleotide polymorphisms (SNPs). Functional SNPs are SNPs which predict if the presence of a variant will be deleterious to the biological function of a gene or gene product [24]. The deleterious influence of SNPs can affect the biomolecular function of a gene through transcription, splicing, translation and post-translation mechanisms [24]. SNPs that are

located in the regulatory region influence the level gene expression if a SNPs occurs in the transcription factor binding site [24]. Functional SNPs are able to have an effect on a disease as they are able to affect the coding of the amino acid sequence of a protein and as a result causes alteration to the protein structure and function [25]. Disease-causing SNPs occur in the functional genomic areas such as the regulatory or the protein coding regions, which result in function distortion [24].

There have been several studies that have demonstrated that there are some genetic variations in genes that encode coagulation factors. Commonly found and studied thrombophilic gene polymorphisms include: Factor V Arg506Glu c.1691 C>T (rs6025), prothrombin gene G20210A (rs1799963) and fibrinogen gamma 10034>T (rs2066865) [25]. Several SNP's of significance in FXIII-A include the Val34Leu located on exon 2 c.103 G>T (rs5985) [25], Tyr204Phe located on exon 5 c.614 A>T (rs3024477) and Pro564Leu located on exon 8 c.1694 C>T (rs5982) [26]. The 204Phe variant is associated with decreased FXIII-A levels and the early break down of FXIII before activation [27], thus it affects the formation of the fibrin network. 34Leu has been linked with the increase in FXIII-A levels, resulting in a more rapid fibrin network formation, which can lead to thrombotic diseases [28-31].

Shastry (2007) stated in a review article that the report by Halushka *et al* (1999) showes that 50% of SNPs are located in the noncoding region, where 25% result in missense mutations, while the other 25% result in silent mutations which do not affect the amino acid translation [32 33]. There are synonymous (silent SNPs) and nonsynonymous SNPs (change-encoded amino acids) which are able to cause diseases [34]. Both synonymous and nonsynonymous SNPs are able to have an effect on the promoter activity and the pre-mRNA stability [34]. In addition they can also influence the binding ability of a protein to a substrate [33] or an inhibitor or change the cellular location of proteins [34].

Aims and Objectives

Polymorphisms in the promoter and encoding regions are able to affect the stability of mRNA and post-transcriptional modification of the mRNA [35]. In addition SNPs that occur in the splicing regulatory region can affect the amount of mRNA produced [35]. Gene expression can be affected by where the gene is regulated, if it is regulated at transcription level, the total amount of the mature and unprocessed pre-mRNA levels will be affected by SNPs that interfere with transcription regulation [35]. If it is regulated post-transcriptionally, the amount of exonic mRNA will be altered by SNPs that are involved in splicing of exons and introns [35]. Furthermore, SNPs are able to affect mRNA and protein levels depending if they are synonymous or nonsynonymous [34].

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 FXIII-A mRNA levels and clot kinetics was assessed in this study. The mRNA levels of coagulation factors (FXII, FXIII-A and tissue factor) were also assessed.

Objective 1: Evaluate clot kinetics in T2DM and healthy controls using thromboelastography (TEG)

Objective 2: Assess mRNA levels of coagulation factors XII, XIII-A, and TF in whole blood (WB) using quantitative polymerase chain reaction (PCR)

Objective 3: Identify Val34Leu (rs5985) and Tyr204Phe (rs3024477) SNP's in FXIII-A using PCR– restriction fragment length polymorphism (RFLP)

Objective 4: Evaluate the effect of the presence or absence of each SNP on clot kinetics and FXIII-A mRNA levels.

Chapter 2

Literature Review

2.1 Background

Type 2 diabetes mellitus (T2DM), constitutes 90% of diabetes commonly found worldwide, and is classified as a chronic disease, frequently associated with cardiovascular risk [36]. The underlying cause of the disease is multifactorial, and many of these are still under investigation. It is characterized by hyperglycaemia that has a significant impact on the regulation of carbohydrates, fat and protein metabolism, which leads to dysfunction in insulin secretion [37]. One of the hallmarks of T2DM is inflammation, and it is characterized by oxidative stress and an increase in circulating inflammatory markers, including various interleukins, our research group has discussed this in various papers [38-42]. Accompanying oxidative stress and inflammation frequently is hypercoagulation and hypofibrinolysis, with resulting coagulopathies, and this is also true for T2DM [42]. Many circulating inflammagens, as well as genetic factors (or predisposition), may together, result in coagulopathies, and Factor XIII is one such role player. Therefore, this literature review is on the various mechanisms involved in coagulopathy (focussing on the cellular (haematological) changes associated with it) and the role of specifically Factor XIII-A, single nucleotide polymorphism in T2DM.

There are known associations between the disease and genetic predisposition that influences the rate, progression and the onset of T2DM [43]. Genome-wide association studies (GWAS) have shown that the risk of developing T2DM is influenced by a number of genes (polygenic) [44]. It has also been suggested that most T2DM-linked genes affect the function of the pancreatic islet and beta cells which are supported by genetic epidemiology studies [44]. Previously, 40 non-synonymous mutations from two melatonin receptor 1B (MTNR1B) coding exons were identified [45]; of which 36 of the mutations were rare mutations, that had a frequency <0.1% (OR=3.31) and were strongly associated with the risk of developing T2DM. This combination of the loss-of function of genetic variants that have a frequency <0.1% increased the risk of developing T2DM by 5.5 folds higher [45].

Genome-wide association studies have also shown that there is a “complex genetic landscape” for disease states such as T2DM and metabolic genes [43]. There are also genes that have been identified that are involved in the function of ß-cells, insulin action and other metabolic pathways that increase the risk of developing T2DM [46]. To date, 50 genes have been identified, and these genes are associated with the development of T2DM [46-47]. Peroxisome proliferator-activated receptor gamma (PPARγ*),* ATP binding cassette subfamily C member 8 (ABCC8) and calpin 10 (CALPN10) are some of the most promising genes that have an association to the risk of developing T2DM [46-47]. Some of these genes cause early development of T2DM and T1DM such as the eukaryotic translation initiation factor 2-alpha kinase 3 (EIF2AK3) genes, which are important in the unfolded protein stress response [43].

There are also different genetic markers which can be used to determine the association between a disease and a gene. One such marker is telomeres, which are repetitive DNA-protein complexes that are found at the end of chromosomes and serve to protect and maintain genetic stability [48]. The length of these telomeres is reduced by age, factors such as oxidative stress and inflammation [48]. There is evidence of increased oxidative stress and inflammation in individuals with T2DM [48]. Njajou and colleagues have demonstrated that the shortening of telomeres length may also play a key role in the development of T2DM [49]. Because our focus is on the role of a pathological coagulation system, together with role of specifically Factor XIII, the next paragraphs will now first focus on inflammation, oxidative stress and the pathological coagulation system present in T2DM. This will be followed by a review of literature pertaining to Factor XIII, in particular.

2.2 T2DM, pathological insulin signalling, inflammation and dysregulation of lipid metabolism

The hallmarks of T2DM is insulin resistance, oxidative stress and inflammation [42]. There is substantial evidence that demonstrates that cluster of differentiation (CD4+) T cells play a key role in the pathogenesis in obesity and insulin resistance [50]. Based on the function and cytokine secretion, CD4+ effector T cells are differentiated into 4 sub-populations, which are T-regulatory cells (T-reg), T-helper 1 (Th1), T-helper 2 (Th2) and T-helper 17 (Th17) [50]. In adipose tissue of obese individuals, macrophages

are found to be increased [50]. The balance between pro-inflammatory (Th1 and Th17 lymphocytes) and anti-inflammatory (Th2 and T-reg lymphocytes) CD4+ cells, in addition to the differentiation of macrophages, can be modified during obesity and T2DM [50]. As a result, there is activation of adipose tissue macrophages [51].

The Th17 sub-population, has been shown to play a role in the dysregulation of lipid metabolism, insulin resistance and diabetic nephropathy [50,52]. T-reg cells are responsible for the inhibition and prevention of an inflammatory response, through the suppression of Th1, Th2 and Th17 cells [50]. And in T2DM individuals, T-reg cells suppression of the other CD4+ cells can improve insulin resistance [53]. However, the levels of this CD4+ sub-population is shown to be decreased in T2DM individuals [54]. In addition Zeng and colleagues, demonstrated that there is a decrease in the T-reg/Th1 ratio and T-reg/Th17 in T2DM individuals [55]. Thus, adipose tissue inflammation can be taken into account in the incidence of T2DM, metabolic syndrome, atherosclerosis and cardiovascular diseases [50]. Inflammation in T2DM is characterized and attenuated by the presence of hyperglycaemia, oxidative stress and obesity (see Figure 2.1).

Inflammation and the presence of hypercoagulation, are characterized by the dysregulation and presence of various circulating cytokines [42,56]. In addition, the hyperglycaemic state (and its associated molecules), plays an important role in the development of hypercoagulation. During hypercoagulation, elevated glucose levels may induce the formation of the fibrin network which then leads to a clot being formed [57]. These individuals have elevated levels of coagulation factors, pro-inflammatory cytokines and chemokines, which are used as predictors of T2DM. Both the presence of inflammation and upregulated coagulation markers, have shown to be predictors of coronary heart disease, in T2DM observational studies [51].

Upregulated inflammatory cytokines also affect various cells, tissues and organs. Particularly Tumour necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and IL-1β are important role players in T2DM. Inflammation present in the pancreatic islets of T2DM individuals, is characterized by amyloid deposits, fibrosis, β- cells apoptosis and excessive secretion of pro-inflammatory cytokines (IL-1β) and chemokines by macrophages [51]. Cytokine IL-1β plays a key role in the progression and regulation of inflammation in islet cells of T2DM, as it induces the upregulation and recruitment of

other cytokines and chemokines to the pancreas [51]. The secretion of IL-1β is regulated by the c-Jun N-terminal kinase (JNK) and Nuclear factor kappa-B (NF- κB) pathways. IL-1β signalling through the IL-1 receptor, that results in the activation of NF-κB pathway [51]. Activation of this pathway, leads to the transcription and expression of other inflammatory mediators in addition to IL-1β, such as TNF-α and this results in cytokine amplification [51]. TNF- α, is involved in the expression of vascular adhesion cell molecules such as E- and P-selectin and Vascular cell adhesion molecule (VCAM) [58].

Pro-inflammatory cytokines, pattern recognition receptors (PRRs) and cellular stresses induce the activation of the JNK and NF-κB pathways, as well as ROS and endoplasmic reticulum (ER) stress [59]. Both pathways play a key role in inflammation-activated insulin resistance. JNK is known as a stress kinase, which under normal conditions phosphorylates c-Jun of the transcription factor AP-1. Studies have also demonstrated that JNK plays a role in the progression of insulin resistance as it phosphorylates the serine amino acid on insulin receptor substrate-1 (IRS-1) [59-64].

Elevated levels of lipid deposition in adipocytes results in the expression of TNF-α, IL-6, IL-1β and these cytokines are able to cause the activation of JNK and NF-κB through a feed forward mechanism [59]. This can also cause the expression of transcription factors, chemokines and receptors which through signalling lead to the recruitment of monocytes and the stimulation of monocytes differentiation into macrophages which produce even more pro-inflammatory cytokines [59]. An increase in the size of adipocytes induces the production of cytokines and chemokines, with subsequent recruitment of macrophages [51]. There have been a number of studies that have shown that abdominal obesity is closely linked to systemic low-grade inflammation, and this may be one of the factors that play a role in insulin resistance and metabolic disorders [51].

There have been epidemiological studies done, that have shown that the incidence of T2DM is also correlated high levels of inflammatory markers such as C-reactive protein (CRP), IL-6, plasminogen activator inhibitor-1 (PAI-1) and fibrinogen to name a few [59]. Endothelial cells and macrophages take part in the alteration of vasoreactivity and the development of a pro-coagulant state, by the upregulation of PAI-1 and tissue factor [8].

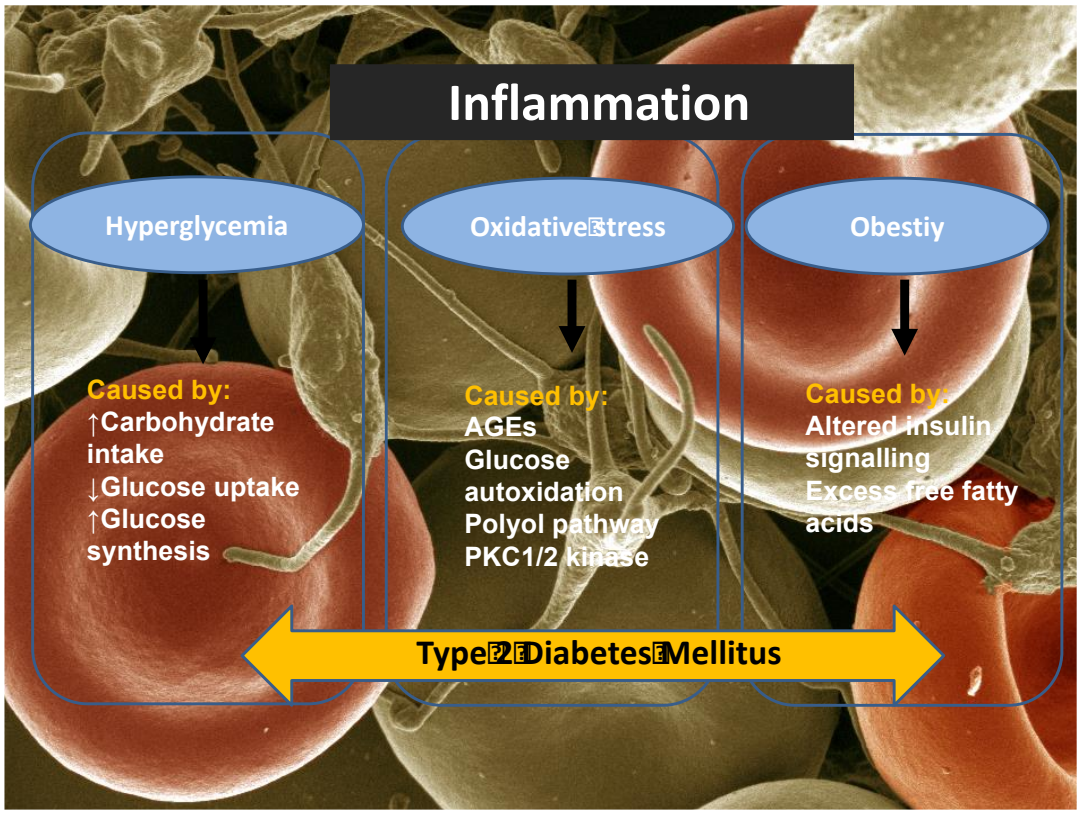

Figure 2.1: Summary of key pathophysiology factors in type 2 diabetes mellitus. AGEs: advanced glycation end products, PKC: protein kinase C

Atherosclerosis and T2DM dyslipidaemia are also closely linked to each other. Low levels of high density lipoproteins (HDL) cholesterol, elevated levels of triglyceride-rich lipoproteins are amongst the characteristics of T2DM [65]. Each of these features plays a role in the progression of the disease, mainly by enhancing the inflammatory state. A pro-inflammatory state observed in endothelial cells and macrophages are stimulated by triglyceride-rich lipoproteins [65]. In addition, these lipoproteins are involved in the expression of TNF-α by macrophages and the expression of adhesion receptors by monocytes that adhere to the endothelial cells [65]. The production of pro-inflammatory cytokines by macrophages is induced by triglyceride-rich lipoproteins [65]. If there is an increase in free fatty acids that is transported to the peripheral tissue, there will be activation of toll-like receptors (TLRs), that then stimulate the expression of pro-inflammatory mediators and the subsequent progression of insulin resistance in individuals with T2DM [65]. The next paragraphs will discuss hyperglycaemia, glycation end-products (AGEs) and how it causes oxidative stress

2.3 Effects of hyperglycaemia, advanced glycation end-products and oxidative stress

Elevated glucose levels in the body can induce oxidative stress, which is a prominent physiological response in inflammation [42]. Oxidative stress is induced due to several mechanisms such as advanced glycation end products (AGEs) formation, glucose autoxidation, polyol pathway and PKCβ1/2 kinase activation [66]. Reactive oxygen species (ROS) are molecules which can reduce several biological molecules (lipids, protein and nucleic acids), where they donate an electron to those biological molecules. They are neutralized or balanced by antioxidants in living organism systems; any imbalance in ROS and antioxidants leads to oxidative stress state and cellular disruption and damage [66]. In patients with T2DM, ROS production is increased by the activation of many harmful pathways such as AGEs formation and PKCβ1/2 kinase pathways [66]. The combination of increased oxidative stress, hyperglycaemia and the formation of AGEs contributes to the initiation and progression of inflammation and hypercoagulation observed in patients with T2DM [67]. Activation of pathways such as the PKCβ1/2 leads to the alteration of structures and biochemical components in the blood of T2DM patients. The pathway activated by diacylglycerol, which is a second messenger that is involved in the activation of proteins involved in cellular signalling pathways [68]. Which then leads to the dysfunction of the endothelial contractility and altered haemodynamics [66].

2.4 Hypercoagulation and hypofibrinolysis in T2DM

The presence of hyperglycaemia in individuals with T2DM increases their odds of having thrombotic conditions such as strokes, myocardial infarction (MI) and venous thromboembolism (VTE) [69]. There have been a few mechanisms that have been proposed highlighting the role of hyperglycaemia in the progression of hypercoagulation;

1. There is inhibition of the production and expression of tissue-plasminogen activator (tPA) by elevated PAI-1 caused by hyperglycaemia and hyperinsulinemia. As a result, there is impairment of fibrinolysis [70].
2. The glycocalyx, glycoproteins found on the surface of the vascular endothelium is affected by hyperglycaemia, as there is an increase in the attachment

of platelets that adhere to endothelial cells, enhancing the production of coagulation factors [71].

3. In an *in vitro* study, the presence of both insulin and hyperglycaemia induces the activity of transcription factor NF-κB in human hepatocyte cells, increasing the expression of PAI-1 [72].

4. The expression of tissue factor on monocytes can be stimulated by AGEs [73].

5. Platelet calcium homeostasis is disrupted by the elevated glucose resulting in cytoskeleton dysfunction and as a result there are increased pro-aggregant factors [74].

The balance between clot formation and lysis is critical since any imbalances may result in coagulopathies, and in T2DM we see hypercoagulation and hypofibrinolysis, as a result of hyperglycaemia [9]. This leads to an increased risk for the development of atherothrombotic events in individuals with T2DM. This results from increased production of coagulation factors and dysregulation of the fibrinolysis process in the coagulation cascade.

There have been different proposed mechanisms that demonstrate the imbalance of clot formation and lysis that can lead to prothrombic events [69]. The proposed mechanisms include the disruption of gene transcription of coagulation factors that may be caused by the hyperglycaemia-induced oxidative stress, dysfunction of the endothelial glycocalyx layer, which contains some of the coagulation factors and the effect of glycation of the factors that lead to altered activity [69]. There is also an increase in the levels of prothrombin fragment 1 and 2, linked to cardiovascular diseases that are observed in T2DM individuals [75].

2.5 Platelet activation

Platelets provide a bridge between inflammation, haemostasis and immunity [56]. Megakaryocytes and platelets contain mRNA for factors involved in the coagulation cascade and platelets activation [56]. Tissue factor is expressed during both the coagulation cascade, inflammation and when there are different external stimulating factors such as lipopolysaccharide (LPS), TNF-α, IL-1, IL-2 and IL-6 [76]. Platelets are one of the integral components in the coagulation cascade. The formation of fibrin network fibres is dependent on platelet interaction and aggregation through the glycoprotein receptors (GPIIb/IIIa) [77]. There is an increase in platelets activation in

diabetic individuals due to the reduction in NO production and hyperglycaemia increase the adhesion of platelets to the endothelia wall by increasing the expression of surface receptors [77]. This occurs during the activation phase of the platelets, whereby there is transfer of P-selectin that is located in the α-granules and weibel-palade bodies of the endothelial cells to the cell membrane of the platelets [78]. For the fibrinogen to be able to bind to the platelet surface there is a change in the receptors, GPIIb/IIIa conformation [78]. Glycoprotein GPIIb/IIIIa is involved in platelet aggregation as they bind to fibrinogen; GPIb is involved in the binding of platelets to von Willebrand factor (vWF) which helps the platelets adhere to the wound site [78].

