University of Pretoria
Faculty of Health Sciences School
of Medicine

The association of methylglyoxal-adducts with kinetics and ultrastructure of fibrin clots in coronary artery disease patients with type 2 diabetes mellitus

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DECLARATION OF ORIGINALITY

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Topic of work: Investigation of the ultrastructure of fibrin network, the physiological clot properties, and the methylglyoxal influence on clot morphology in coronary heart diseases patients with and without type 2 Diabetes Mellitus.

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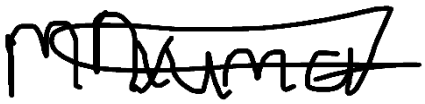

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ABSTRACT

Background: Glycation influences the ultrastructure and clot kinetics of fibrin clots due to the post-translational modifications in fibrinogen. Methylglyoxal (MG) is used to measure the level of glycation which has been associated with the pathogenesis of type 2 diabetes Melilites (T2DM) and coronary heart disease (CHD). The aim of the study was to determine the role of MG on clot kinetics and fibrin clot structure in CHD

patients with and without T2DM to provide insight into the mechanism of pathogenesis of atherosclerosis in T2DM which results in the development of CHD.

Methodology: Scanning electron microscopy (SEM) was used to evaluate the morphology of fibrin clots. Thromboelastography (TEG) was used to assess the physiological clot properties (kinetics). Enzyme-linked immunosorbent assay (ELISA) was used to determine the levels of methylglyoxal-adducts.

Results: The morphology of clots from controls analysed using SEM showed thick and thin fibres which created an organised mesh of fibrin fibres. In T2DM, CHD with T2DM and CHD some alterations in the morphology were observed. The ultrastructure micrographs in CHD shows that some of the fibrin fibres formed have individual fibres with both thick and thin fibres as well as a thick mass of fibres with a net-like structure that forms dense-matted deposits. In addition, the fibrin fibres are not organised. The densitometry analysis between controls and patient groups' (CHD: mean (standard deviation) 0.42±0.11; CHD+T2DM: 0.31±0.08 and T2DM: 0.29±0.08) was found to be significantly lower in all groups compared to the control which had a mean of 0.57±0.1, $p<0.0001$.

There are no significant differences in the alpha angle between CHD, T2DM, CHD with T2DM and controls (60.88±2.321˚ vs. 60.81±2.385˚ vs. 59.09± 3.185˚ vs. 66.47±1.300˚, $p=0.5279$). There was no significant difference found in the K-value between T2DM, CHD with T2DM, CHD and control subjects (3.458±0.446mins vs. 5.118±1.589mins vs. 3.758±0.450mins vs. 2.839±0.2156mins, $p=0.0102$). The maximum amplitude was higher in T2DM patients compared to CHD, CHD with T2DM and controls (40.51±1.914mm vs. 34.10±2.127mm vs. 33.12±3.365mm vs. 33.60±1.525mm, $p=0.0102$). The MRTG was higher in CHD compared to T2DM, CHD with T2DM and controls (10.74±3.335 dyn cm^{-2} s^{-1} vs. 4.268±0.690 dyn cm^{-2} s^{-1} vs. 5.046± 0.927 dyn cm^{-2} s^{-1} vs. 6.535±0.664 dyn cm^{-2} s^{-1}, $p=0.0096$). The reaction time was higher in CHD with T2DM patients compared to T2DM, CHD and controls (32.58±4.005min vs. 23.92±2.793min vs. 21.29± 2.383min vs. 8.322±0.886min, $p<0.0001$). There was no significant difference found in the TTG between T2DM, CHD with T2DM, CHD and control subjects (231.3±28.68 dyn cm^{-2} vs. 258.5±38.15 dyn cm^{2} vs. 343.7±71.92 dyn cm^{-2} vs. 287.7±21.37 dyn cm^{-2}, $p=0.8421$). The TMRTG was higher in T2DM patients compared to T2DM, CHD with T2DM, CHD and controls

(23.91±2.409mins vs. 20.46±3.411mins vs. 14.14±1.287mins vs. 10.16±0.751mins, p<0.0001).

To assess if an association between MG-adducts and clot kinetics exists, the Spearman r correlation was completed for each clot parameter. The reaction time (p=0.0047, 95% CI: 0.138 to 0.665) and time taken before maximum speed of the clot growth to be achieved (p=0.3958, 95% CI: 0.072 to 0.644) was significant. This indicates the relationship between the parameters i.e., the higher the level of MGadducts present, the longer it takes for clotting to begin and reach maximum speed of formation.

Conclusion: This study showed that there are ultrastructural differences in fibrin fibres formed in CHD patients with T2DM. The viscoelastic parameters indicated that haemostasis was irregular in CHD and T2DM. The levels of MG-adducts were much higher in T2DM, CHD with T2DM and CHD and may be a contributing factor to the pathogenesis associated with altered coagulation in these patients.