2.6 Endothelial dysfunction

There are several factors that contribute to endothelial dysfunction such as increased production of vasoconstrictors, thromboxane A2, decreased production of prostacyclin (PGI2) and impairment of NO availability [74]. Endothelial dysfunction together with hypercoagulation, hyperglycaemia, and insulin resistance increases the risk of T2DM individuals of developing a prothrombic state that will contribute to cardiovascular diseases. Insulin resistance as a factor is involved in the upregulation of PAI-1 and fibrinogen and the downregulation of tissue plasminogen activator [74]. The expression of tissue factor on monocyte, vascular endothelium in diabetic individuals can be stimulated by low-grade inflammation and hyperinsulinemia leading to elevated production of thrombin and also contributes to atherothrombosis, Figure 2.2 [74].

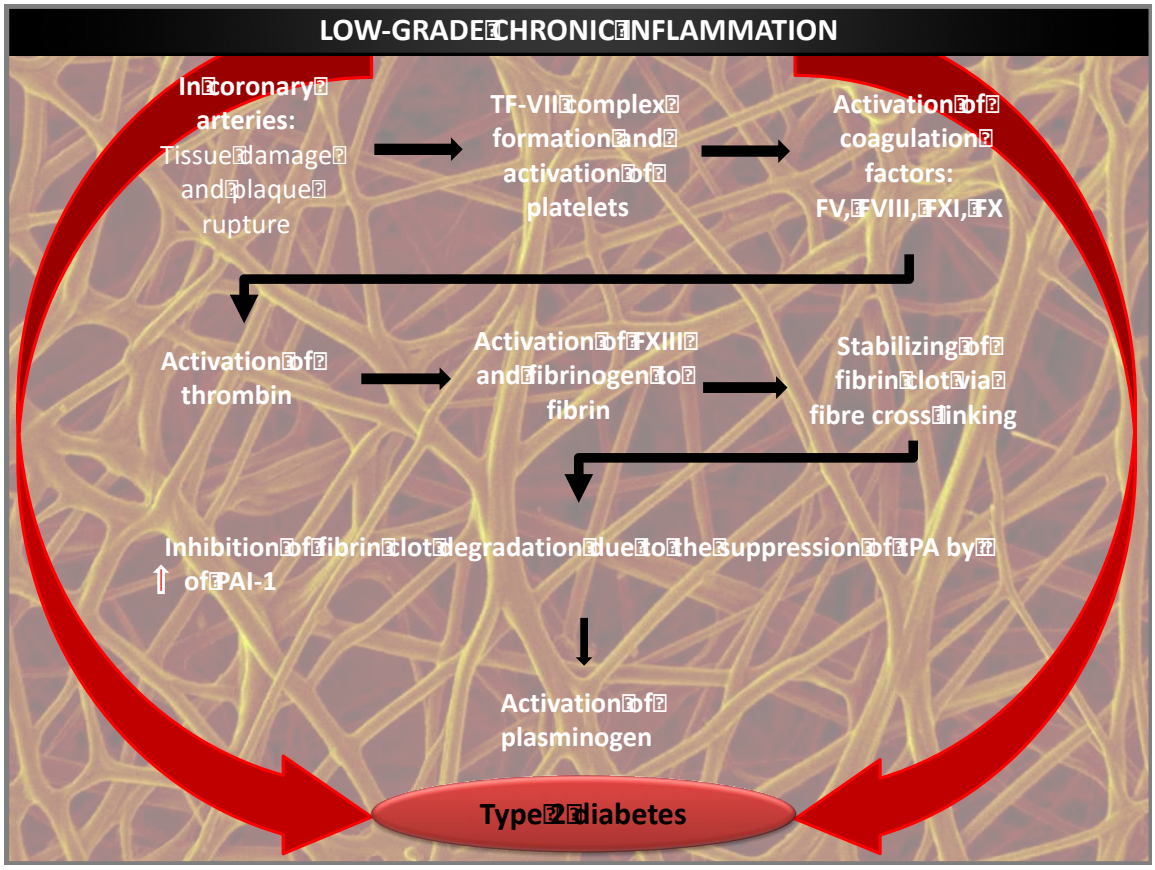

Figure 2.2: Coagulation process in T2DM individuals with atherosclerosis. Clot formation increases the risk of atherothrombosis. TF-VIII: Tissue factor- factor seven, FV: factor five, FVIII: factor eight, FXI: factor eleven, FX: factor ten, FXIII: factor thirteen, tPA: tissue plasminogen activator, PAI-1: plasminogen activator inhibitor 1.

Damage to the endothelial wall is a stimulator of thrombotic events as it creates a platform for platelet adhesion. Following adhesion, platelets change shape, undergo degranulation and are activated [17]. In T2DM individuals, there is a decrease in the sensitivity of platelets to prostacyclin (PGI2) which inhibit platelet activity and along with NO act as vasodilators [78]. The shape of platelets, platelets aggregation and secretion and the production of thromboxane A2 depend on the presence of calcium [79]. Production of thromboxane A2, platelet secretion, aggregation and shape is regulated by calcium [80]. Altered levels of magnesium and calcium in T2DM lead to an increase in platelet aggregation and adhesion [79].

2.7 Coagulation factors

In the coagulation cascade the contact (intrinsic) and tissue factor (TF) (extrinsic) pathways come together to activate factor X. The contact pathway can be activated during contact of blood with a negatively charged material or surface. This stimulates the activation and production of thrombin and fibrin fibre formation. The contact pathway consists of factor XII, XI, plasma pre-kallikrein (PPK), plasma kallikrein (PK) and high molecular weight kininogen (HK) [18-19]. Activation of factor XII leads to the activation of factor XI, which then furthers activates factor IX. Factor IXa binds to factor VIIIa on the surface of the activated platelet, and together activate factor X [19].

Several studies have suggested that tissue factor serves as a bridge that links inflammation and coagulation. In the coagulation cascade tissue factor is a co-factor, that activates factor VIIa initiating the extrinsic pathway of the coagulation cascade. Tissue factor-FVIIa complex is involved in the activation of a number or serine proteases which can induce the activation of inflammation through the cleavage of protease activated receptors (PARs) [81]. AGEs and oxidative stress factors play a key role in the expression and production of tissue factor on endothelial cells. Kim and colleagues showed that there is an increase in the levels of coagulation factors II, V, VII, VIII and X and that there is a reduction in anticoagulation factor protein C in T2DM [82].

Factor XIII is a key factor that is involved in the final step in the formation of fibrin network during coagulation. It is a transglutaminase that is composed of two subunits, the A and two carrier B-subunits (FXIII-A2B2). The activation of FXIII by thrombin through cleavage of the activation peptide separates the A and B-subunits [22]. FXIII can be located in the cytoplasm, platelets, megakaryocytes, monocytes and tissue macrophages (cFXIII-A) [23,83-84]. Platelets contain an abundance of FXIII in the α-granules [23]. In WB, cFXIII mRNA only codes two subunit A chains[85]. During the fibrin network formation, activated FXIII (FXIIIa) plays a role in stabilizing the clot through the formation of covalent bonds in the cross-linking of fibrin monomers and also include the α-2-antiplasmin into the clot structure illustrated in figure 2.3 [86]. Increased expression of FXIII is linked to the development of strokes and cardiovascular diseases. Studies have shown that factor XIII is involved in thrombus formation that may result in deep vein thrombosis [22].

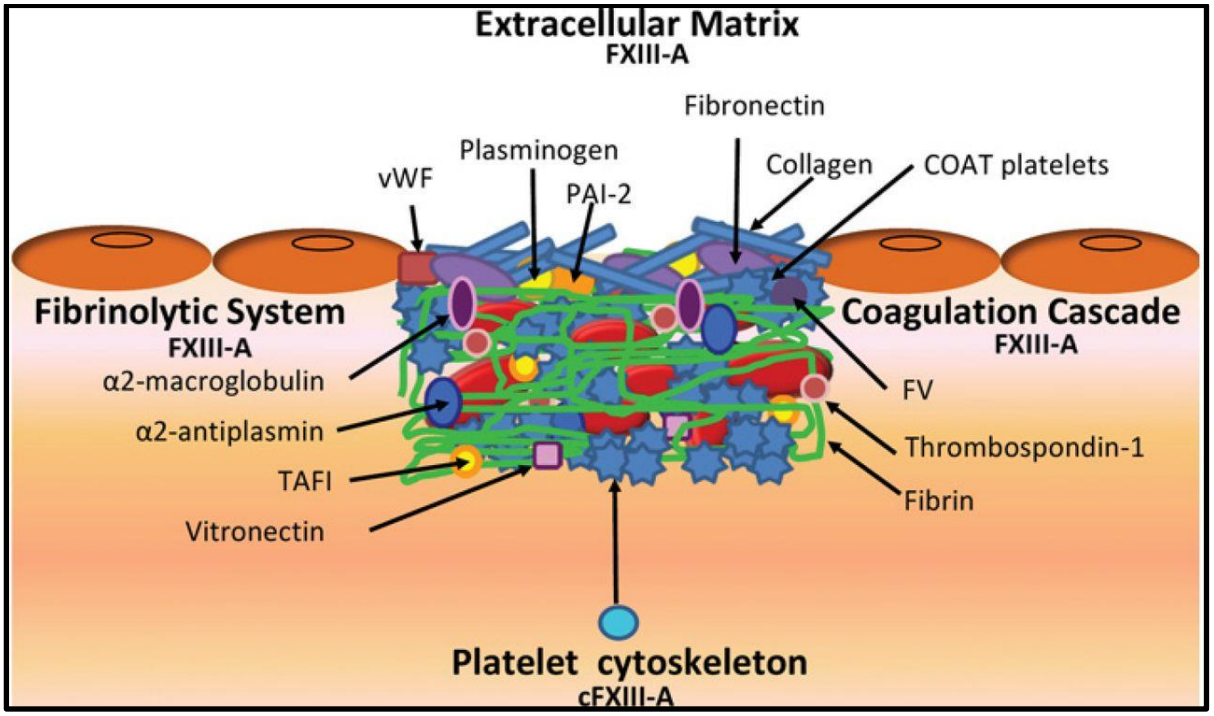

Figure 2.3: During the fibrin network formation thrombin plays an important role in the cleavage of fibrinogen to fibrin and activates platelets for the release and activation of plasma FXIII-A. Platelet activation results in the platelets undergoing conformational change that is caused by the cross-linking of the cytoskeleton of proteins caused by cFXIII-A [86].

2.8 Single nucleotide polymorphism (SNPs) that affect coagulation factor XIII

The human genetic makeup consists of genetic variations such as inversions, substitutions, copy number variations and single nucleotide polymorphism (SNPs). SNPs are one of the most commonly found variation, and common SNPs are SNPs which have allele frequency of ≥ 5% in a population [87]. Most common observed SNP alleles in a disease, may serve as an indication of having a higher predisposition to developing that given disease. The concept of linkage equilibrium and linkage disequilibrium is important in determining the association of a given disease with a SNP [88].

In linkage equilibrium there is an association of the SNP variant with a disease where different alleles at a nearby position can remain on the same haplotype with that disease [88]. On the other hand, in linkage disequilibrium there might not be a direct effect or association between a SNP and a disease [89], and this may be due to only few nucleotide changes in history [88]. The effect of SNPs on a disease occurs during

the coding of the amino acid sequence of a protein. As there is a change in nucleotide, which may result in alteration to the amino acid code and this may result in a change in the function of the protein [25]. In addition, there might be an introduction of a stop codon that will lead to a truncated protein.

Protein function such as catalytic activity, ligand binding or DNA binding properties maybe impaired if there is a SNP. In complex diseases the SNP is commonly located in non-coding regulatory regions such as the promoter region, enhancer, silencer and intronic regions [25]. SNPs located in the non-coding region may also influence how the post-transcriptional processes such as splicing of the mRNA, mRNA stability and translation occurs [25]. Therefore, SNPs that are found in genes that are involved in the coagulation cascade may result in imbalances of coagulation and anticoagulation factors [25].

Several studies have demonstrated that there are some genetic variations in genes that encode coagulation factors and they then play a significant role in the pathophysiology of certain diseases. Commonly found and studied thrombophilic gene polymorphisms include factor V Leiden (rs6025), prothrombin gene G20210A (rs1799963), fibrinogen gamma 10034>T (rs2066865) and FXIII-A Val34Leu located on exon 2 c.103 G>T (rs5985) [25]. These variants have been shown to be involved in increased or decreased risk of having a venous thrombosis (VT) in the future [25]. The prothrombin G20210A and fibrinogen gamma have been shown to increase the risk of developing VT, while FXIII Val34Leu has been shown to reduce the risk of developing VT [90-91].

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 [92]. The presence of this SNP increases the rate at which factor XIII is activated, and affects how the fibrin network forms [93]. FXIII-B subunit also has been a site where two major SNPs have been identified. The first one is located on exon 3 where there is a transversion His95Arg c.344 G>A (rs6003) [94]. The variant is present in Caucasians (9%) and Black Africans (68%).

The second FXIII-B subunit SNP is located on intron K, c.1952 + 144 C>G (rs12134960) [95]. The presence of this SNP in the intronic region results in a new splice acceptor site [92]. It is found in the Caucasian population (17%) [96]. Studies have

reported that there is an interaction between the FXIII-B His95Arg and FXIII-A Val34Leu polymorphisms which then affects thrombotic diseases [58,97].

The effect of FXIII Val34Leu has been investigated in recurring pregnancy loss (RPL), and in a study done by Jung and colleagues they found that there is no linkage between RPL and the SNP variant in European and South American populations [98]. However, there was an association found in the Asian population [98]. FXIII deficiency has been associated with severe bleeding disorders and the risk of having miscarriage in female individuals [99]. 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 [99-101]. One study demonstrated that there is a protective effect of the FXIII Val34Leu variant against the development of arterial thrombosis [99]. Table 2.1 shows studies that have been done to show an association between the FXIII Val34Leu variant and thrombotic and haemorrhage disease states.

Table 2.1: Association studies of FXIII-A Val34Leu and thrombotic/ haemorrhage diseases.

Reference	Risk of having a thrombotic disease	Association
[30]	Brain infarction	Negative
[29]	Arterial or venous thrombotic events; 1. Deep venous thrombosis 2. Cerebrovascular disease 3. Coronary heart disease	None
[102]	Myocardial infarction	None
[103]	Recurrent pregnancy loss	Positive
[104]	Fast fibrin stabilization and increased risk of recurrent myocardial infarction	Positive
[105]	Intracerebral haemorrhage	None
[106]	Intracerebral haemorrhage	None
[107]	Umbilical cord bleeding	None
[108]	Coronary heart disease and Cardiovascular disease Intracerebral haemorrhage	Negative Positive

A study conducted by 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 [109]. 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 [110-111]. Ariens *et al*. (2000) not only showed microscopic changes in clot structure (Figure 2.4) 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 $(mmol/L)^{-1} \times sec^{-1}$ for FXIII 34Leu than for 34Val [111]. 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 [110].

Fibrin ultrastructure

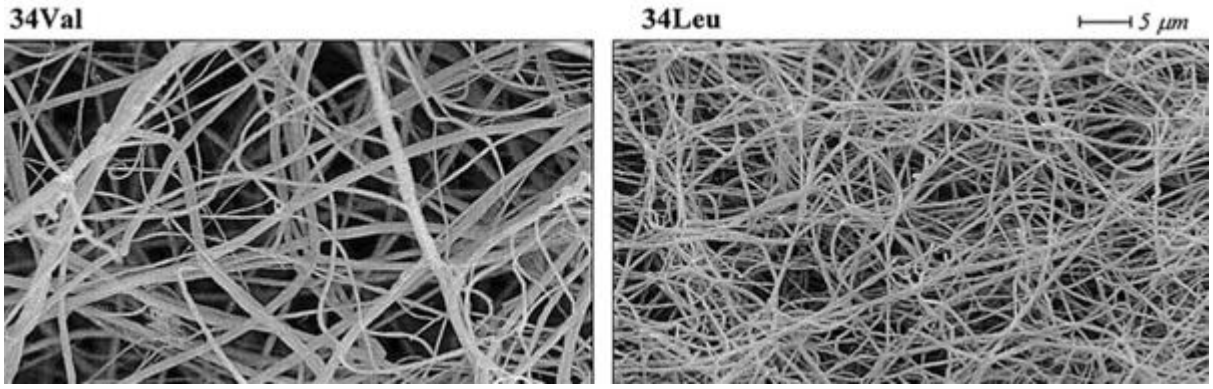

Figure 2.4: Ultrastructural changes of fibrin clots prepared from subjects homozygous for FXIII 34Val and FXIII 34Leu. Reproduced from [111].

Other FXIII subunit-A SNP variants are Tyr204Phe located on exon 5 (rs3024477) and Pro564Leu (rs5982) located on exon 8 [26]. These variants are associated with a decrease in the plasma levels of FXIII [112]. Furthermore, it is suggested that Pro564Leu is involved in the reduced specific activity of FXIII [112]. However, in other studies this Pro564Leu SNP variant is also shown to increase the activity of FXIII [113]. A study done by Reiner and colleagues, suggested that the Tyr204 and 564Leu

variants can be used as genetic marker of haemorrhagic stroke in women [112]. This has also been demonstrated by Pruissen and colleagues, and they showed that there is a strong association between the Tyr204Phe variant and the risk of young women having ischaemic strokes [31], and it increases the risk by 9-fold [26].

There are several well described factors that affect the pathophysiology and progression of T2DM. Hyperglycaemia, the resulting oxidative stress and inflammation have been shown to have a significant impact on the impairment and disturbance of many of the metabolic pathways. Central to the disease is pathological hypercoagulation and hypofibrinolytic pathways which are impaired, and which may contribute to thrombotic events that increase mortality in T2DM. However, genetic predisposition should not be ignored, and this includes variants of FXIII. Genetic analysis of variants that may be present should be investigated as such changes may play a crucial role and contribute significantly to the etiology of T2DM.

Chapter 3

Materials and Methods

3.1 Materials

Cups and pins (catalogue number (cat. no.: 6211)) and 0.2M 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); for cDNA synthesis (cat. no.: 1708891) and quantitative PCR (cat. no.: 1725121); Bio-Rad (Hercules, California, 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 nucletoides from Bio-Line (London, UK).

3.2 Patient Recruitment and Sample Collection

Following statistical (appendix 4), MSc committee (appendix 5) and ethical approval (Ethical approval no.: 269/2017, appendix 6), 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 (appendix 6), 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.

3.3 Thromboelastography

Thromboelastography (TEG) is a technique that was developed by Dr. Hellmut Hartert in 1948 and it is used to measure the quality of coagulation in WB [114]. It assesses the viscoelasticity parameters of clot formation [114-116]. There are a number of parameters that the TEG measures [115,117];

1. Reaction time (R; sec)-the time elapsed until the first measurable clot forms.

2. Clot kinetics (K; sec)- the time elapsed until the clot reaches a fixed strength (20 mm).

3. α-angle (º)- reflects the speed of fibrin accumulation.

4. Maximum amplitude (MA; mm)- reflects the strength of the clot.

5. Coagulation index (CI; dynes/sec)- provides a representation of a patients coagulable state.

6. Lys 30 (% of the lysed clot)- lysis of a clot within 30 min.

7. Clot firmness (G; dynes/cm²)- the shear elastic modulus strength.

8. Maximum rate of thrombus generation (MRTG; Dyn cm^{-2} s^{-1})- the maximum speed of the clot growth.

9. Time to maximum rate of thrombus generation (TMRTG; min)- the time period before the maximum speed of the clot growth.

10. Total thrombus generation (TTG; Dyn.cm^{-2})- the total clot resistance.

The TEG instrument assesses the different parameters through a pin which is suspended in a cup from a torsion wire, that is linked with a mechanical electrical transducer [116]. The changes in the rotation of the pin are then converted into electrical signals that will be displayed and graphically and numerically represented on the computer screen [115-116]. Changes in the parameters can be used to assess and diagnose coagulation related disorders. If the R time and clot kinetic time is prolonged in combination with a decrease in MA and α-angle can be used to diagnose disorders such as hypofibrinogenaemia, thrombocytopenia or thrombocytopathy [114]. Combination of a decrease clot kinetics, R time and increase MA and α-angle [114]. The technique can be used during cardiac surgery, liver transplantation and obstetric haemorrhage [114].

Graphical representation of TEG tracing

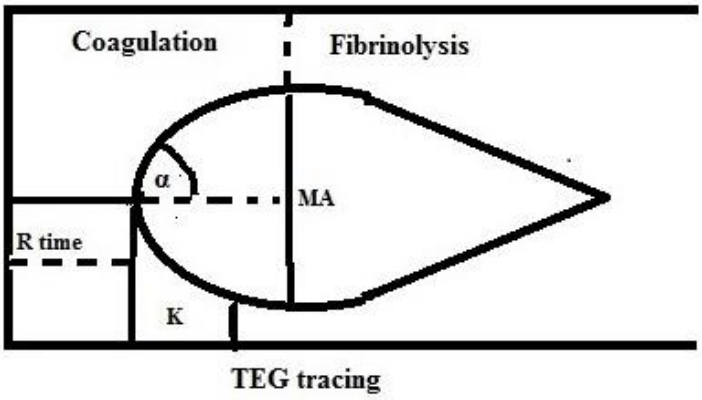

Figure 3.1: TEG tracing showing viscoelasticity parameters; R-time (mins), K-time (mins), MA (mm) and alpha angle (º).

After blood collection, 2 ml of WB was centrifuged (2000 xg, 10 min) to get platelet rich plasma (PRP), then the PRP was centrifuged (2000 xg, 10 min) to get platelet poor plasma (PPP) which was used to asses clotting parameters. A cup and a pin was mounted into the TEG instrument (TEG®6S 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 3.1. A thromboelastograph (appendix 1) was generated from which the clotting kinetics was obtained for each sample.

Table 3.1: TEG parameters normal value ranges

Parameters	Normal ranges [117]
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

3.4.1 Ribonucleic acid extraction

Total RNA was extracted from WB of T2DM patients and healthy controls using the QIAzol kit (cat. no.: 79306, QIAGEN, USA) QIAzol lysis reagent was added to the appropriate vessel for disruption and homogenization and subsequent centrifugation; 500 µl QIAzol lysis reagent was added per 500 µl WB. The tube containing the homogenized ruptured WB was placed on the bench top at RT for 5 min and 0.1 ml of chloroform per 0.5 ml QIAzol lysis reagent was added to the tubes and vortexed for 15 s.

The tubes were incubated (RT, 2-3 min) then centrifuged (12 000 xg, 15 min, 4°C). The upper, aqueous phase was transferred into a new 1.5 tube and 0. 1 ml isopropanol was added to the tubes and mixed thoroughly. The tubes were then centrifuged (12 000 xg, 10 min, 4°C). The supernatant from each tube was carefully aspirated and discarded.

A 0.5 ml aliquot of 75% ethanol was added and centrifuged (7400 xg, 5 min, 4°C). The supernatant was removed completely, and the RNA pellet was briefly air-dried. Re-dissolving of the RNA was done in 12.5 µl nuclease-free water. The total isolated RNA was quantified (1 µl) spectrophotometrically (NanoDrop2000c, Thermo-Fischer, USA). The quantified RNA was then standardized to 200 ng/µl and used to prepare cDNA which was then used for qPCR.

3.4.2 cDNA synthesis and Quantitative Polymerase Chain Reaction (qPCR)

Standardized RNA was converted to cDNA (iScript Reverse Transcriptase for RT-qPCR, Bio-Rad, South Africa) and was used for qPCR. Primers identified as shown in table 3.2 and checked for specificity using National Center for Biotechnology Information(NCBI) nucleotide blast, was used to assess the mRNA levels of FXII, FXIII and TF using a master mix (iTaq Universal SYBR Green Supermix, Bio-Rad, South Africa) containing SYBR Green fluorescence dye. The fluorescence that was detected per tube, was directly proportional to the efficacy of the PCR and the relative number of templated cDNA that was present.

Before qPCR was done on the control and T2DM samples genes were optimized to specific primer concentrations and annealing temperature (Ta) to avoid nonspecific binding and primer dimers. The thermal cycling conditions for optimization were done as followed: denaturation at 95˚C for 2-5 sec, annealing/extension were at different temperature according to the gene primers for 35-40 cycles and the melt curve analysis was done at 65-95˚C for 2 sec/step. The concentration range of the primer pairs was from 100nM-1000nM. The optimized primer concentration for tissue factor was [600nM] at Ta=54˚C, FXIIIa [500nM] at Ta=54˚C, FXII [650nM] at Ta=54˚C. See appendix 2 for melt curves of the different optimization concentrations. This was done using a Roche Light Cycler (Roche, South Africa). The mean cycle threshold was used to calculate the relative fold changed in the expression between groups using the method described by Livak and Schimittgen, 2001 [118].

Table 3.2: Primer sequences for quantitative polymerase chain reaction

Gene	Primer sequence (5´-3´)	Annealing Temperature (º)	Optimized concentration (nM)
Tissue factor Sense Anti-sense	CTACTGTTTCAGTGTTCAAGCAGTGA CAGTGCAATATAGCATTTGCAGTAGC	54	600

Factor XIII-A Sense Anti-sense	CCCAGAAACAGACACGTACATTCT TTCTCATTGTCCAGATACACAGCAT	54	500
Factor XII Sense Anti-sense	GGGCCACCACGCATTTT TGTCGCCACTCCAGACGAA	54	650
18S RNA Sense Anti-sense	ACACGGACAGGATTGACAGA CAAATCGCTCCACCAACTAA	49.1	500

3.5.1 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 seconds (s) and incubated [55ºC, 10 minutes (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 -20ºC, quantified using spectrophometrically (Nanodrop 2000c, Thermo-Fischer, South Africa), standardised to 50 ng/µl and used for polymerase chain reaction (PCR).

3.5.2 Polymerase Chain Reaction

Polymerase chain reaction is a powerful scientific technique that is used in molecular biology, through the amplification of small amounts of DNA. It was developed by Kary Mullis a biochemist in 1984 [119]. This technique is mainly based on the separation of a double helix DNA strand into two single DNA strands and in the presence of DNA polymerase and building blocks, nucleotides [adenine (A), thymine (T), cytosine (C) and guanine (G)] each strand can be replicated. There are 3 steps that are involved for the amplification of the strands, denaturation, annealing and extension [119]. During the first step, denaturation of the provided DNA strand is denatured through the increase of temperature (90-97 ºC). During the second step, the nucleotide building blocks anneal to the single stranded DNA and this occurs at a low temperature (50-60 ºC). In the final step, there is extension of the new DNA strand being formed by DNA polymerase to form complementary strands and this is done at 72 ºC. The three steps are repeated for 20-40 cycles and during each cycle DNA is amplified by two folds.

The PCR products are then electrophoresed on an agarose gel using gel electrophoresis. For visualization the gel is stained with GR green and the gel is viewed under an ultraviolet (UV) light. To check the size of the amplicon a DNA ladder (cat. no.: 33045, Bio-Line, London, UK) are electrophoresed together with the PCR products.

3.5.3 Polymerase Chain Reaction optimization and cycle conditions

To avoid non-specific binding and primer dimers, primer concentration of the two of FXIII-A SNPs (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, the detail of which is shown in Table 3.3 The other components were kept constant and only the primer

concentration was varied. Primer concentration range was between 100-1000 nM. For verification, the PCR products were electrophoresed on a 1.8% GR Green stained agarose gel (120 V; 30 min) and the gels were viewed under UV light (Vilber Lourmat, France) and a gel documentation system with a UV transilluminator was used to detect the bands in the agarose gel following electrophoresis. Concentration selection was based on the PCR band that had the appropriate weight PCR product and no primer dimers.

Table 3.3: Mastermix components

Optimized primer conditions (25 µl reaction)	**Val34Leu**	**Tyr204Phe**
GoTaq® Hot Start (2X)	1 X	1 X
dNTPs (each)	400 µM	400 µM
MgCl2	4 mM	4 mM
Forward primer	200 nM	500 nM
Reverse primer	0.2 µM	0.5 µM
DNA template	50 ng/µl	50 ng/µl

Val34Leu [192 base pairs (bp)] and Tyr204Phe (113bp) were amplified using the forward and reverse primer concentrations listed in table 3.1, in a 25 µl reaction.

The primer sequences were:

Val34Leu: Forward: 5'- CATGCCTTTTCTGTTGTCTTC-3'

Reverse: 5'- TACCTTGCAGGTTGACGCCCCGGGGCACTA-3'

Tyr204Phe: Forward: 5'- GGAAACAGTCTGGTTTGGTAA-3'

Reverse: 5'- ACCCCGATGTCATTCAGGACG-3'

The primers were subjected to PCR with the following PCR conditions;

1. Initial denaturation at 95ºC for 2 minutes

2. Denaturation at 95ºC for 30 seconds

3. Annealing, each SNP primer pair had its own annealing temperature for 30 seconds

* Val34Leu: 48.2ºC

*Tyr204Phe: 48.9ºC

4. Extension at 74ºC for 30 seconds

5. Final extension at 74ºC for 5 minutes

3.5.4 Polymerase Chain Reaction- Restriction Fragment Length Polymorphism

Restriction fragment length polymorphism (RFLP) is based on the enzymatic properties of a restriction endonuclease (RE), which cleaves DNA into small fragments if there is an nucleotide change (polymorphism) in the sequence [120]. The fragmentation of the sequence is then assessed by means of electrophoresis, where the fragments are run on an agarose gel and the fragments are separated by means of size or molecular weight.

The restriction of the 2-above mentioned FXIII-A SNPs were done in a 25 µl reaction (3 µl PCR product, 5 µl 1X NE buffer and 1 µl RE (1 000U) (New England BioLabs) stated in table 3.4 and their specific RE cleavage sites. The restriction fragments were then analysed using gel electrophoresis on a 3% agarose gel (GR Green stained) for 30 min and then visualised.

Table 3.4: 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	37ºC and inactivation at 65ºC for 20 min	37ºC and inactivation at 65ºC for 20 min

3.6 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 was considered to be significant when the P-value < 0.05. The Hardy-Weinburg equilibrium was calculated using the Court-lab calculator, and the association analysis was conducted using the Fischers Exact Test.

Chapter 4

Results

4.1 Cohort demographics

The mean age of the controls was 43.52 (22-86) years old and the T2DM group was 59.64 (32-76) 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 checked in patient hospital records and confirmed by co-supervisor (Dr Soma, medical doctor). 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. In order to eliminate gender and ethnicity bias, the samples selected to assess clot kinetics and mRNA levels were matched accordingly, all samples genotyped were also evaluated as a total cohort and stratified according to ethnicity to calculate an association [odds ratio (OR)] using the Fischer's exact test.

4.2 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 (Figure 4.1, $p<0.0001$). 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 (Figure 4.2, $p=0.1960$ and Figure 4.3 $p=0.246$, respectively). No significant difference in the speed of fibrin accumulation was observed between T2DM patients and controls (Figure 4.4, $p=0.0565$). There was a significant decrease in the maximum speed of the clot growth (MRTG) in T2DM compared controls (Figure 4.5, $p= 0.0074$). The time period before the maximum speed of the clot growth (TMRG) was significantly increased (Figure 4.6, $P<0.0001$) and the total clot resistance (TTG) was significantly decreased (Figure 4.7, $p= 0.0385$) in T2DM.

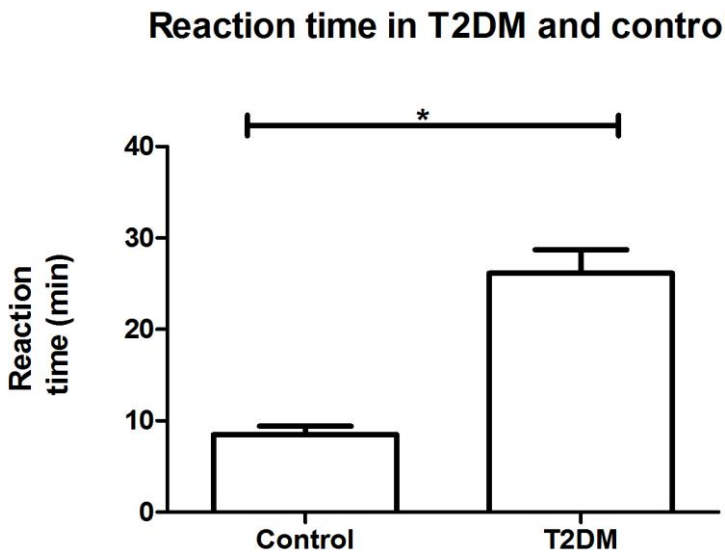

Figure 4.1: Bar graph illustration of the reaction time between T2DM patients and controls subjects. Reaction time was higher in T2DM patients compared to controls (26.15±2.56 min vs. 8.48±0,92 min, p<0.0001).

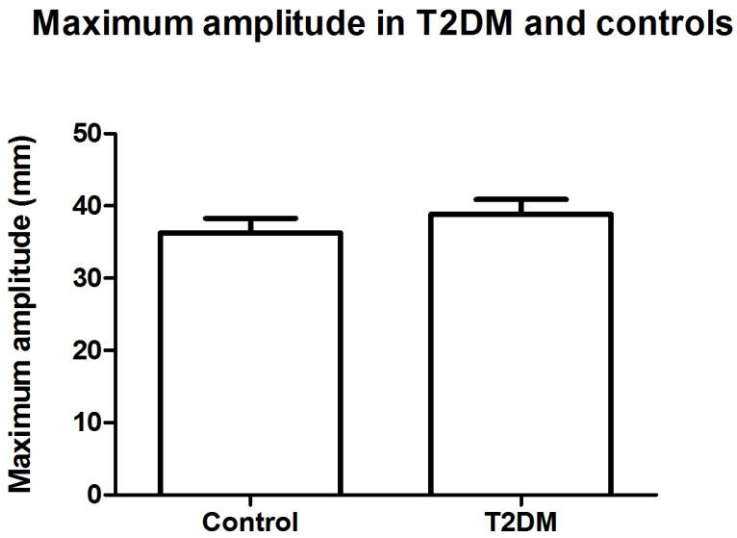

Figure 4.2: Bar graph illustration of the maximum amplitude between T2DM patients and control subjects. There was no significant difference between T2D patients and controls for the maximum amplitude (38.84±2.04mm vs. 36,29±1.98 mm, p=0,1960).

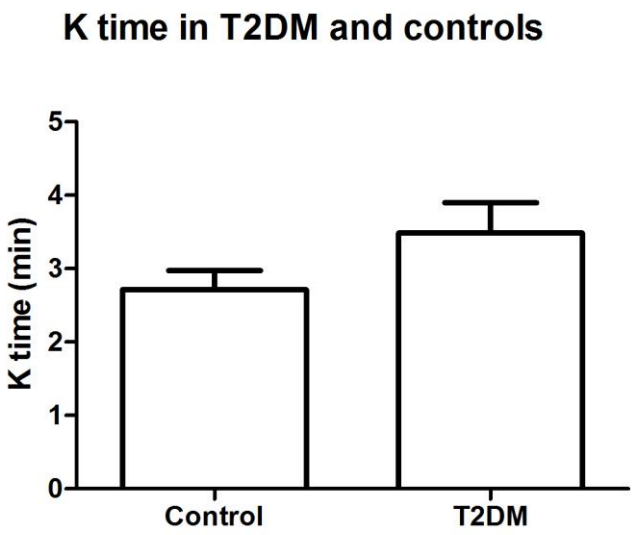

Figure 4.3: Bar graph illustration of the clot kinetics (K time) between T2DM patients and control subjects. No significant difference in K time was found between T2DM patients and control subjects (3.48±0.42 min vs. 2.71±0.26 min, p=0.246).

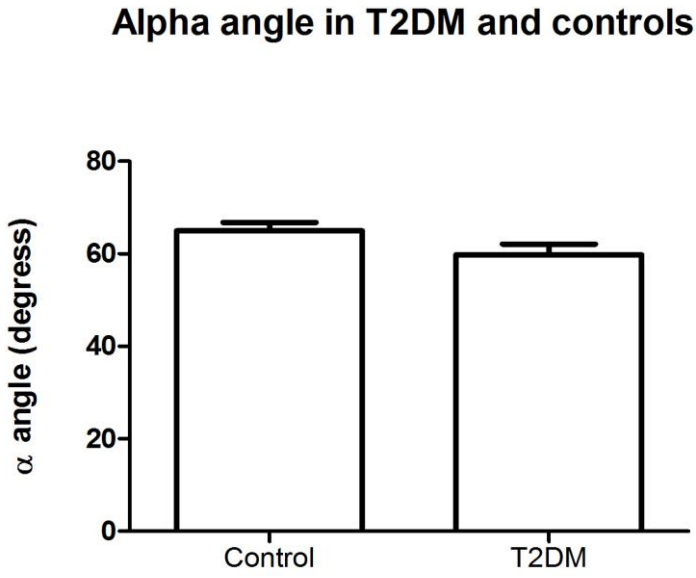

Figure 4.4: Bar graph illustration of the speed of fibrin accumulation (alpha angle) between T2DM patients and control subjects. No significant difference in the alpha angle was found between T2DM patients and control subjects (59.78±2.30° vs. 64.99±1.79°, p=0.0565).

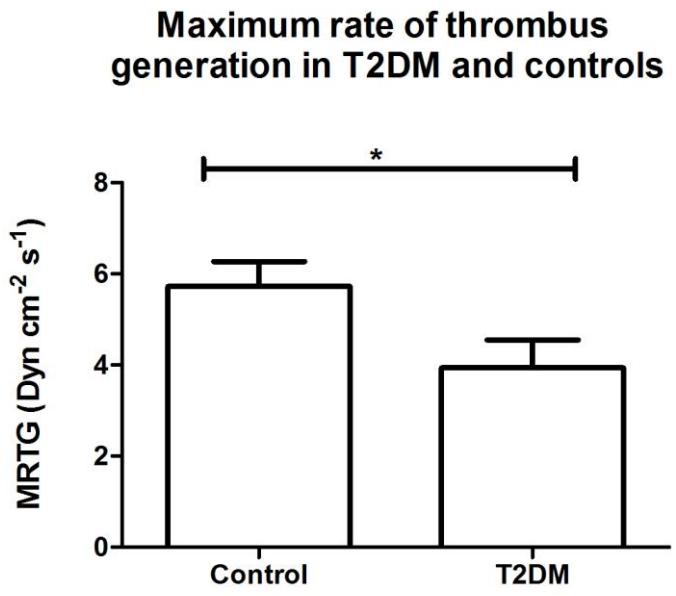

Figure 4.5: Bar graph illustration of the maximum speed of the clot growth (MRTG) between T2DM patients and control subjects. MRTG was significantly decreased in T2DM compared to controls (3.94±0.61 dyn cm^{-2} s^{-1} vs. 5.73±0.54 dyn cm^{-2} s^{-1}, p=0.0074).

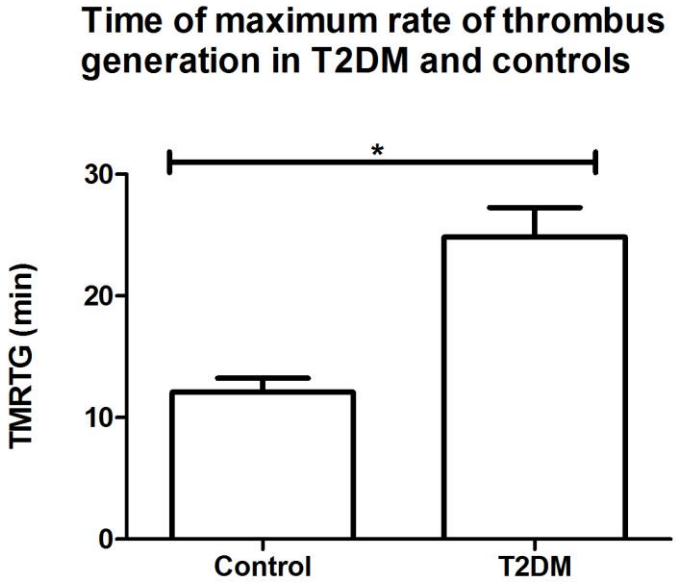

Figure 4.6: Bar graph illustration of the time period before maximum speed of the clot growth (TMRTG) between T2DM patients and control subjects TMRTG was significantly increased in T2DM compared to controls (24.85±2.41 mins vs. 12.09±1.15 mins, p<0.0001).