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LIST OF ABBREVIATIONS

AGEs	Advanced glycation end-products
aPTT	activated partial thromboplastin time
DCs	Dendritic cells
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease

CHD	Coronary heart disease
CVD	Cardiovascular disease
ECG	Electrocardiogram
ELISA	Enzyme-linked immunosorbent assay
FIX	Factor IX
FVII	Factor VII
FVIII	Factor VIII
FX	Factor X
FXI	Factor XI
FXII	Factor XII
FXIII	Factor XIII
H1	Hydroimidazolones
HMDS	Hexamethyldisilazane
HTA	Hemi-thioacetal
K	Clotting time
LDL	low-density lipoprotein cholesterol
ox-LDL	oxidized low-density lipoprotein cholesterol
MA	Maximum amplitude
MG	Methylglyoxal
MRTG	Maximum rate of thrombus generation
NK	Natural killer
NHANES	National Health and Nutrition Examination Survey
NO	Nitric oxide
PBS	Phosphate buffered saline
PPP	Platelet poor plasma
PT	Prothrombin time
PRP	Platelet rich plasma
PKC1/2	protein kinase C
R	Reaction time
ROS	Reactive oxygen species
SANBS	South African National Blood Service
SEM	Scanning election microscope

SRs	scavenger receptors
T2DM	Type 2 diabetes mellitus
TEG	Thromboelastography
TF	Tissue factor
TMRTG	Time to the maximum rate of thrombus generation
tPA	Tissue-plasminogen activator
TTG	Total thrombus generation

CHAPTER 1 INTRODUCTION

According to the National Health and Nutrition Examination Survey (NHANES) estimated data there are 462 million T2DM individuals globally and the number is rising at a high rate in developed regions.(1) The worldwide prevalence of T2DM is estimated to increase by 7079 individuals per 100 000 by the year 2030.(1) In Africa there are 19 million adults that have diabetes with 95% being T2DM individuals. It has been postulated that if it is not controlled, it will rise to 47 million by the year 2045.(2) Type 2 diabetes mellitus is characterized by insulin resistance, inflammation, obesity, central adiposity, hypertension and hypertriglyceridemia.(3, 4)

Cardiovascular diseases (CVD) is the main contributor to the high mortality in patients with T2DM.(3) It is estimated that there were 17.9 million deaths in 2016 due to heart diseases worldwide.(5)

Atherosclerosis causes CVD as it promotes chronic inflammation in arteries which results in plaque formation.(6) The continual growth of the plaque leads to a narrowing of the arterial lumen causing a blockage that results in ischemia.(7) The regulation of the rate of blood clot formation and lysis is important for the maintenance of haemostasis .(6) However, haemostasis is altered in T2DM due to factors that affect clot formation and lysis.(7, 8)

In T2DM, reactive oxidative species (ROS) is increased due to the high concentration of advanced glycation end products (AGEs) and this plays an important role in inflammation and oxidative stress (4). The link between inflammation, oxidative stress and insulin resistance leads to irregular activation of coagulation cascade (4). The increased levels of glucose in T2DM as well as the formation of AGEs induce oxidative stress which initiates an inflammatory response and hypercoagulation.(9, 10)

Methylglyoxal (MG) is a by-product of glycolysis and is highly reactive leading to the formation of AGEs resulting in increased oxidative stress.(11-14) In T2DM, MG is abundant, and its formation is higher than the detoxification causing complications resulting in mitochondrial and proteasomal dysfunction.(15, 16)

Fibrinogen is a glycoprotein that is converted into fibrin during the coagulation cascade.(17) The structure of fibrinogen can be modified by glycation which leads to

the formation of fibrins that have morphological changes which is seen in the pathological state.(18) Fibrin clots formed from modified fibrinogen are difficult to lyse which results in an increased prevalence of CVD.(19) The post-translational modifications in T2DM result in the production of different structural and functional properties when compared to healthy individuals.(20)

The increasing rate of mortality due to CHD indicates the need to discover novel drug treatments that will help reduce the risk of CHD and high mortality rates in individuals with T2DM. This study will investigate the role of MG on clot kinetics and fibrin clot structure in CHD patients with T2DM in order to provide insight into mechanism of pathogenesis of atherosclerosis in T2DM which results in the development of CHD.

CHAPTER 2 LITERATURE REVIEW

Type 2 diabetes Mellitis

Diabetes can be classified into three main types namely Type 1, Type 2 and gestational.(21) The most common type of diabetes is Type 2 Diabetes Mellitis (T2DM), which will reach prevalence rates that will make it an epidemic disease by 2040 with 625 million individuals.(22) The prevalence of T2DM has increased worldwide. The data from the International Diabetes Federation (IDF) shows that in the year 2011 there were about 336 million individuals with T2DM, 41 million in South Africa in 2017 and the number is most likely to increase to 552 million by the year 2030.(23) In developing countries, the prevalence is likely to be higher due to urbanisation and the introduction of Western life style.(24)

The key feature in the pathogenesis of T2DM is that of insulin resistance and reduced insulin secretion. Insulin is responsible for complex metabolic processes in peripheral tissues, thus, impaired insulin secretion and uptake results in hyperglycaemia.(25) Insulin resistance occurs due to oxidative stress causing dysregulation in insulin signals and adipocytokines.(8) Insulin resistance may pre-exist for several years before the onset of T2DM.(8) The increase of adipocytokines happens as a result of the modern lifestyle and lack of physical activity leads to insulin resistance.(8)

The balance between blood clot formation and lysis is important to maintain blood haemostasis.(6) However, in T2DM, haemostasis is altered due to factors that affect clot formation and lysis.(7) These factors include: hyperglycaemia, low-grade inflammation, increased oxidative stress and the production of advanced glycation end products (AGEs).(7)