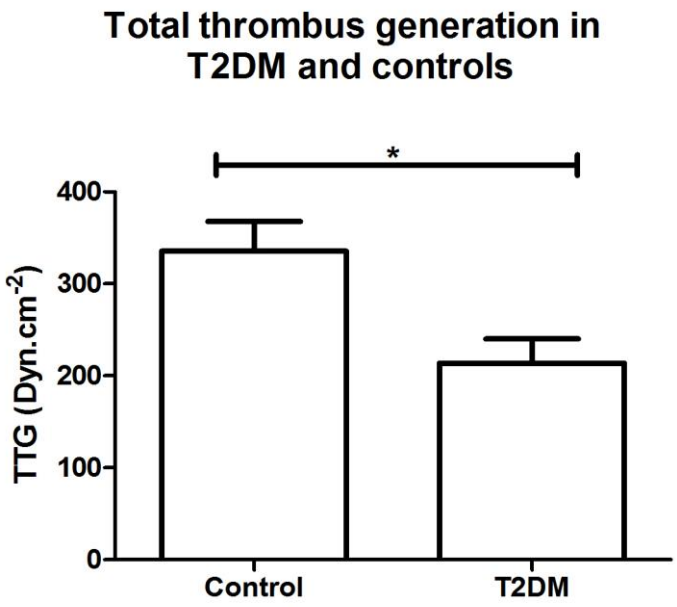

Figure 4.7: Bar graph illustration of the total clot resistance (TTG) between T2DM patients and control subjects. TTG was significantly decreased in T2DM compared to controls (213.8±26.29 dyn cm^{-2} vs. 335.5 ± 32.33 dyn cm^{-2}, p=0.0385).

4. 3 Coagulation factors expression levels

A comparison of delta cycle threshold when analysing mRNA levels of FXIII-A was higher, which translates to a 4.38-fold decrease in T2DM patients compared to controls (Figure 4.8, p=0.0033). The fold decrease observed in T2DM for Factor XII and tissue factor mRNA was not significantly different compared to controls (Figures 4.9 and 4.10, p>0.05).

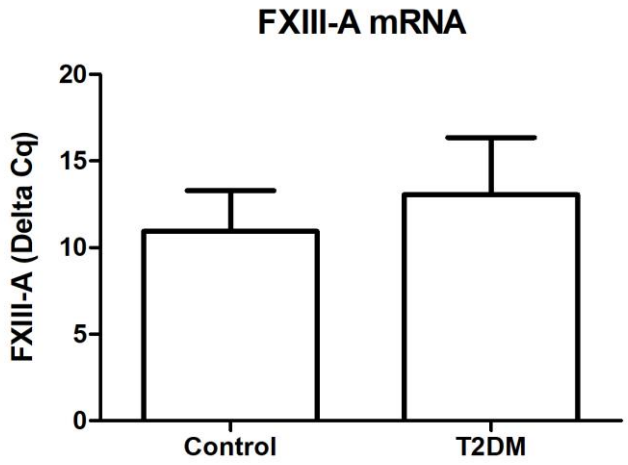

Figure 4.8: Bar graph illustration of delta cycle threshold of FXIII-A. Factor XIII-A mRNA levels were 4.38-fold lower in T2DM patients compared to controls (p=0.0033).

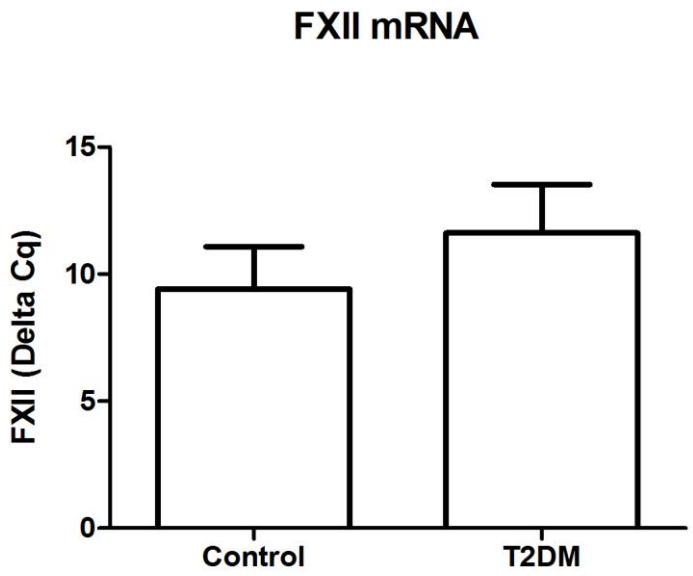

Figure 4.9: Bar graph illustration of delta cycle threshold for FXII in T2DM and controls, p>0.05.

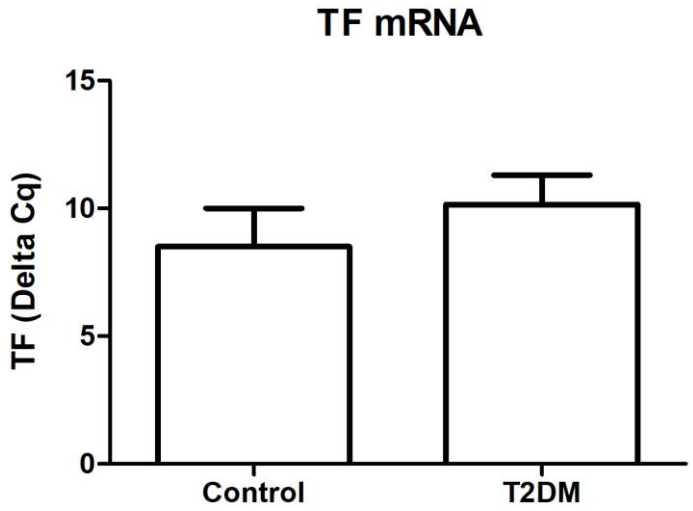

Figure 4.10: Bar graph illustration of delta cycle threshold for tissue factor (TF) mRNA in T2DM patients compared to control subjects, p>0.05.

4.4 Single nucleotide polymorphisms

Confirmation of the presence of the SNPs was done using PCR and RFLP gels (see Appendix 3). Control and T2DM genotype distribution conformed to Hardy-Weinberg equilibrium (HWE) ($p>0.05$). There was a higher prevalence of the wildtype genotype of both SNPs assessed in T2DM, Val34Val (G/G) (Odds ratio (OR)=1.670, 95% confidence interval (CI)=0.97-2,89, p=0.0747) and Tyr204Tyr (A/A) (OR=3.57, 95% CI=2.37-5.45, $p<0.0001$). Table 4.1 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=1.047, 95% CI=0.32-3.41, p=1.00) and Caucasians (OR=1.813, 95% CI=0.90-3.65, p=0.0979). The 204Phe variant was more frequent in the Caucasian population (OR=2.306, 95% CI=1.37-3.89, p=0.0023) compared to the Black population (OR=1.933, 95% CI=1.00-3.72, p=0.0693).

Table 4.1: Genotype distribution in T2DM patients and control subjects.

	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**
T2DM (100)	81	16	3	57	5	38
Controls (100)	70	22	8	23	12	65
Caucasians (128)	86	32	7	41	14	67
Blacks (65)	55	5	4	26	2	36
Black Controls (25)	21	1	2	4	0	20
Black T2DM (40)	34	4	2	22	2	16
Caucasian Controls (74)	49	21	6	19	12	45

Caucasian T2DM (54)	37	11	1	25	2	22

4.5 The effect of the presence of Val34Leu and Tyr204Phe SNPs on Factor XIII-A mRNA levels

Analysis of the presence of the Val34Leu polymorphism the mRNA levels of FXIII-A was not significantly altered in both controls and T2DM (Figure 4.11, p=0.2178, Figure 4.12 and p=0.5059 respectively). The presence of the polymorphic variant 204Phe (Tyr/Phe and Phe/Phe) had significantly higher levels, (14.46±3.68 vs. control delta cycle threshold:12.03±2.57, 5.39-fold increase) of FXIII-A mRNA in T2DM (Figure 4.13, p=0.0105). No significance was observed in control subjects for the 204Phe variant (Figure 4.14, p=0.7383).

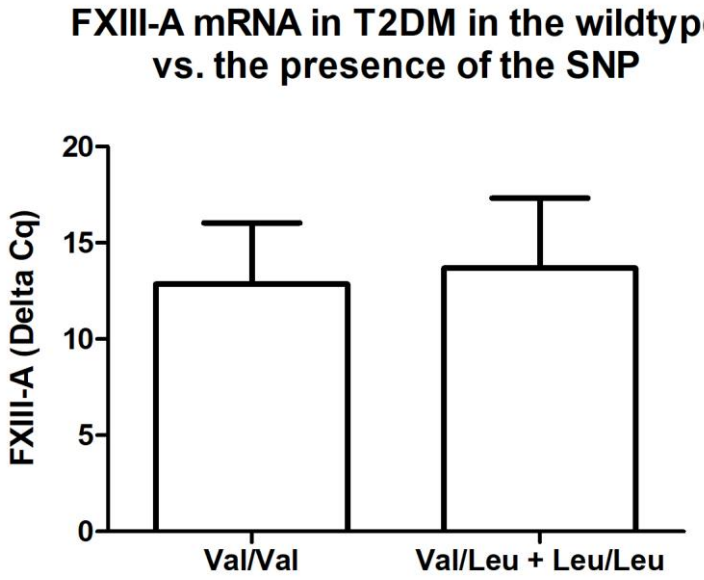

Figure 4.11: Bar graph illustration of cycle threshold for FXIII-A mRNA in the presence or absence of the Val34Leu variant in T2DM, p>0.05.

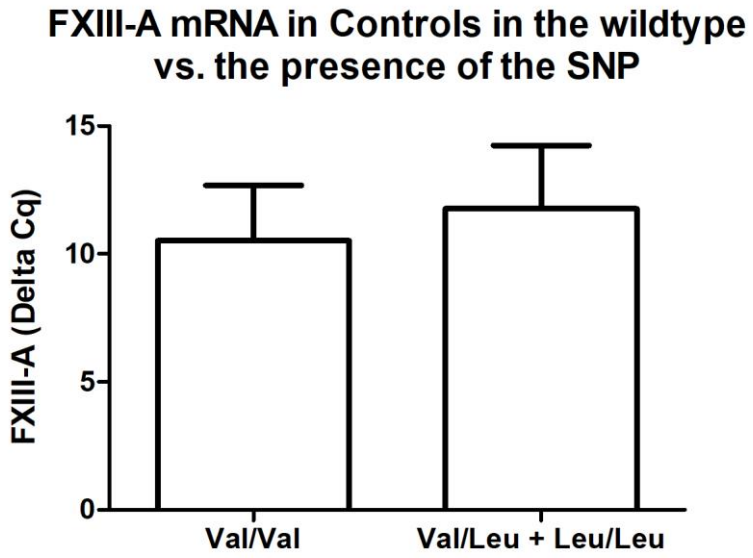

Figure 4.12: Bar graph illustration of delta cycle threshold FXIII-A mRNA in the presence or absence of the Val34Leu variant in control subjects, p>0.05).

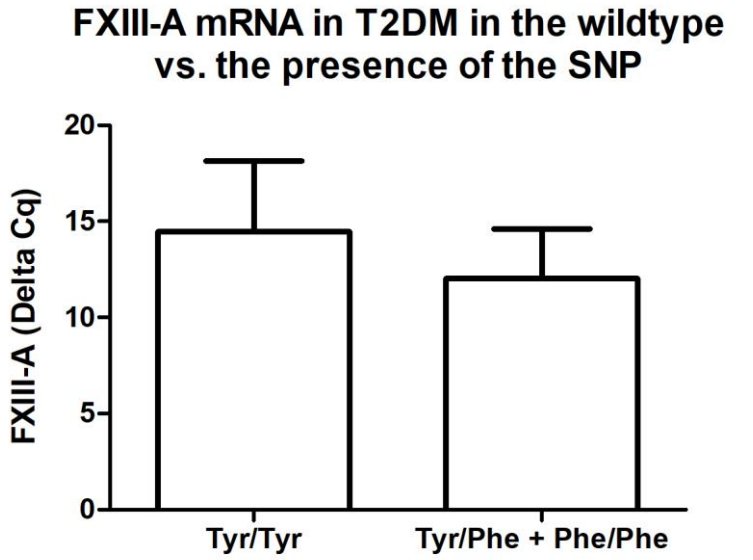

Figure 4.13: Bar graph illustration of delta cycle threshold for FXIII-A mRNA in the presence or absence of the Tyr204Phe variant in T2DM patients. A significant 5.39-fold increase was found in the presence of the 204Phe variant compared to the wildtype (p=0.0105).

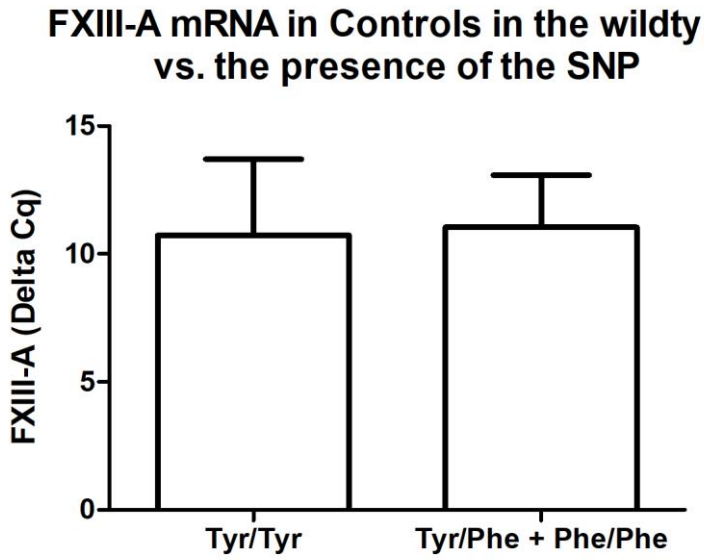

Figure 4.14: Bar graph illustration of FXIII-A mRNA levels in the presence or absence of the Tyr204Phe variant in control subjects, p>0.05.

4.6 The effect of the presence of Val34Leu and Tyr204Phe on clot formation

Analysis on the effect of FXIII-A SNPs on the clotting parameters was done and there was no significant variation for the R-time (figure 4.15A, p=0.7868) and MA (figure 4.16A, p=0.8987) found between the Val34Leu genotypes. However, there was a significant increase in the maximum rate of thrombus generation (MRTG) (Figure 4.17A, p=0.0457) and total thrombus generation (TTG) (Figure 4.19A, p=0.0367), while there was a slight decrease in the time before the maximum rate of thrombus generation (TMRTG) (Figure 4.18A, p=0.4864) in T2DM patients that had the 34Leu variant present.

The reaction time was longer in T2DM patients with the homozygous wildtype genotype Tyr/Tyr compared to the heterozygous Tyr/Phe and homozygous variant Phe/Phe genotypes (10.47±1.63min vs. 6.56±0.73min, respectively p=0.033, Figure 4.15B). The clot strength (MA) was slightly higher in T2DM patients with the homozygous wildtype genotype compared with patients with the heterozygous Tyr/Phe and homozygous variant Phe/Phe genotypes (38.17±2.39mm vs. 34.5±2.68mm, respectively, p=0.55 Figure 4.16B). In addition, in the presence of

204Phe there was a significant increase in the maximum rate of thrombus generation (Figure 4.17B, p=0.0020) and significant decrease in the TMRTG (Figure 4.18B, p=0.0017), while TTG was not significantly affected by the presence of the variant in T2DM patients.

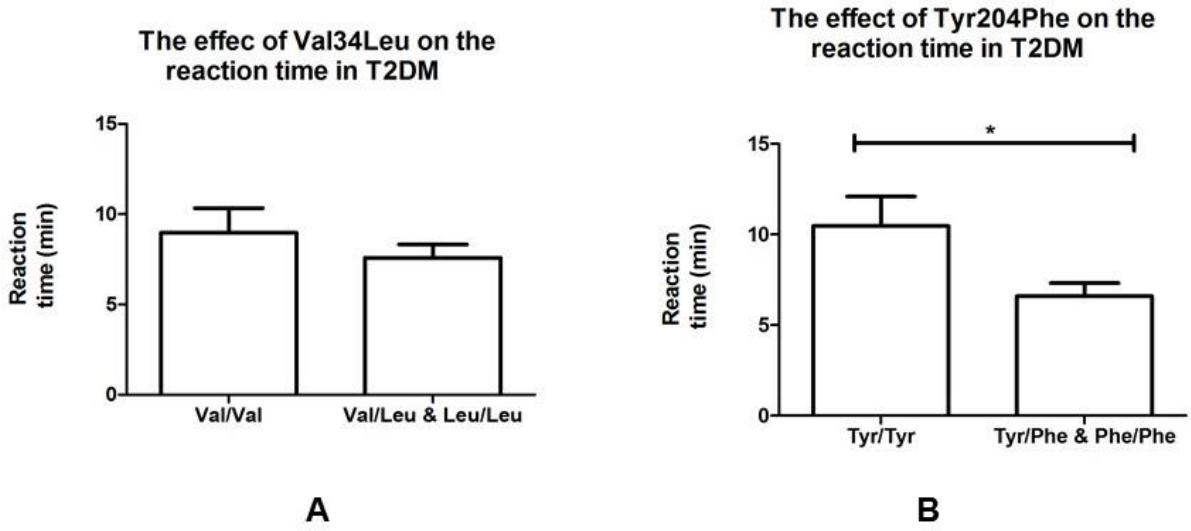

Figure 4.15: Bar graph illustration of reaction time in the presence or absences of the Val34Leu and Tyr204Phe variants in T2DM patients. A: In T2DM there was a slight decrease in the time for the first measurable clot to form (R time) in the presence of the 34Leu variant (7.66±0,74min vs. 8.97±1.45min, p=0.7868). B: Presence of FXIII-A 204Phe altered the reaction time where there was a decrease in the reaction time (6.68± 0.73min vs. 10.47± 1.63min, p= 0.0334).

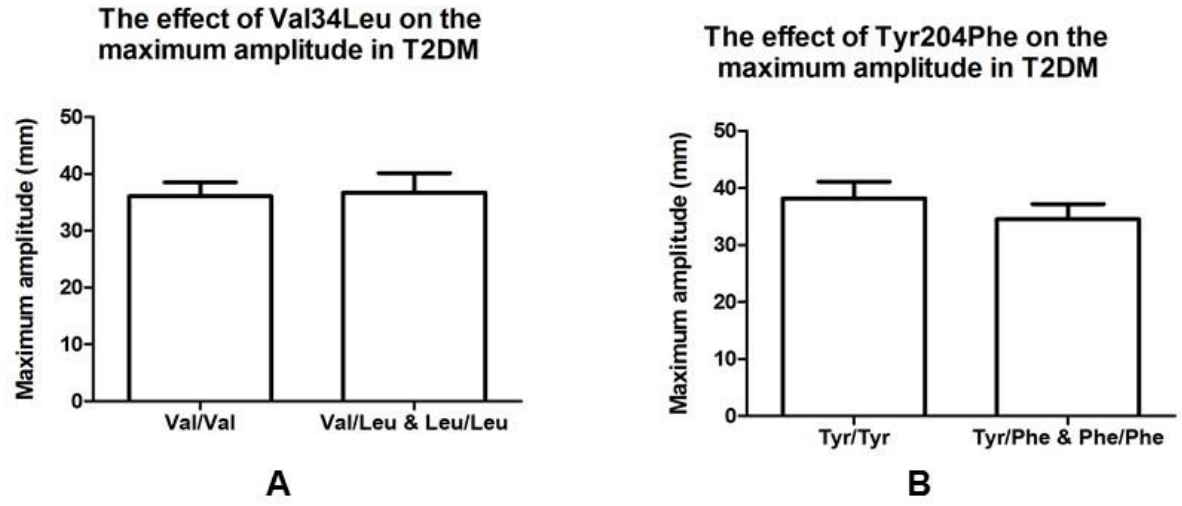

Figure 4.16: Bar graph illustration of maximum amplitude in the presence or absences of the Val34Leu and Tyr204Phe variants in T2DM patients. A: No difference in clot strength was found between the Val34Leu genotypes (36.71± 3.43mm vs. 36.06± 2.47mm, p= 0.8987). B:

There was a slight decrease in the clot strength in the presence of 204Phe (34.5± 2,68mm vs. 38.17± 2.93, p= 0.5535).

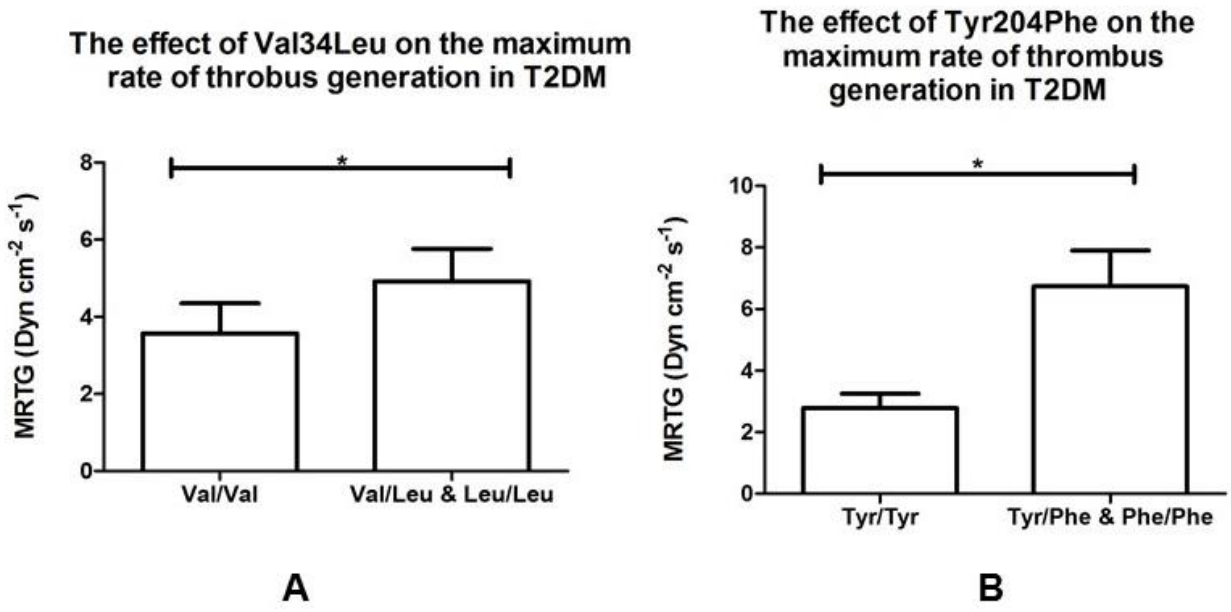

Figure 4.17: Bar graph illustration of the maximum speed of clot growth in the presence or absences of the Val34Leu and Tyr204Phe variants in T2DM patients. A: The maximum speed of clot growth was significantly increased in the presence of 34Leu (4.92±0.84 Dyn.cm^{-2}s^{-1} vs. Val/Val 3.57±0.78 Dyn.cm^{-2}s^{-1}, p=0.0457). B: The maximum speed of clot growth was higher in T2DM patients with the 204Phe variant (6.74±1.17 Dyn.cm^{-2}s^{-1} vs. Tyr/Tyr 2.79±0.47 Dyn.cm^{-2}s^{-1}, p=0.0020).

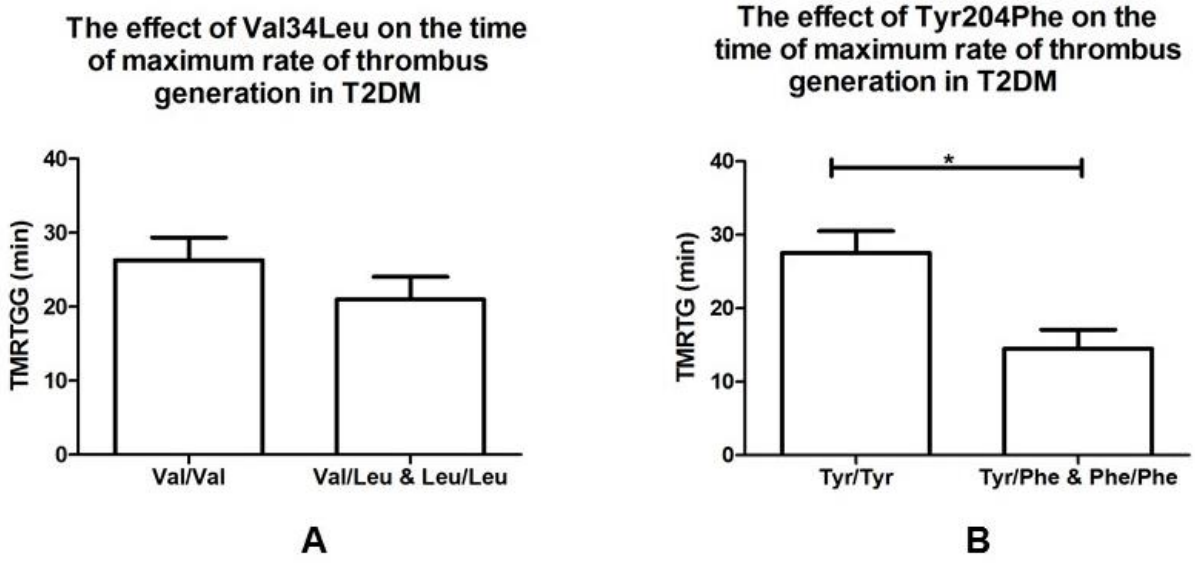

Figure 4.18: Bar graph illustration of the time period before maximum speed of clot growth in the presence or absences of the Val34Leu and Tyr204Phe variants in T2DM patients**.** A: The

presence of 34Leu did not alter TMRTG (20.99±3.0 min vs. Val/Val 26.28±3.09min, p=0.4864). B: In the presence of the 204Phe polymorphism TMRTG was significantly reduced (14.49±2.59 min vs. Tyr/Tyr 27.52±3.01 min, p=0.0017).

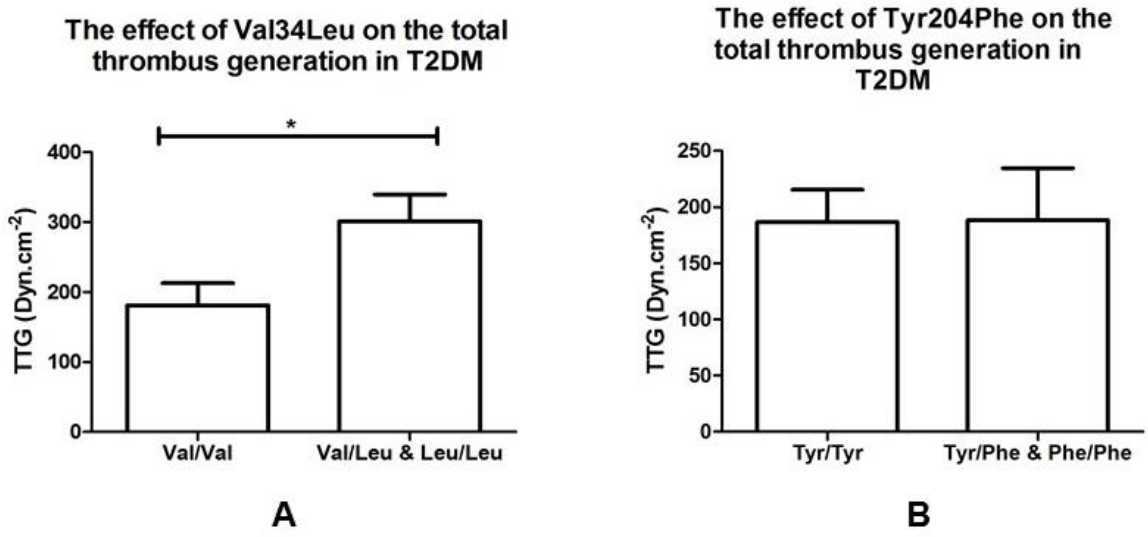

Figure 4.19: Bar graph illustration of the total clot resistance in the presence or absences of the Val34Leu and Tyr204Phe variants in T2DM patients. A: TTG was significantly increased by the presence of the 34Leu polymorphism (301.3±38.22 Dyn.cm^{-2} vs. 181.3±31.68 Dyn.cm^{-2}, p=0.0367). B: There was no difference in TTG in the presence of the 204Phe polymorphism (188.4±46.28 Dyn.cm^{-2} vs. 186.8±28.66 Dyn.cm^{-2}, p=0.2788).

In control subjects the 34Leu polymorphism had no significant effect in all the clotting parameters assessed reaction time (figure 4.20A, p= p=0.8398), MA (figure 4.21A, p=0.8987), MRTG (figure 4.22A, p=0.0596), TMRTG (figure 4.23A, p= p=0.6876) and TTG (figure 4.24A, p= p=0.6876). The 204Phe polymorphism only had an effect on the R time, where there was a reduction in the time for the first measurable clot to form (Figure 4.20B, p=0.0334), while the other parameters MA (figure 4.21B, p=0.5535), MRTG (figure 4.22B, p= p=0.4513), TMRTG (figure 4.23, p=0.0654) and TTG (figure 4.24, p=0.7696) were not significantly affected by the polymorphism.

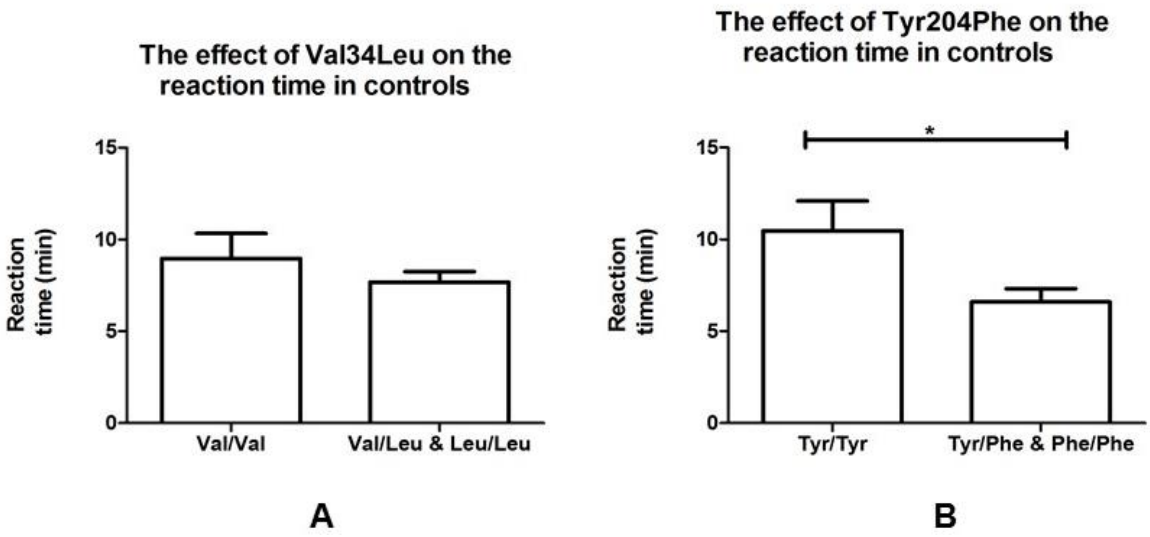

Figure 4.20: Bar graph illustration of the reaction time in the presence or absences of the Val34Leu and Tyr204Phe variants in control subjects. A: In control subjects the 34Leu polymorphism had no significant effect on the R time (7.68±0.57 min vs. 8.97±1.36 min, p=0.8398). B: The 204Phe polymorphism only had a significant decrease on the R time (6.59±0.73 min vs. 10.47±1.63 mins, p=0.0334).

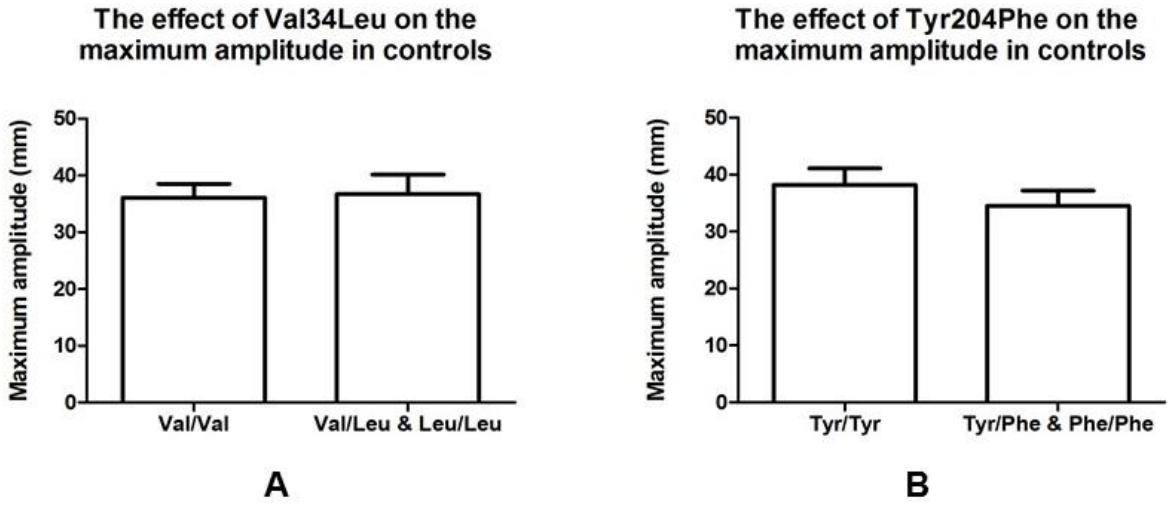

Figure 4.21: Bar graph illustration of maximum amplitude in the presence or absences of the Val34Leu and Tyr204Phe variants in control subjects. A: The 34Leu polymorphism did not affect MA (36.71±3.43mm vs. 36.06±2.47, p=0.8987). While the 204Phe polymorphism slightly decreased the MA, however it was not significantly affected (34.50±2.68mm vs. 38.17±2.93mm, p=0.5535).

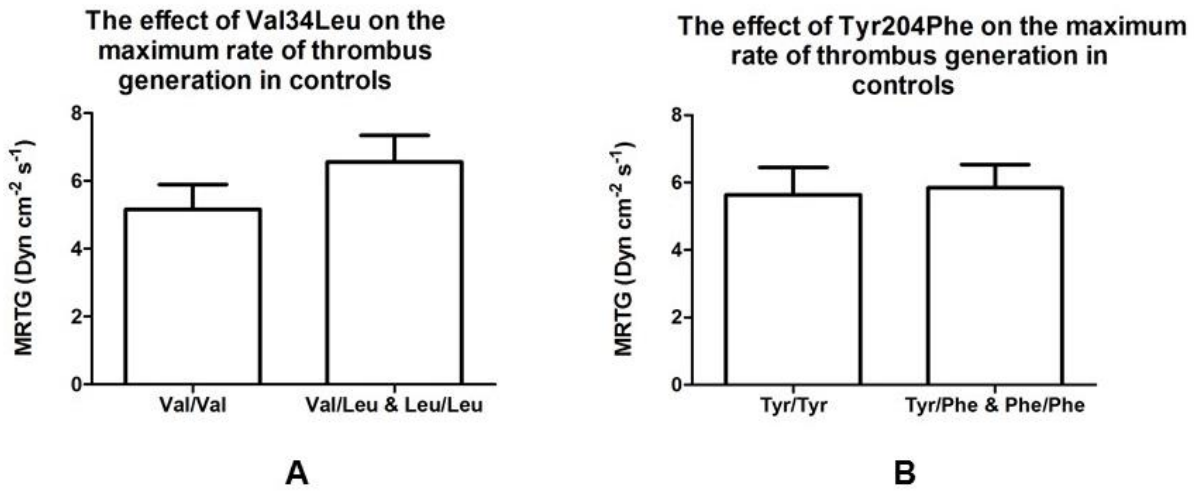

Figure 4.22: Bar graph illustration of the maximum speed of clot growth in the presence or absences of the Val34Leu and Tyr204Phe variants in control subjects. A: The 34Leu polymorphism slightly increased the MRTG (6.57±0.77 Dyn.cm^{-2}s^{-1} vs. 5.17±0.73 Dyn.cm^{-2}s^{-1}, p=0.0596). B: There was no significant effect on MRTG by the 204Phe polymorphism (5.84±0.69 Dyn.cm^{-2}s^{-1} vs. 5.64±0.82 Dyn.cm^{-2}s^{-1}, p=0.4513).

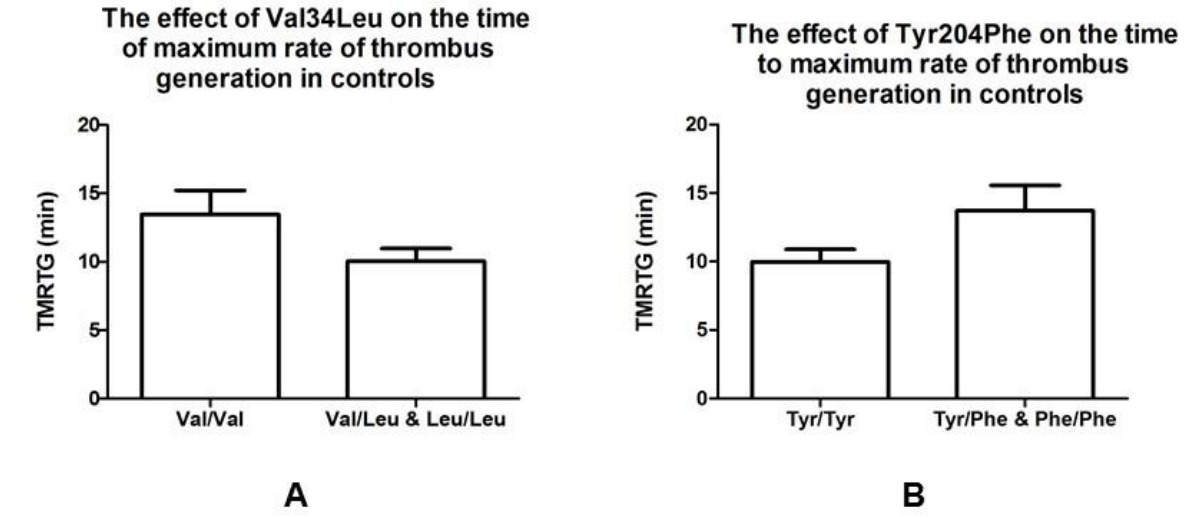

Figure 4.23: Bar graph illustration of the time period before maximum speed of clot growth in the presence or absences of the Val34Leu and Tyr204Phe variants in control subjects. A: Presence of the 34Leu polymorphism slightly decreased the TMRTG (10.05±0.92 min vs. 13.46±1.77 mins, p=0.6876). B: The 204Phe polymorphism slightly increased the TMRTG (13.72±1.84 min vs. 9.96±0.92 mins, p=0.0654).

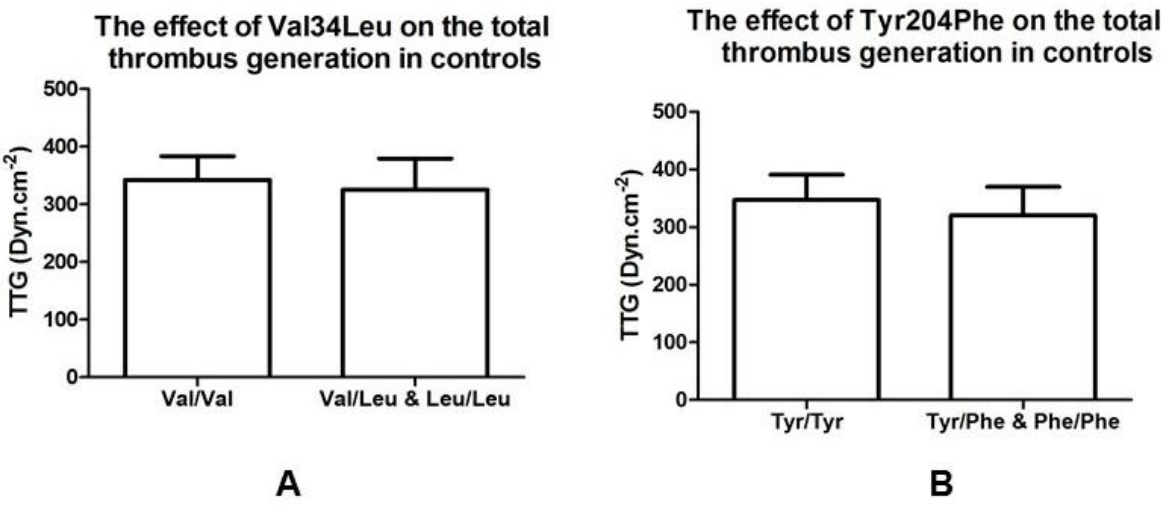

Figure 4.24: Bar graph illustration of the total clot resistance in the presence or absences of the Val34Leu and Tyr204Phe variants in control subjects. A: The 34Leu and B: 204Phe polymorphisms slightly decreased the TTG (325.1±54.11 Dyn.cm^{-2} vs. 342,4±41.25 Dyn.cm^{-2}, p=0.6876; 320.1±49.70 Dyn.cm^{-2} vs. 347.2±43.66 Dyn.cm^{-2}, p=0.7696).