Heart disease

In South Africa, CVD is the second leading cause of death, with up to 43% deaths in adults each year. Data published in 2017 by the World Health Organization estimated that 8.12% of deaths in South Africa were due to coronary artery disease (CAD).(26) The most common CVD is CAD, which is the one of the main causes of the increase in mortality worldwide, even though there have been many advances in diagnosis and treatment of the diseases.(10) In CAD, the electrocardiogram (ECG) electrical wave patterns are abnormal and different when compared to normal physiology.(13) The intervals between ventricular depolarization and repolarization (ST segments) seen when using ECG either show depressed or elevated waveforms in patients with

myocardial infarction (MI) or CAD therefore, the evaluation of ECG waveforms is important for MI and CAD diagnosis.(13)

Atherosclerosis is the main factor that results in the occurrence of CAD, it promotes chronic inflammation of the arteries which leads to the formation of an atherosclerotic plaque.(10) Coronary artery disease is the gradual narrowing of blood vessels that provide oxygenated blood for the myocardium and if the narrowing continues it results in ischemia.(14) The atherosclerotic plaque is commonly formed in the epicardial coronary arteries.(27) The risk factors of atherosclerosis include smoking, T2DM, hypertension, high levels of plasma low-density lipoprotein and low plasma highdensity lipoprotein which results in the abnormal functioning of the vascular endothelium.(27) In normal physiology, the endothelium prevents thrombosis, regulates inflammation and controls vascular tone.(27) However, in atherosclerosis, the collection of fat, fibroblasts, smooth muscle cells and intercellular matrix leading to plaque formation.(27)

The initial formation of an atherosclerotic lesion is known as a fatty streak, composed of lipid deposits, foam cells, as well as T-cells.(28) As the disease gradually develops, it leads to an interaction between the immune system and vessel cell walls leading to plaque formation.(28) Growth of plaque narrows the arterial lumen leading to an obstruction that prevents blood supply, which contributes to ischemia.(28) The plaque formed can rupture exposing the thrombogenic components catalysing the thrombotic response causing the plaque to detach from the lumen leading to ischemia that is distal to the point of plaque initiation.(28)

Atherosclerosis is characterized by the increase in lipids and inflammatory cells in arteries with large as well as medium sized walls.(29, 30) The development of atherosclerosis is due to the triggering of pro-inflammatory signalling pathways, accumulation of oxidative stress and the expression of cytokines.(31) Oxidative stress is when the production of ROS increases or when the body is not able to remove ROS through antioxidants.(29) The production of ROS is responsible for inflammation, apoptosis, cell growth and change in the vascular tone, hence, increase in ROS in arterial walls leads to increased risk of CVD.(29) However, under normal conditions ROS plays an important role transducing intercellular signals in different mechanisms.(31)

The mechanism of atherogenesis begins when endothelial vascular injury occurs, this leads to the increase in low-density lipoprotein cholesterol (LDL) in the sub-endothelial

space.(32) The increase in LDL, increases the levels of oxidized LDL (ox-LDL) as LDL particles are prone to oxidative damage. The mechanism of ox-LDL uptake by macrophages is shown in Figure 2.1.(29) When these lipoproteins are modified to oxLDL it results in increased cell adhesion molecules like P- and E-selectin thus increasing the concentration of monocytes and T-lymphocytes.(29) Monocytes that are recruited change into macrophages expressing scavenger receptors (SRs) and they also internalise ox-LDL.(32) The macrophages that contain lipids are called foam cells because of their appearance and their presence is important for the initial formation of an atherosclerotic lesion.(29) The formation of foam cells causes a disruption in the vascular endothelium.(33) Moreover, as the foam cells and macrophages accumulate it triggers the inflammatory and thrombotic pathways due to an increase in oxidative stress.(33) Mast cells and T-lymphocytes move to the intima along with the foam cells leading to the release of cytokines which increase inflammation and the production of ROS.(29) The plaque formation is promoted by the release of growth factors as well as ROS activation of smooth muscle cells and collagen deposits.(29)

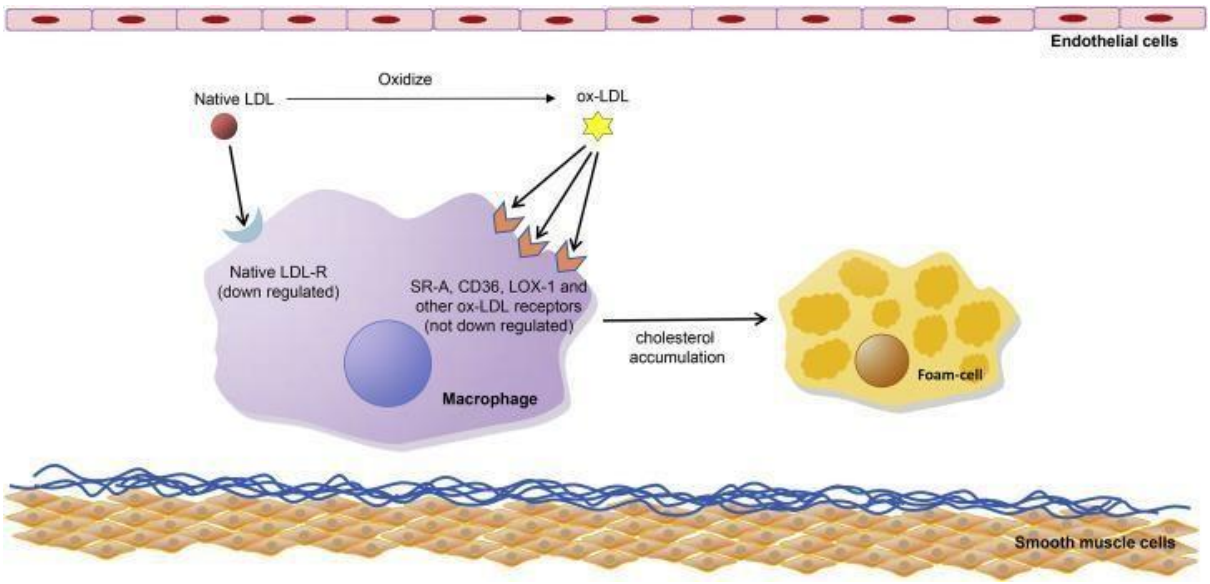


Figure 2.1 Mechanisms of ox-LDL update by macrophages. Native LDL can hardly induce foam-cell formation because of the down regulation of LDL-R. Ox-LDL induces cholesterol accumulation in macrophages through rapid uptake by the SRs, which are not down regulated to an increase intracellular cholesterol.

LDL: low-density lipoprotein; ox-LDL: oxidized low-density lipoprotein; LDL-R: lowdensity lipoprotein receptor; SR-A: scavenger receptor A; CD36: cluster differentiating
36; LOX-1: lectin-like oxidized low density lipoprotein receptor 1. (34)

Advanced Glycation End-products

Increased levels of glucose as seen in T2DM present in the body, induces oxidative stress, as an inflammatory response.(9) There are several other mechanisms that are able to induce oxidative stress such as the formation of AGEs, glucose autoxidation and the activation of protein kinase C (PKCβ1/2).(8) In T2DM, there is an overproduction of ROS due to the activation of AGEs formation and PKCβ1/2 kinase pathway.(8) In combination, elevated levels of oxidative stress, hyperglycaemia and the formation of AGEs, all play an important role in the initiation and progression of inflammation and hypercoagulation observed in T2DM.(35)

AGEs are chemical compounds known as glycotoxins which form through a nonenzymatic interaction between a carboxyl group of reducing sugars and amino groups in proteins, nucleic acids, lipids and peptides.(21) This reaction is known as the Maillard reaction which forms Schiff bases and Amadori products.(21, 36) Most AGEs formation is by the Maillard reaction where the carbonyl part of the reducing sugar reacts with the amino groups. Other reactions that take place are dehydration and oxidation forming complex structures due to increased crosslinking.(10)

Glycation is a process that results in post-translational modifications of proteins on the lysine, arginine and cysteine residues leading to the formation of AGEs.(37) Proteins that are stable and have a long half-life are mainly targeted by glycation agents which causes permanent dysfunction to cells leading to tissue remodelling. The production and accumulation of AGEs is a slow process; however, long-lived proteins are the main targets which leads to increased levels of AGEs resulting in severe impairment to tissue in the body.(21) Advanced glycation end-products are formed both intercellularly and extracellularly, the increase of AGEs in the human body may result in abnormal nitric oxide (NO) signalling and the production of oxidative stress which leads to endothelial dysfunction, arterial and myocardial stiffness and atherosclerotic plaque formation.(38) In addition, AGEs have been associated with the pathogenesis of T2DM.(11)

The advanced glycation end product reactions take place extra- and intracellularly, which can change the physiological properties of elastin, collagen and laminin by crosslinking or by the production of intermolecular bonds which decreases the elasticity and mechanical characteristics of the targeted tissue.(10) In the endoplasmic reticulum the accumulation of AGEs induces abnormal folding of protein and stress responses which leads to inflammation and apoptosis in cells.(10) The crosslinking of AGEs to mitochondrial proteins decreases the production of ATP and increases the formation of free radicals.(10)

The measurement and analysis of AGEs can help develop novel therapeutics to prevent metabolic disorders, however, AGEs are found both in free and bound form in blood and tissue respectively. Therefore, it is hard to find a method that can be used as a measurement to prove that AGEs are responsible for the pathology seen.(21)

Methylglyoxal is a very reactive by-product of glycolysis and it results in the production of AGEs which increases the oxidative stress.(11, 12) The reactivity of MG is about 20 000-fold more than glucose and the formation of MG is by the degrading of glyceraldehyde-3-phosphate, intermediates of glycolytic triose phosphate and dihydroxyacetone phosphate.(16) The high reactivity of MG during glycolysis is as a result of the strong interaction between MG and arginine residues on the proteins, however, the reaction with glucose is less reactive due to the reaction with lysine residues.(21) Methylglyoxal is the main compound involved in fast formation glycation adducts on protein and reacts with arginine and lysine.(16, 39)