Chapter 5

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 [121]. The main coagulation factors production site is found in the hepatocytes and the sinusoidal endothelial cells in the liver [122]. 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) [122]. Lallukka *et al*. 2017 demonstrated that insulin-resistant individuals have increased clotting factor activity [122]. 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 [122].

Insulin resistance affects endothelial cells, macrophages and platelets, which influences atherosclerosis and vascular disease [123]. Hyperglycaemia induce changes to the structure and function of proteins such as fibrinogen by glycation [124]. The degree of progression of T2DM is influenced by insulin resistance that then leads to dyslipidaemia, hypertension, hypofibrinolysis and hypercoagulation [125]. Increased cardiovascular disease occurrence and mortality is linked with hyperinsulinemia [125]. 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 [114-126].

Premature atherosclerosis in T2DM patients plays an important role in increasing the risk of cardiovascular complications in these patients [126]. In T2DM, hypofibrinolysis and prothrombic characteristics are associated with increased clot time [126]. There are studies that have shown that the antifibrinolytic factor TAFI is upregulated in T2DM patients which is one of the factors that contribute to hypofibrinolysis and it is also associated with enhanced risk of developing cardiovascular diseases [10-14].

Low grade inflammation influences the development of atherothrombosis [126]. Altered metabolism in T2DM patients enhances the development of atherosclerosis [127].

Mechanisms such as “low-grade inflammation, impaired insulin signalling, increased ROS production and altered glucose metabolism” result in endothelial dysfunction [127]. In poorly controlled T2DM patients, increased levels of plasminogen activator (PAI-1) inhibitor-1, and low levels of tissue-type plasminogen activator (tPA) which causes decrease fibrinolysis activity [127].Development of atherosclerosis is also triggered by obesity, as it results in the enhancement of proinflammatory cytokine secretion resulting in an inflammatory state [127].

The exact mechanisms of how hyperglycaemia and impaired insulin secretion plays a role in the pathogenesis of the thrombotic milieu is still unclear [128]. In a study done by Zheng *et al*. (2015), they found that low-grade inflammatory markers and clot formation was increased in patients with T2DM with microvascular complications compared to T2D patients without microvascular complications [128]. Hyperglycaemia increases the incidence of thrombotic events due to the up-regulation of coagulation factors and antifibrinolytic factors [126].

T2DM patients are prone to develop premature atherosclerosis. They also have hyper-reactive platelets, high levels of procoagulant factors and impaired fibrinolysis all of which enhance thrombotic events [129]. Procoagulant factor, tissue factor expression level is low during resting condition however it is upregulated during a low-grade inflammatory condition as is observed in T2DM patients [129]. Expression level of tissue factor in atherosclerotic plaques is elevated in patients with unstable angina and also in T2DM patients [129]. In addition, tissue factor levels cause the formation of advanced end products and reactive oxygen species, which leads to the development of a clot [129].

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 [130]. Deficiency of coagulation factors FXII, FXI,FVIII,FII (thrombin) and FI (fibrinogen) (see figure 5.1) affects the reaction time (R time) and clot strength (MA) [131]. Absence of FXII results in “no clot formation”, while the absence of FXI results in an increased R-time and decreased α-angle [131]. Interestingly, the absence of FIX decreases the R time, whereas FVIII absence was increased and the α-angle was decreased [131]. 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

[131]. The clot strength and fibrin crosslinking is mainly affected by FXIII and FI (fibrinogen), in addition to FII, FVII, FX and FXII [131]. FI also plays an important role in the speed of fibrin accumulation (α-angle) [131].

In a study done by 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 [132]. They also found a decrease in the clot strength [132]. In another study done by 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±8mm; p<0 001) which is associated with an increased risk of T2DM patients developing myocardial infarction [130]. They also found no significant difference for the R time and the K time in T2DM patients [130].

Coagulation cascade

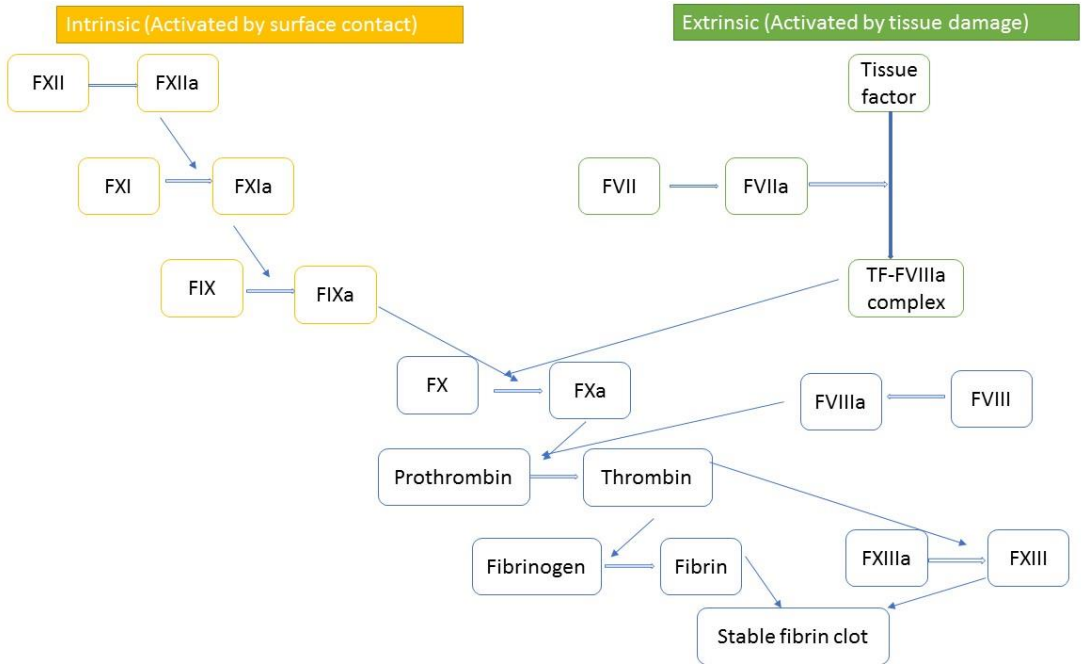

Figure 5.1: The coagulation cascade illustrating the intrinsic and extrinsic pathways and the individual factors involved.

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.* (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) [117]. 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 [117]. 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 activation of the intrinsic pathway of the coagulation cascade is caused by surface contact which results in the activation of factor XIII, while the activation of the extrinsic pathways is caused by tissue damaged which results in the activation of tissue factor. In this study mRNA levels of FXIII-A were found to be low, while FXII and tissue factor were not significantly different in T2DM compared to controls.

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)[30]. During FXIII activation by thrombin, the transglutaminase forms covalent e-(g-glutamyl)-lysly bonds between the fibrin monomers and this results in a stable fibrin clot which is resistant to fibrinolysis [30]. In the current study the expression levels of this factor was lower in T2DM patients and clot kinetics indicate a hypocoaguable state.

One study showed that FXIII cross-linking decreased the fibrin fibre diameter, which increases resistance to fibrinolysis [133]. 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 [133]. One of the causes of FXIII deficiency is caused by the presence of mutations, which are then regarded to have a protective effect against thrombotic diseases and in other cases lead to the development of haemorrhagic disorders [134].

The coding gene of the FXIII-A subunit is composed of 15 exons, mapped on the 6p24-25 chromosome, with a size of 160kb, 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 28kb [26]. 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) [26]. There are different SNP target sides on subunit A as shown on figure 5.2

Factor XIII structure

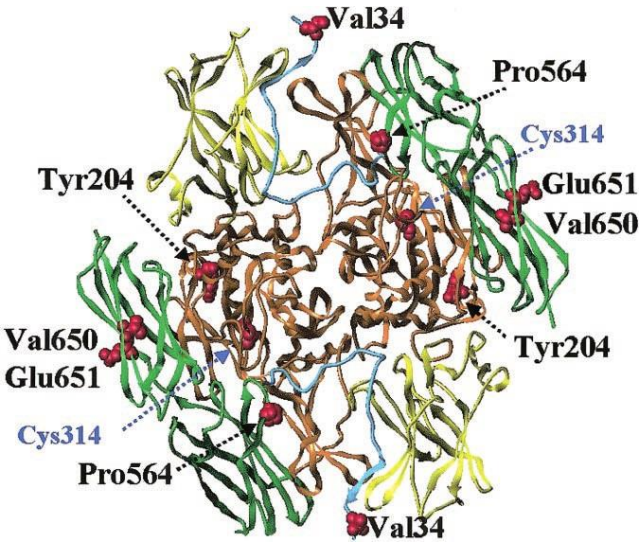

Figure 5.2: Factor XIII structural illustration of subunit A [20].

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 [28,30]. It has been suggested to result in the increased activity of the transglutaminase and increased fibrin cross-linking [28-31]. 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[135]. 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 [135]. 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 fibers[27].

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 [27]. 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 [27]. In this study significantly higher FXIII-A mRNA levels was observed in T2DM in the presence of the 204Phe variant.

Many FXIII-A Val34Leu studies have been done on conditions such as vascular thrombosis[29], myocardial infarction[30,136-137], deep venous thrombosis[30], ischaemic stroke[31], coronary artery disease [138] and recurrent pregnancy loss [27]. Many of these studies focused on the association of the variant with the investigated condition. The variant had a protective effect against myocardial infarction[136], deep venous thrombosis, brain infarction [30] and vascular thrombosis[29]. 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 [30]. Another proposed hypothesis is that there is linkage disequilibrium between FXIII-A Val34Leu and other functional FXIII-A variants [30].

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) [134]. One study proposed that due to the location of the polymorphism, it serves as a risk factor for the development of thrombotic disorders [139]. A study conducted by Yildirm *et al.* (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%) [140]. 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 [140]. In this study both Val34 and the Tyr204 wildtype alleles were more prevalent in control subjects compared to T2DM patients.

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 [137]. In a study done by 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) [27].

In another study done by Pruissen *et al.* (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 [31]. The presence of both allele copies (Phe/Phe) increased the risk much higher by 77-fold compared to the wild-type (Tyr/Tyr) [31], while there was a 8-fold increase risk for the intermediate genotype (Tyr/Phe)[31]. 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 [141].

The prevalence of the Val34Leu variant differs amongst different ethnic groups. Hancer *et al.* (2006) found that the variant is high in Western countries where it occurred at 48% and 51% in England [100,142], 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 [136]. In addition, it is rarely found in the Asian population [136]. 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)[28]. The study also found that the prevalence of the variant in the Black population was lower compared to other populations [28].

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.* (2004) found that the occurrence of the Tyr204Phe was not different between patients that have recurrent miscarriages and controls [143]. They also showed that the Tyr204Phe variant is rare in the Brazilian population[143]. In another study they found that there is no association of recurrent miscarriages and Val34Leu polymorphisms [144].

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) [112]. While carriers of either 1 or 2 copies of the 34Leu allele had a reduced risk of developing

haemorrhagic stroke (OR=0.7) [112]. 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 . 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 clot 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.* (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 [145].

Nucleotide substitutions that a located in the coding region can result in the alteration of gene mRNA and protein levels [32]. An increase or decrease in mRNA level of a gene is influenced by the ability transcription factor to bind to the regulatory region, also by post-transcriptional modification, which affect the early degradation of mRNA [34]. In addition, nucleotide changes are able to influence the translation of proteins [34]. Changes in nucleotide alter the coding of amino acid and determine the functionality of the protein [33].

Chapter 6

Conclusion

6.1 Conclusion

This study as 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 of clots assessed using PRP and WB. The exclusion of these components in the current study enabled the assessment of how fibrin influences clot kinetics.

In addition, mRNA levels of key coagulation factors, FXII and TF which are key in the initiation of coagulation were not significantly different in T2DM compared to controls. Only FXIII-A the key factor involved in the fibrin network formation, was significantly lower in T2DM.

An association of the Val34Leu SNP and T2DM was found, with an odds ratio of 1.670. An even stronger association was found with the Tyr204Phe SNP and T2DM with an odds ratio of 3.57, with an indication that the polymorphic variant, 204Phe, offers a protective effect being more prevalent in controls. The Val34Leu did not influence FXIII-A mRNA levels while the presence of Tyr204Phe polymorphic variant had significantly high levels of FXIII-A mRNA in T2DM.

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.

6.2 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 attempted to match the controls accordingly.

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.

The clot kinetics was conducted on PPP specifically in order to assess the effect of FXIII-A on the cross-linking process and therefore the influence of platelets which play a key role in coagulation was not considered in the results discussed.

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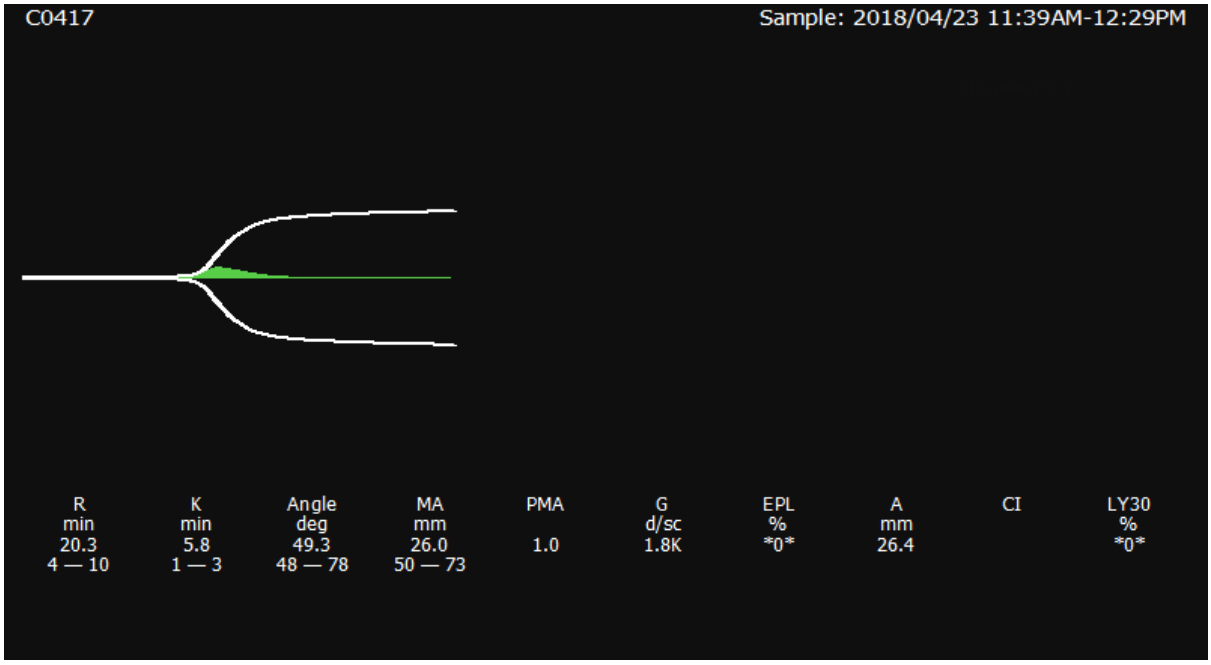
Appendix 1