Methylglyoxal is an alpha-oxoaldehyde which is formed by many different pathways in the cell.(40) It is formed as a by-product of glycolysis after the fifth reaction which is a spontaneous process of degrading triose phosphate intermediates, glyceraldehyde-3phosphate (G3P) and dihydroxyacetone phosphate (DHAP).(40) The formation of MG is also as a result of acetone oxidation, the breakdown of threonine and proteins that are modified through glycation as seen in figure 2.2.(41) The daily production of MG is about 125 µmol/kg cell mass.(41)

The levels of MG in healthy humans has been estimated to be about 50-150 nM extracellular and 1-4 µM intracellular.(40) During hyperglycaemic conditions, the concentration of MG increases intercellularly as well as extracellularly by 2-4 fold.(21) When the levels of MG go beyond the normal levels it leads to dicarbonyl stress and this occurs as a result of a decreased glyoxalase activity which is an enzyme that detoxifies MG and decreases the level of enzymes that form MG from

aminoacetone.(16, 40) The accumulation of MG is decreased by the reduction of MG to D-lactate due to the metabolism of the glyoxalase system with involves glyoxalase 1 (GLO1), glyoxalase 2 (GLO2) and glutathione.(42) In the process of MG detoxification, glutathione is the catalyst and it binds to MG to form a hemi-thioacetal (HTA) so that the GLO1 reaction can take place. The enzyme GLO1 reacts with HTA to form S-D-Lactoylglutathione (SDL) then GLO 2 reacts with SDL to form Dlactate.(21) In T2DM, GLO 1 and 2 enzyme activity decreases due to a hyperglycaemic state which results in accumulation of MG, as the MG production is increased it forms hydroimidazolones (MG-H1) which are adducts that cross-link to form AGEs.(21) The formation of MG-H1 is due to the interaction with the arginine protein residue.(43)

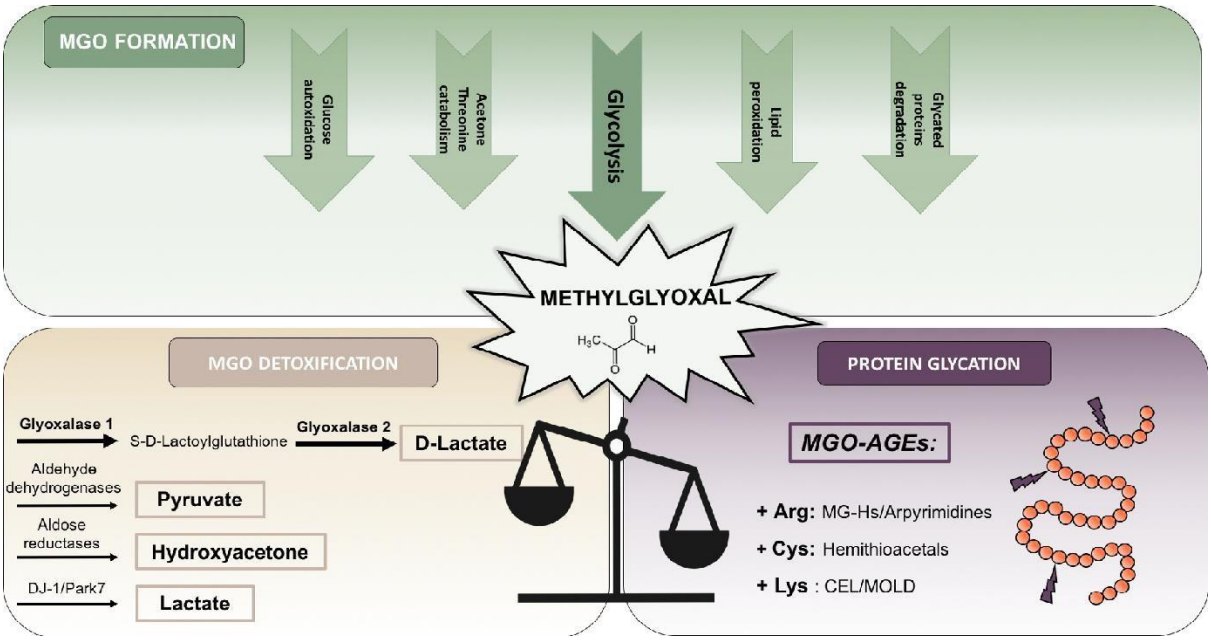


Figure 2.2 The formation, detoxification of methylglyoxal (MGO) and the formation of glycated proteins. The synthesis of MGO occurs during glycolysis, autoxidation, during the breakdown of acetone and threonine, from the peroxidation of lipids and the breakdown of glycated proteins. The glyoxalase system induces the detoxification of MGO as well as other pathways such as aldehyde dehydrogenases, aldose reductases and DJ-1/Park7 enzymes. When the regulation of MGO production and detoxification is abnormal and the production of MGO is favoured it results in the production of MGOAGEs.