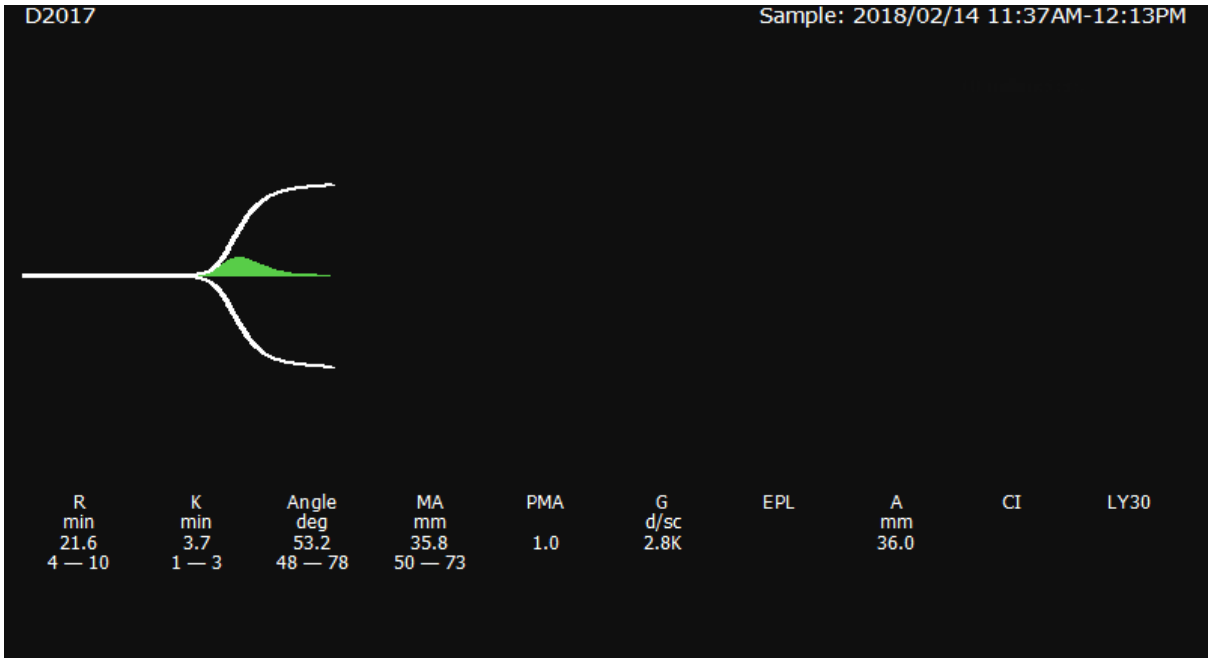
Thromboelastography Traces

Thromboelastography Traces

Control trace

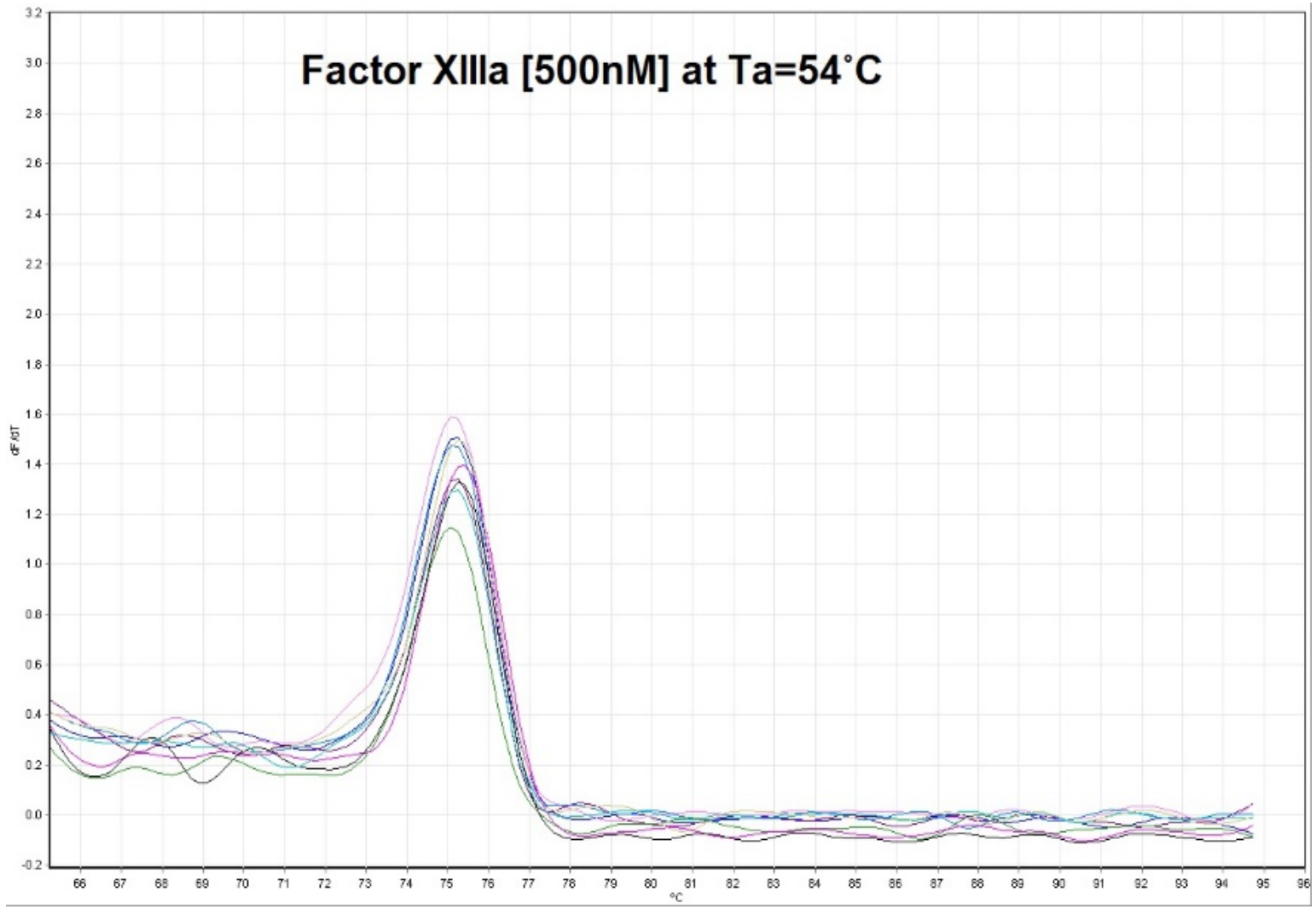


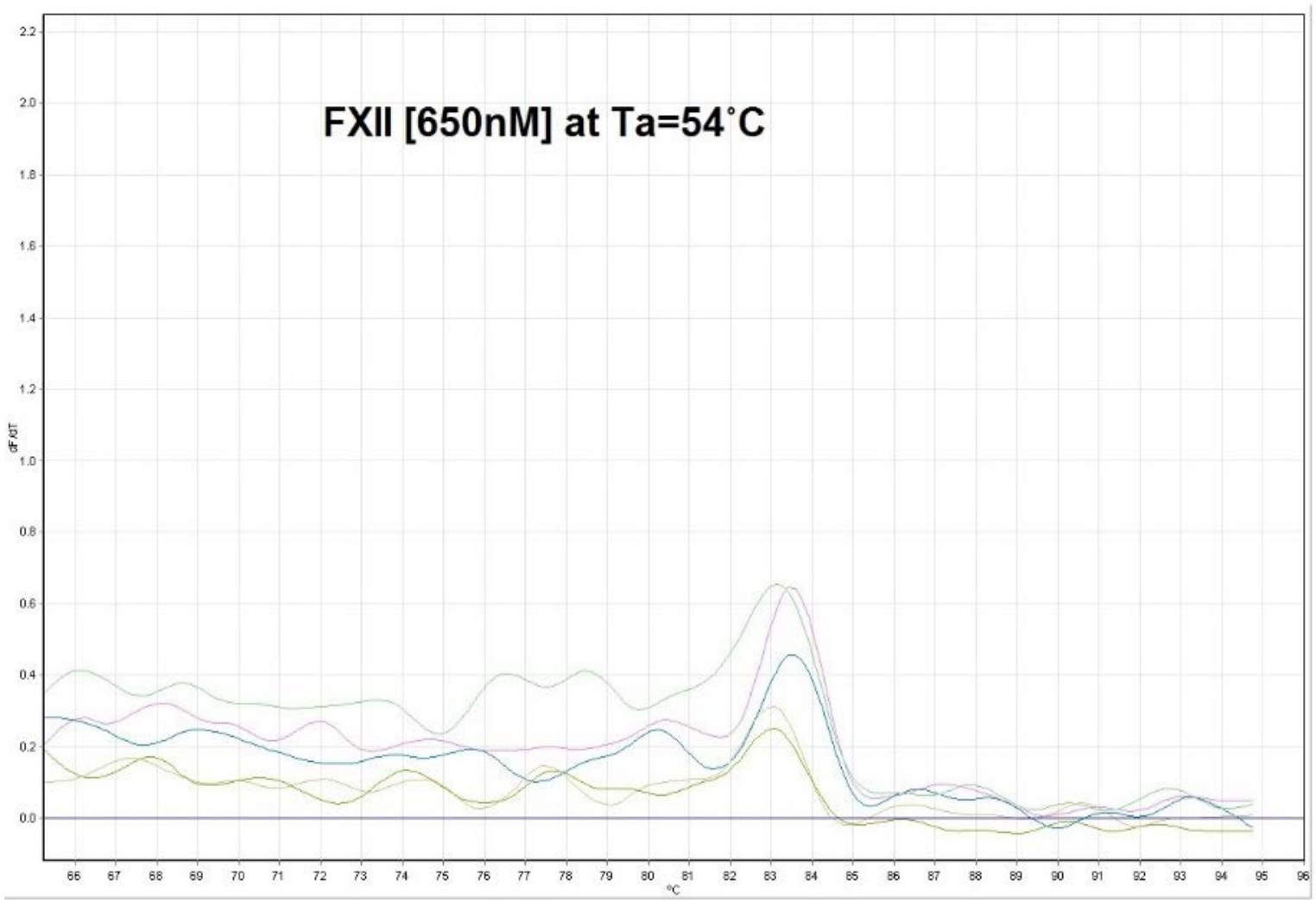
T2DM trace

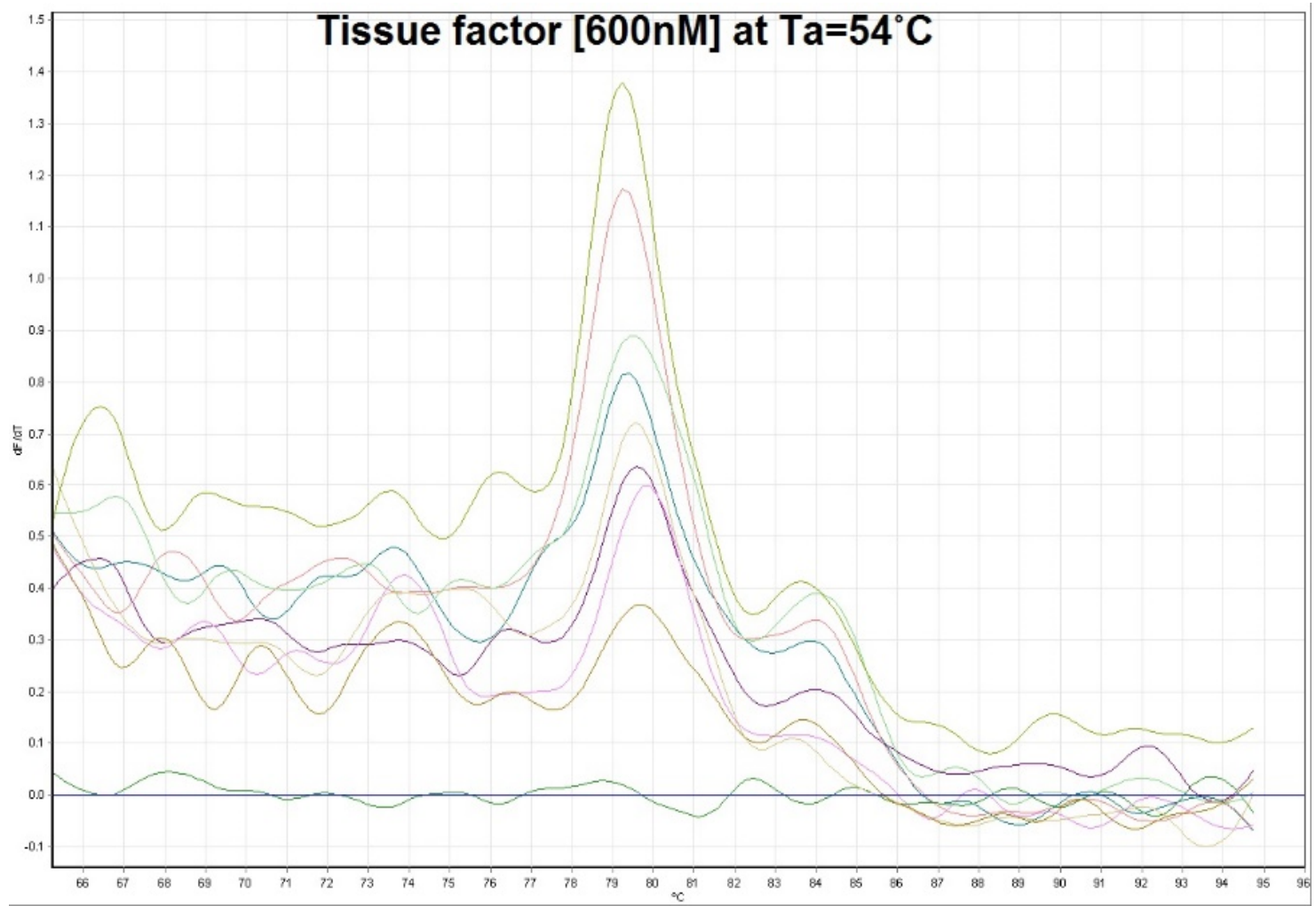


Appendix 2

Quantitative PCR melt curves

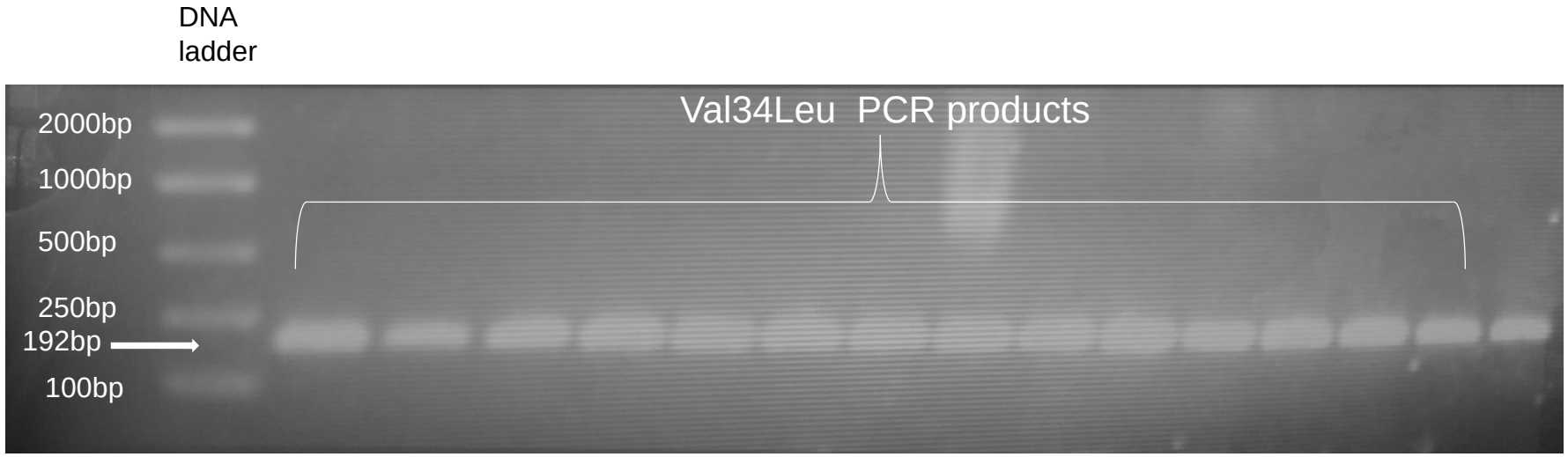

Factor XIIIa [500nM] at Ta=54˚C
dF/dT
°C


FXII [650nM] at Ta=54˚C
-dF/dT
°C

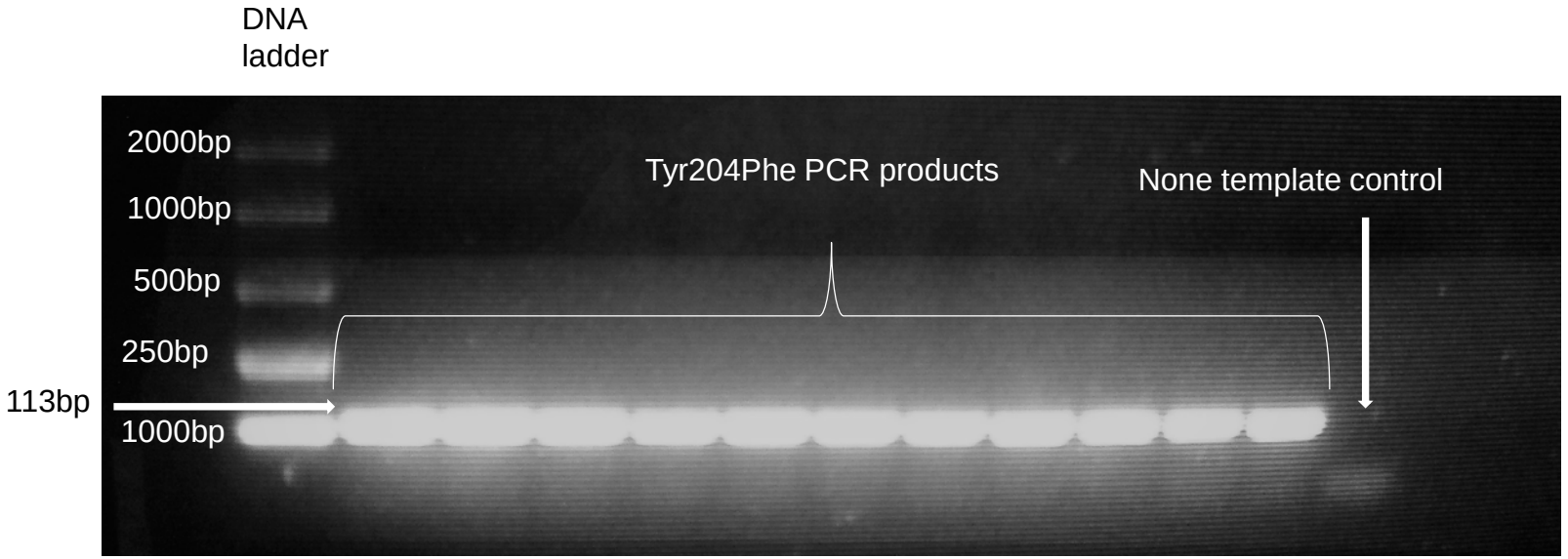

Tissue factor [600nM] at Ta=54˚C
-dF/dT
°C

Appendix 3
PCR and RFLP gel images

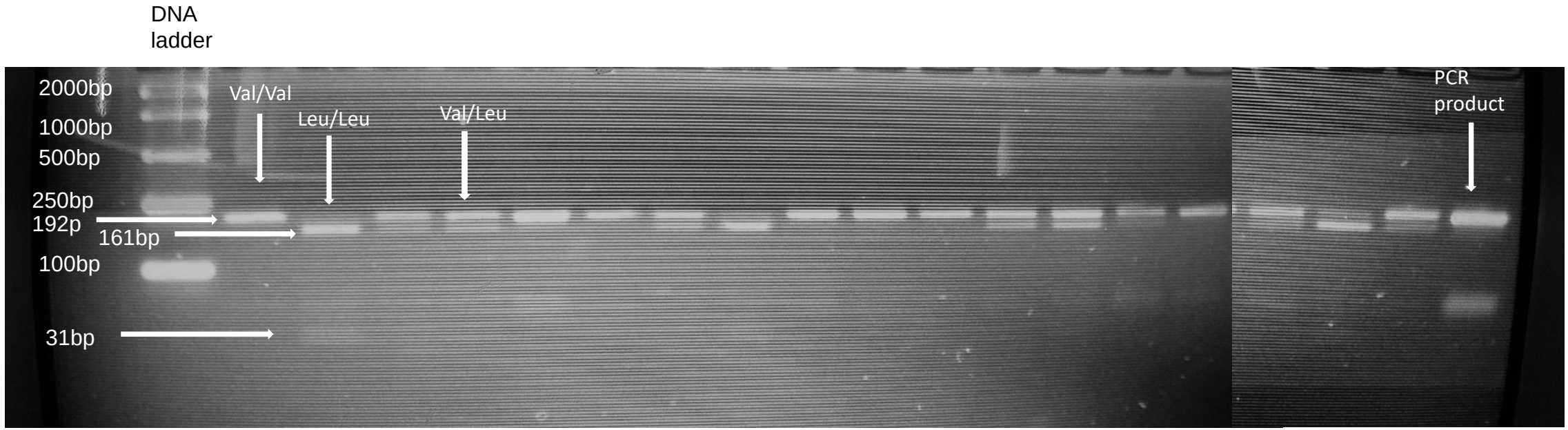
Val34Leu PCR gel



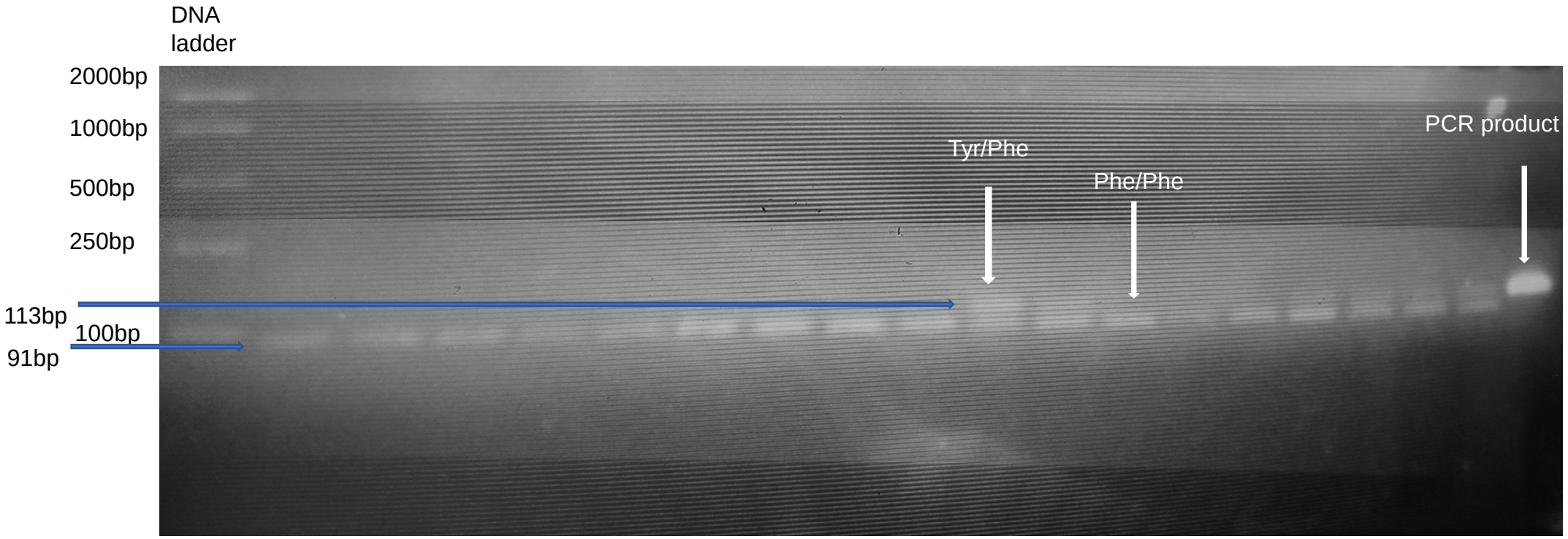
Tyr204Phe PCR gel



Val34Leu RFLP gel



Tyr204Phe RFLP gel



Appendix 4
Statistical Approval Letter

BIOSTATISTICS UNIT
MEDICAL RESEARCH COUNCIL
Biostatistics Unit
Private Bag X385
Pretoria
0001
Tel: 012 339 8523 / Fax: 012 339 8582

LETTER OF STATISTICAL SUPPORT

8 March 2017

This letter is to confirm that M Phasha from **Faculty of Health Sciences**, University of Pretoria discussed the Project with the title "**A molecular investigation into regulation of coagulopathy and pro-inflammatory pathways in patients with type 2 diabetes".** We therefore confirm that we are aware of the project and also undertake to assist with the statistical analysis of the data generated from the project.

Data Analysis

The aim of the study is to assess the expression of molecules that are involved with coagulation and inflammation in untreated and LPS-treated whole blood and identification of factor XIIIa SNPs between diabetic and control subjects in South African Cohort. Mean, median, and the standard deviation will be given for continuous variables and proportions together with their associated 95% confidence intervals will be given for SNPs. T-test will be used to compare the difference in mRNA levels in whole blood of controls and type 2 diabetes patients or otherwise the non-parametric equivalent will be used if data is not normal. ANOVA will be undertaken to determine the effect of LPS on genes. Data will be captured into Excel and all statistics will be done using STATA, where p-value<0.05 will be considered statistically significant.

Sample size

For the purpose of this study, a sample of 100 from both diabetic and control group is required.

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Appendix 5

MSc Committee Approval Letter

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

MSc Committee
School of Medicine
Faculty of Health Sciences

MSc Committee
22 February 2018

Dr A Phulukdaree
Department of Physiology
Faculty of Health Sciences

Dear Dr,

Ms MN Phasha, Student no 13113047

Please receive the following comments with reference to the MSc Committee submission of the abovementioned student:

Student name	Ms MN Phasha	**Student number**	13113047
Name of study leader	Dr A Phulukdaree		
Department	Physiology		
Title of MSc	Assessing gene expression of coagulation factors and Factor XIII polymorphisms in type 2 diabetes mellitus patients		
Date of first submission	March 2017		
April 2017	• Please make the changes as requested at the previous meeting. Check that the document adheres to the rules of good scientific writing. • Correct budget table. • Change objectives to include genes that will be studied.		
May 2017	• Thank you for submitting the revised protocol.		
February 2018	• Thank you for submitting the ethics clearance certificate. • Thank you for nominating the examiners.		
Decision	This protocol has approved. Ethics approval has been obtained. The examiners have been approved.		

Yours sincerely

Prof Marleen Kock
Chair: MSc Committee

MSc Committee, School of Medicine
Faculty of Health Sciences
University of Pretoria,
Private Bag X323
Pretoria 0001, South Africa
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Appendix 6

Ethics Approval Letter

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

18/07/2017

Approval Certificate
New Application

Ethics Reference No: 269/2017

Title: Assessing gene expression of coagulation factors and Factor XIII polymorphisms in type 2 diabetes mellitus patients.

Dear Miss Marry-Ann Phasha

The **New Application** as supported by documents specified in your cover letter dated 4/07/2017 for your research received on the 10/07/2017, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 17/07/2017.

Please note the following about your ethics approval:
- Ethics Approval is valid for 1 year
- Please remember to use your protocol number (**269/2017**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of **6 monthly written Progress Reports**, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed,PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

☎ 012 356 3084 deepeka.behari@up.ac.za / fhsethics@up.ac.za http://www.up.ac.za/healthethics
✉ Private Bag X323, Arcadia, 0007 - Tswelopele Building, Level 4, Room 60, Gezina, Pretoria

Appendix 7

Informed Consent Forms

Informed Consent Forms

Participants information and information leaflet and information consent form

(Type 2 diabetes mellitus Group)

Ethics No:269/2017

Title of the Project: Assessing gene expression of coagulation factors and Factor XIII polymorphisms in type 2 diabetes mellitus patients

Principal Investigator: Marry-Ann Phasha, Department of Physiology University of Pretoria (under the supervision of Dr Phulukdaree, Dr P Soma and Prof Pretorius)

Introduction (invitation to participate in a Research study)

Introduction (invitation to participate in a Research study)

You are invited to participate in a research study in the Department of Physiology Faculty of Health Sciences, University of Pretoria. You must be 18 years or older to participate in the study. This information leaflet is to help you understand what the study is about should you like to participate in the study. The researcher will explain the reason for the drawing of blood.

The blood drawn will be used to look at the amount of molecules involved in the clotting of blood, how the blood clots and if there are any genetic change affecting blood clotting. One tube (5 ml) of blood will be drawn to be used for to check blood clotting and genetic changes. The drawn blood will be used for this study only and the remaining amount will be stored at -80˚C for the experiments to be repeated. The blood will be stored for the duration of the study which is two years. You are more than welcomed to ask any questions, should you not fully understand the information provided in this leaflet. You should not agree to take part unless you are completely happy about all the procedures involved.

Purpose of the study?

The main aim of this study is to look at important molecules that are involved in blood clotting, to measure blood clotting parameters and to look at the presence of any genetic changes in molecules involved in blood clotting between type 2 diabetes mellitus patients and healthy controls.

There are small molecules that help regulate blood during damage or injury of the blood vessel. The amounts of the molecules the body produces is different in everyone depending if a person has a medical condition or is healthy. To do the study, blood sample will be needed. Three procedures will be used to assess the blood: (1)Thromboelastography (TEG) which will be used to measure how the blood clots, (2) Quantitative Polymerase Chain Reaction (qPCR) which will be used to look at the amounts of important molecules involved in blood clotting, (3) Restriction Fragment Length Polymorphism- Polymerase Chain Reaction (RFLP-PCR) which will be used to look at the presence of any genetic changes in one of the molecules involved in blood clotting.

Who will draw the blood*?*

Dr Prashilla Soma will draw the blood

Description of your involvement

Should you be interested and agree to participate in this research study, you will be asked to provide your personal details such as your name, surname, gender and age. Furthermore, you will be asked about your health condition for example, if you are on any chronic medication, if you smoke

Ethical approval

The protocol of this study will be sent to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria to ensure that the study meets the requirements of Declaration of Helsinki (updated: October 2013) which has served as a guideline directing methods and intensions of this study. A copy of the Declaration may be obtained from the investigator should you wish to review it. Should you want to find out if the study has been approved by the Faculty of Health Sciences Research Ethics Committee here is their contact details: 012-3563085

Research knowledge obtained in this study

The laboratory part of this study is not intended to benefit you, but will add more our knowledge on how the clotting process is regulated on a genetic level and will form part of a MSc dissertation in the Department of Physiology Faculty of Health Sciences, University of Pretoria.

May procedures result in discomfort or side effects?

Drawing the blood may result in a bruise at the puncture site, swelling of the vein or infection or bleeding from the site. For your protection, the procedure will be performed under sterile conditions by a qualified phlebotomist.

Confidentiality

All information obtained during this study is strictly confidential. Data that may be reported in scientific journals will not include any information which identifies you as a patient in this study.

Any information uncovered regarding your test results or state of health will be held strictly confidential in this study. If there are any findings of importance to your health, you will be informed but this information will not be disclosed to any third party without your written permission.

What are your rights as a participant in this study?

Your participation in this study is completely voluntary. Should you agree to take part in the study, you may change your mind and discontinue your participation without stating any reason. Even if you decide to withdraw before the study is completed, it will be acceptable. The investigator has the right to withdraw you from the study if it is in your best interest.

Informed consent

I hereby confirm that I have been informed by the investigator, MN Phasha about the nature, conduct benefits and risks of this study. I have also received, read and understood the above written information (Patient Information Leaflet and Informed Consent) regarding the research. I am aware that the results of the study including personal information regarding my sex, age, date of birth, initials and diagnosis will be anonymously processed into a study report. I may at any stage, without prejudice, withdraw my consent and participation in the study. I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.

Patient's name (Please print)…….

Patient's signature……
Date……

Medical practitioner's signature……
Date……

Principal investigator signature……
Date……

<u>Participant information</u>

(Type 2 diabetes mellitus Group)

Ethics No: 269/2017

Title of the Project: Assessing gene expression of coagulation factors and Factor XIII polymorphisms in type 2 diabetes mellitus patients

Principal Investigator: Marry-Ann Phasha, Department of Physiology University of Pretoria (under the supervision of Dr Phulukdaree, Dr P Soma and Prof Pretorius)

Information filled in by the investigator

Sample ID	
Age	
Sex	
Inflammation condition	
Does the patient smoke?	
Is the patient on the pill if female	
Any other chronic condition (including anaemia)?	
Pills that the patient uses?	
Pills that the patient used 2 weeks prior?	
Was the patient diagnosed with type 2 diabetes 3 months (or more) prior?	
Has the patient been previously diagnosed with cancer, or received immunosuppressive treatment for any other condition?	

Information leaflet and Informed consent form for study participants

[Control Group]

Ethics No:269/2017

Title of the Project: Assessing gene expression of coagulation factors and Factor XIII polymorphisms in type 2 diabetes mellitus patients

Principal Investigator: Marry-Ann Phasha, Department of Physiology University of Pretoria (under the supervision of Dr Phulukdaree, Dr P Soma and Prof Pretorius)

Introduction (invitation to participate in a Research study)

You are invited to participate in a research study in the Department of Physiology Faculty of Health Sciences, University of Pretoria. You must be 18 years or older to participate in the study. This information leaflet is to help you understand what the study is about should you like to participate in the study. The researcher will explain the reason for the drawing of blood.

The blood drawn will be used to look at the amount of molecules involved in the clotting of blood, how the blood clots and if there are any genetic change affecting blood clotting. One tube (5 ml) of blood will be drawn to be used for to check blood clotting and genetic changes. The drawn blood will be used for this study only and the remaining amount will be stored at -80˚C for the experiments to be repeated. The blood will be stored for the duration of the study which is two years. You are more than welcomed to ask any questions, should you not fully understand the information provided in this leaflet. You should not agree to take part unless you are completely happy about all the procedures involved.

Purpose of the study?

The main aim of this study is to look at important molecules that are involved in blood clotting, to measure blood clotting parameters and to look at the presence of any genetic changes in molecules involved in blood clotting between type 2 diabetes mellitus patients and healthy controls.

There are small molecules that help regulate blood during damage or injury of the blood vessel. The amounts of the molecules the body produces is different in everyone depending if a person has a medical condition or is healthy. To do the study, blood sample will be needed. Three procedures will be used to assess the

blood: (1)Thromboelastography (TEG) which will be used to measure how the blood clots, (2) Quantitative Polymerase Chain Reaction (qPCR) which will be used to look at the amounts of important molecules involved in blood clotting, (3) Restriction Fragment Length Polymorphism- Polymerase Chain Reaction (RFLP-PCR) which will be used to look at the presence of any genetic changes in one of the molecules involved in blood clotting.

Who will draw the blood?

Dr Prashilla Soma will draw the blood.

Description of your involvement

Should you be interested and agree to participate in this research study, you will be asked to provide your personal details such as your name, surname, gender and age. Furthermore, you will be asked about your health condition for example, if you are on any chronic medication, if you smoke .

Ethical approval

The protocol of this study will be sent to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria to ensure that the study meets the requirements of Declaration of Helsinki (updated: October 2013) which has served as a guideline directing methods and intensions of this study. A copy of the Declaration may be obtained from the investigator should you wish to review it. Should you want to find out if the study has been approved by the Faculty of Health Sciences Research Ethics Committee here is their contact details: 012-3563085.

Research knowledge obtained in this study

The laboratory part of this study is not intended to benefit you, but will add more our knowledge on how the clotting process is regulated on a genetic level and will form part of a MSc dissertation in the Department of Physiology Faculty of Health Sciences, University of Pretoria.

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Drawing the blood may result in a bruise at the puncture site, swelling of the vein or infection or bleeding from the site. For your protection, the procedure will be performed under sterile conditions by a qualified phlebotomist.

Confidentiality

All information obtained during this study is strictly confidential. Data that may be reported in scientific journals will not include any information which identifies you as a patient in this study.

Any information uncovered regarding your test results or state of health will be held strictly confidential in this study. If there are any findings of importance to your health, you will be informed but this information will not be disclosed to any third party without your written permission.

What are your rights as a participant in this study?

Your participation in this study is completely voluntary. Should you agree to take part in the study, you may change your mind and discontinue your participation without stating any reason. Even if you decide to withdraw before the study is completed, it will be acceptable. The investigator has the right to withdraw you from the study if it is considered to be in your best interest.

Informed consent

I hereby confirm that I have been informed by the investigator, MN Phasha about the nature, conduct benefits and risks of this study. I have also received, read and understood the above written information (Patient Information Leaflet and Informed Consent) regarding the research. I am aware that the results of the study including personal information regarding my sex, age, date of birth, initials and diagnosis will be anonymously processed into a study report. I may at any stage, without prejudice, withdraw my consent and participation in the study. I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.

Patient's name (Please print)…….

Patient's signature…….

Date……

Medical practitioner's signature……

Date……

Principal investigator signature…….

Date……

Participant information

[Control Group]

Ethics No: 269/2017

Title of the Project: Assessing gene expression of coagulation factors and Factor XIII polymorphisms in type 2 diabetes mellitus patients

Principal Investigator: Marry-Ann Phasha, Department of Physiology University of Pretoria (under the supervision of Dr Phulukdaree, Dr P Soma and Prof Pretorius)

Information filled in by the investigator

Sample ID	
Age	
Sex	
Does the participant smoke?	
Is the participant on the pill if female?	
Are you on any chronic medication?	

Appendix 8

Submission of Manuscript to Scientific Journal

Format: Abstract

Curr Diabetes Rev. 2019 Jan 29. doi: 10.2174/1573399815666190130113328. [Epub ahead of print]

Coagulopathy in Type 2 Diabetes Mellitus: pathological mechanisms and the role of Factor XIII-A single nucleotide polymorphisms.

Phasha MN[1], Soma P[1], Pretorius E[2], Phulukdaree A[1].

Author information

Abstract

The prevalence of type 2 diabetes mellitus (T2DM) has quadrupled within three decades since 1980 affecting 422 million adults in 2016. It remains one of the most common non-communicable chronic diseases and underlying risk factor for cardiovascular diseases worldwide. There are different underlying mechanisms that play a role in the development of pathologies associated with the disease such as hyperglycaemia, oxidative stress, obesity, inflammation and hypercoagulation. Each of which are interlinked. Hyperglycaemia, oxidative stress and obesity play a huge role in the activation of inflammation and coagulation. Activation of inflammatory pathways increases the production of thrombin which predisposes the development of thrombotic related diseases. One of the factors that contribute to the increase of thrombin is the impairment of the fibrinolysis process due to decreased expression of tissue-plasminogen activator (tPA) by increased levels of plasminogen activator inhibitor-1 (PAI-1). Coagulation factor XIII (FXIII), a transglutaminase that is composed of subunits A and B (FXIII-A2B2), is essential for the last step of fibrin clot formation in the coagulation pathway. Genetic variation of FXIII-A in the form of single nucleotide polymorphisms (SNPs) alters the activity of FXIII, altering clot properties which influence disease outcomes. This review discusses the link between underlying mechanisms of T2DM, well known FXIII-A variants and coagulation.

KEYWORDS: Coagulation; Factor XIII; Single nucleotide polymorphisms; Type 2 diabetes mellitus

PMID: 30706822 DOI: 10.2174/1573399815666190130113328

Appendix 9

Abstract Acceptance to National and International Conferences

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Alisa Phulukdaree <u04830670@up.ac.za>

Phasha: Abstract Accepted for Oral Presentation; Fibrinogen and Factor XIII workshop

4 messages

Workshop, Fibrinogen <ifrs2018@wfu.edu> 15 March 2018 at 23:44
To: Alisa Phulukdaree <alisa.phulukdaree@up.ac.za>
Cc: Gloria Stickney <stickngd@wfu.edu>

Dear Alisa,

Congratulations! Your abstract has been selected for an oral presentation at the 25th Fibrinogen and 3rd FXIII Workshop! Abstract title: Assessing gene expression of coagulation factors and Factor XIII polymorphisms in type 2 diabetes mellitus patients.

All abstracts were reviewed by a panel of 15 experts, each abstract was evaluated and scored by three independent reviewers.

The workshop will be held June 3 - 7, 2018 at the Graylyn International Conference Center of Wake Forest University. Details can be found at the Workshop websites. The program will be posted in the next few weeks.

Please note that the early bird registration deadline is Wednesday, March 21, 2018.

We are looking forward to seeing you at the Workshop!

Best regards,

Martin Guthold

Alisa Phulukdaree <alisa.phulukdaree@up.ac.za> 16 March 2018 at 08:18
To: "Pretorius, E, Prof <resiap@sun.ac.za>" <resiap@sun.ac.za>, Prashilla Soma <prashilla.soma@up.ac.za>

Dear Prof and Dr Soma

Mary-anne's abstract was accepted. I am going to try and gather some funds so she can attend. Unfortunately I cannot attend since I am pregnant and my gynae suggested I avoid travelling considering that it is a high risk pregnancy.

Kind regards,
Alisa

Dr. Alisa Phulukdaree
Senior Lecturer

Tel +27 (0)12 319 2147
Email alisa.phulukdaree@up.ac.za
www.up.ac.za

Faculty of Health Sciences
Department of Physiology
Room 9-27, Level 9, Basic Medical Science Building,
University of Pretoria, Private Bag X20, Hatfield 0028, South Africa
[Quoted text hidden]

Pretorius, E, Prof <resiap@sun.ac.za> <resiap@sun.ac.za> 16 March 2018 at 08:24
To: Alisa Phulukdaree <alisa.phulukdaree@up.ac.za>, Prashilla Soma <prashilla.soma@up.ac.za>

ALISA!!!!!!! SO so glad to hear about the pregnancy!

Great news about the abstract. Is it a poster or a talk?

Resia

From: Alisa Phulukdaree <alisa.phulukdaree@up.ac.za>
Date: Friday, 16 March 2018 at 8:18 AM
To: "Pretorius, E, Prof <resiap@sun.ac.za>" <resiap@sun.ac.za>, Prashilla Soma <prashilla.soma@up.ac.za>
Subject: Fwd: Phasha: Abstract Accepted for Oral Presentation; Fibrinogen and Factor XIII workshop

[Quoted text hidden]

Alisa Phulukdaree <alisa.phulukdaree@up.ac.za> 16 March 2018 at 08:27
To: "Pretorius, E, Prof <resiap@sun.ac.za>" <resiap@sun.ac.za>

Dear Prof

Thank you so much!

Mary-anne's is an oral presentation, I hope she can attend.

Kind regards,
Alisa

Dr. Alisa Phulukdaree
Senior Lecturer

Tel +27 (0)12 319 2147
Email alisa.phulukdaree@up.ac.za
www.up.ac.za

Faculty of Health Sciences
Department of Physiology
Room 9-27, Level 9, Basic Medical Science Building,
University of Pretoria, Private Bag X20, Hatfield 0028, South Africa

[Quoted text hidden]

Gmail **Mary-anne Phasha <maphasha97@gmail.com>**

Abstract Outcome: Accepted for poster presentation (SASBMB-FASBMB 2018)

7 messages

Claries Roelofsz <claries@londocor.co.za> Fri, Jun 1, 2018 at 8:53 AM
To: maphasha97@gmail.com

1 June 2018

Dear Ms Marry-Ann Phasha

Thank you for having shown interest in the conference of the South African Society of Biochemistry and Molecular Biology (SASBMB), in conjunction with the Federation of African Societies of Biochemistry and Molecular Biology (FASBMB) and for your scientific participation and contribution to the field.

Your abstract entitled *"Assessing gene expression of coagulation factors and Factor XIII polymorphisms in type 2 diabetes mellitus patients"* was peer reviewed by a scientific committee and we are happy to inform you that your abstract has been accepted for **POSTER PRESENTATION:**

- Please confirm that you will be presenting your poster by responding to this email by the 8th of June 2018
- All poster authors are requested to set up their posters during the morning tea or lunch of Monday, 9 July.
- You are requested to be present at your poster during either the first (Mon 9 July, 14:30) or second (Tue 10 July, 15:00) poster viewing session. The session for your specific poster viewing will be confirmed shortly.
- Three (3) posters will be selected for prizes by delegates during the conference.

Poster specifications:

Poster size to be printed as A0, portrait

One poster board will be provided per poster and your space will be clearly designated and Prestik will be provided. Posters will be grouped according to themes.

Gmail **Mary-anne Phasha <maphasha97@gmail.com>**

CoBNeST 2018: Confirmation of Oral Abstract Acceptance

1 message

Snyman, Christelle, Me [csnyman@sun.ac.za] <csnyman@sun.ac.za> Wed, Aug 1, 2018 at 12:23 PM
To: "maphasha97@gmail.com" <maphasha97@gmail.com>

Dear Marry-Ann Phasha

Your abstract titled: **Assessing gene expression of coagulation factors and Factor XIII polymorphisms in type 2 diabetes mellitus patients**has been accepted for presentation at the CoBNeST 2018 conference taking place at Spier Estate, Stellenbosch from 7 – 10 October 2018.

Presentation Method: Oral

Category: PSSA

Please refer to the comment section of your submission to confirm your competition category

- The time allotted for oral presentations is **strictly** 15 minutes (10 minutes presentation, 4 minutes for questions and 1 minute for switching speakers). The presentations will be judged by a panel consisting of national as well as international specialists. English will be the language for all oral and poster presentations. The chairperson will request you to stop your presentation if you exceed your allocated time.
  - Unfortunately any delays to your presentation – for example, loading of your presentation at the commencement of your allocated slot – will result in shorter time for your own presentation.

- We will be able to accommodate presentations formatted for both 4:3 and 16:9 aspect ratios, using VGA and HDMI connections. We will provide a Windows based laptop at the lectern.