Methylglyoxal and Type 2 Diabetes Mellitus

In T2DM plasma, MG is the most abundant dicarbonyl compound.(15) There is an increase in MG formation while the detoxification is decreased as a result of reduced activity in GLO1 and this leads to increased levels of MG.(42) The proteins that are glycated have a different structure and molecular weight which leads to biochemical

dysfunctions.(44) Increased levels of MG cause several other complications which result in mitochondrial and proteasomal dysfunction.(16) For this reason, MG causes some of the T2DM complications and plays a role in the pathogenesis of T2DM.(16)

Glycation can also occur on insulin and this reaction results in insulin resistance and this leads to increased levels of glucose.(21) The modification on insulin molecules have an effect on the signalling pathways resulting in hyperglycaemia.(21) The secretion of insulin from β-cells occurs as a response to increased glucose levels, when there is an interaction between MG, β-cells and endothelial cells it causes endothelial cell dysfunction due to the swelling and apoptosis .(42) The presence of MG in the β-cells promotes the production of reactive oxygen species which results in decreased secretion of insulin. When insulin interacts with MG it causes a disruption in the feedback mechanism of insulin hence the increase in release of MG from the liver resulting in removal of insulin that is not homeostatic.(21) The decrease in pancreatic and duodenal homeobox 1 levels leads to increased levels of MG-derived AGEs and causes the liver, muscles, and adipose tissue to become insulin resistant.(39)

Methylglyoxal and Vascular Function

In recent studies, there has been several claims that MG can play a role in vascular dysfunction, which is one of the factors that lead to the development of cardiovascular diseases.(39) Methylglyoxal is able induce apoptosis in cells, oxidative stress, inflammation and AGEs which leads to the toxification that is seen in endothelial dysfunction.(45) The vascular endothelium acts as an autocrine as well as a paracrine organ and functions as a regulator for vascular homeostasis.(39) Altered haemostasis results in vasoconstriction, leukocyte adherence, platelet activation, thrombosis and impaired coagulation, vascular inflammation and atherosclerosis.(39) In T2DM, the endothelial cells are exposed to hyperglycaemia and frequently changing levels of blood glucose contributes to the dysfunction of endothelial cells. The hyperglycaemia leads to the formation of ROS and MG leading to an increased production of AGEs.(42) Developments made over the years show that apoptosis is one of the mechanisms that result in the dysfunction of endothelial cells leading to the decrease in permeability of the vessel wall and this reduces the rate at which NO is absorbed.(46) The reduced bioavailability of NO results in endothelial dysfunction.(47) Homeostasis of the myocardial layer is regulated by the endothelium and also maintains cell viability and function.(42) Endothelial cell death of cardiomyocytes increases the episodes of

myocardial infarctions and this results in ventricular dysfunction influencing the development of heart failure.(42)

Evidence has shown MG to be one of the main contributors for the development of T2DM complications and other age-related disorders.(11) Hence, if the formation of MG can be neutralized or inhibited it may be a possible novel therapy to reduce agerelated disorders.(11) In T2DM, the increased levels of MG cause inflammation leading to the activation of endothelial cells resulting in vascular damage.(42) Other markers contributing to vascular dysfunction are highlighted in figure 2.3. In T2DM, as the AGE's accumulate, it activates the expression of the receptor of AGE (RAGE).(33) This receptor is commonly located on endothelial cells and smooth muscles cells and increase due to hyperglycaemia. When the AGE-RAGE is activated it results in the increase of cell proliferation as well as the activation of NFKB transcription factor which increases the formation of inflammatory cytokines.(33)

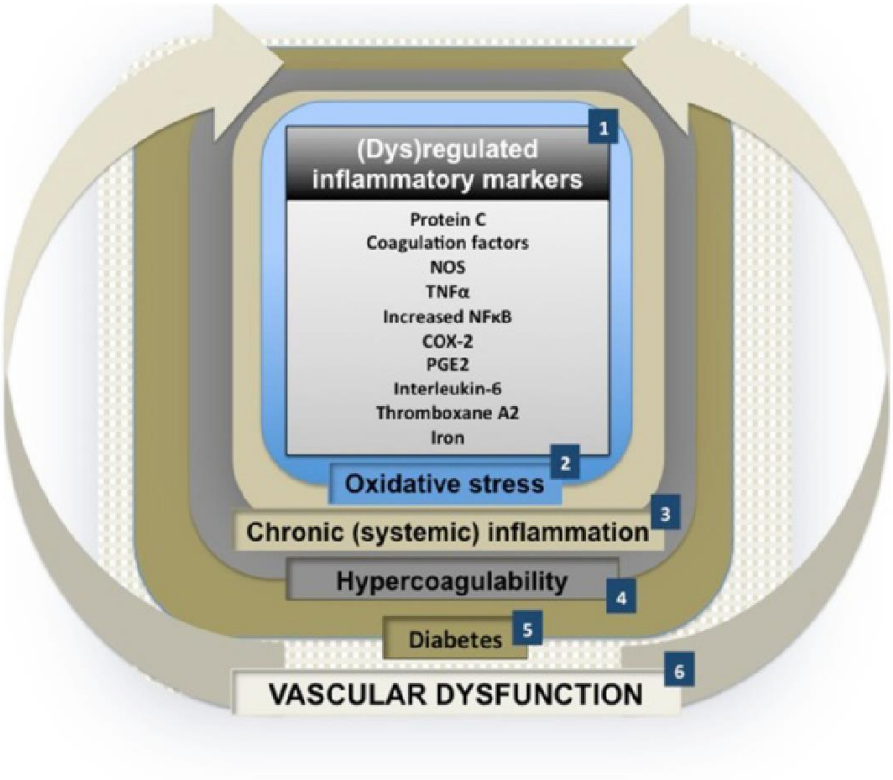


Figure 2.3 Suggested chronological events leading to vascular dysfunction in T2DM. The figure shows the inflammatory markers that are dysregulated in T2DM patients which results in oxidative stress that causes chronic inflammation and hypercoagulation. The dysregulations that occur in 1 to 4 are seen in T2DM and they are used as indicators of vascular dysfunction.(48)

Endothelial dysfunction

Vascular endothelial cells are found in blood vessel walls and their main role is for protection as well as prevention of vascular leakage.(49) The endothelium is composed of a cell layer that lines the luminal surface of blood vessels and one of the other major roles is the regulation of vascular homeostasis.(50) In addition, it regulates which molecules can enter and exit the selective barrier and modulates the balance between vasoconstriction and vasodilation, through the release of endothelin which causes vasoconstriction and NO which causes vasodilation.(51) The vascular endothelium is viewed as an organ due to the role it plays in vascular tone regulation, homeostasis control, metabolism, inflammation and synthesizing secretion molecules.(52) An abnormality in these properties can be the beginning of endothelial dysfunction and the initiation of atherogenesis leading to an increased risk of CVD.(52) Under normal physiological conditions, the endothelium is not damaged, thus, there is decreased leukocyte adhesion and aggregation as well as increased activation of fibrinolysis which prevent plaque formation.(52)

In all the stages of the atherosclerotic process, from the start of atherosclerosis up to the formation of a thrombus, endothelial cell dysfunction occurs.(53) Adhesion molecules found in the cells contribute to the atherosclerotic process with leads to CVD.(53) There are a variety of adhesion molecules such as P-Selectin, E-Selectin, intracellular adhesion molecule 1 (ICAM-1) and VCAM-1 which regulates the communication between thrombotic factors and vessel endothelium.(52) The expression of cellular adhesion molecules is increased in the presence of ongoing oxidative stress.(33) In T2DM, the expression adhesion molecules are also increased due to the hyperglycaemic state as a result of abnormal metabolic activity.(33) As the expression of adhesion molecules increases it triggers the activation of platelets and atherogenesis.(33) Under normal physiology, the adhesion molecules regulate the immunity, signalling pathways and inflammation.(33) In T2DM, there is an elevation of hydrogen peroxide (H_2O_2) and oxygen (O^{2-}) levels which causes more platelets and white cells to adhere to the monolayer by rolling adhesion.(33) This adhesion increases the formation of foam cells, the movement of white cells to the endothelium as well as platelet aggregation increasing the risk of CVD seen in T2DM.(33)

Chronic inflammation is caused by increased oxidative stress which leads to the change in the structure of endothelial cell wall.(29) The formation of lesions in all stages of atherogenesis is influenced by inflammation, the continues inflammation promotes endothelial remodelling which increases intima-media thickness and also

remodels the function of the endothelial cells.(54) Thus, chronic inflammation over time will cause endothelial dysfunction damaging the coronary arterial walls and the ability to repair an injured artery is impaired.(54)

Inflammation

Inflammation is the main factor in the development of CAD.(55) Inflammatory cells and molecules that control the signalling pathways in endothelium play a role in the initiation and progression of CAD by increasing the lipoprotein retention, production of plaque and induces the instability of the arterial wall.(55)

Inflammation is an immune response that occurs as a result of injury or infection, this makes the vascular endothelium more permeable to immune cells.(56) The endothelial cells as well as the platelets activate the inflammatory processes, platelets are responsible for the release of small molecules and cytokines, they also have a physical role of helping in the attachment of neutrophils and in generating neutrophil extracellular traps at the site of injury.(56)

Neutrophils are leucocytes that act as the initial protection against pathogens during the innate immune cell response and play a role in inflammation due to their ability to increase the number of active monocytes at the site of inflammation.(57, 58) These leucocytes are found in circulation and have a short life-span but stay active for a longer period at the site of inflammation.(58) The activation of neutrophils is triggered by pathogens, other leucocytes, cytokines and other immune cell types. The granulocyte colony stimulating factor regulates the formation of neutrophils but the retention of neutrophils is controlled in the bone marrow by two receptors CXCR2 and CXCR4.(57) Unlike other immune cells, neutrophils produce granules before being released from the bone marrow resulting in a quicker response.(58) The neutrophil extracellular traps (NETs) are a unique feature that neutrophils have so that cytotoxicity can occur at the site of injury.(59) At the atherosclerotic lesion site, the neutrophils remove the DNA which looks like a net-like structure, it consists of neutrophil granule proteins, histones and chromatin.(60) Neutrophil extracellular traps (NETs) have been recently discovered and are formed for protection at the site of injury. Atherogenesis is promoted by NETs at the lesion site by increasing ROS and granular proteins as seen in figure 2.4.(58, 59)

In the arterial wall surface, dysfunction of the endothelial cell wall occurs and it is characterised by decreased vasodilation and the cell wall becomes leaky.(57) Neutrophils are recruited and then several different types of chemokine and adhesion

molecules such as E- and P-selection enable the circulating neutrophils to slow, roll and tether on the endothelium.(58) In recent studies it has been shown that after a patient develops CAD, the number of activated neutrophils are increased in circulation.(58) It has been shown that a decrease in neutrophils results in a decrease in monocytes and macrophages, this indicates that neutrophils play an important role in their recruitment at the lesion site.(58)

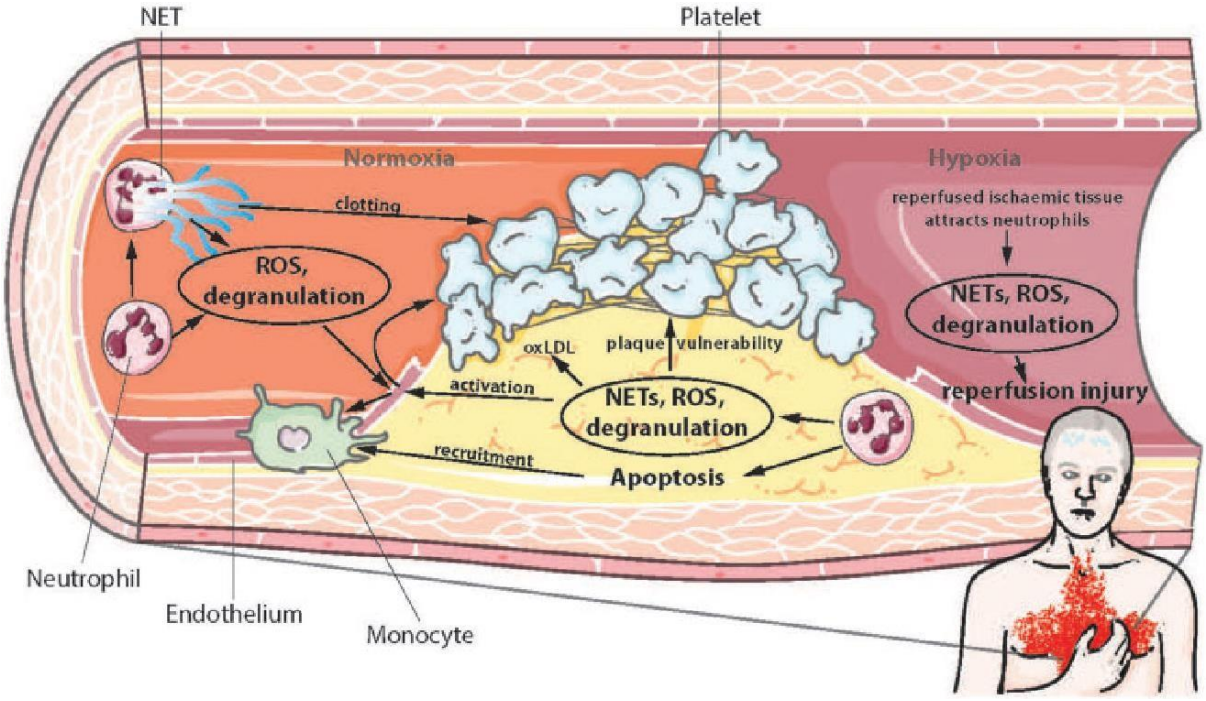


Figure 2.4 Neutrophils play a role in the progression of atherosclerosis and thrombosis when the endothelium is injured.(59)

Macrophages and monocytes are the most abundant and play a major role in the activation of inflammation and the remodelling of tissue at the site of the atherosclerotic lesion.(58, 61) The movement of the monocytes into the subendothelial wall results in the formation of macrophages leading to foam cells which are distinctive features of an atherosclerotic lesion.(62) The proinflammatory macrophage is increased at the lesion site along with dying apoptotic cells and other proinflammatory factors creating a unique microenvironment.(61) As the formation of monocyte into macrophages increases it affects the function of macrophages within the lesion, the macrophages undergo apoptosis leading to secondary necrosis as well as increased inflammation of the plaque.(62) The sub-endothelium is where the monocytes are matured into macrophages and they take up the lipids due to the presence of receptors on the macrophages known as monocyte-derived macrophages .(62) In animal studies it has been shown that macrophages which are active in atherosclerotic lesions release chemokines that promote uptake of neutrophils into the lesion. Neutrophils are

leucocytes that are responsible for protecting the cells against pathogens in the innate immune system.(59) Inflammation is consistently recurring during atherosclerosis which influences the arteries elasticity and muscular cells.(60) Natural killer (NK) cells are the main organizers during inflammation in atherosclerosis and are innate lymphoid cells. The main function is to protect the tissue against any infection.(63) Evidence has shown that the atherosclerotic plaques found in humans contain NK cells and they are found in abundance within deep plaque. The NKs interact with Dendritic cells (DCs) to trigger the T cell response in the adaptive immune system making the NK cells a link between innate and adaptive immunity.(63) Recent evidence has shown that DC play a role in triggering the immune response that leads to atherosclerosis and are found in the site of the lesion.(64)

The atherosclerotic lesion is orchestrated by different immune cell types. The monocytes and macrophages within the lesion release mediators that increase the uptake of the smooth muscle cells from the media as seen in figure 2.5. Once the smooth muscles cells are in the media they migrate, proliferate and release matrix proteins which increases the mass of the plaque. Macrophages and DCs trigger a proinflammatory response by presenting their antigens to T cells and this leads to the continual occurrence of inflammation in the plaque.(65)

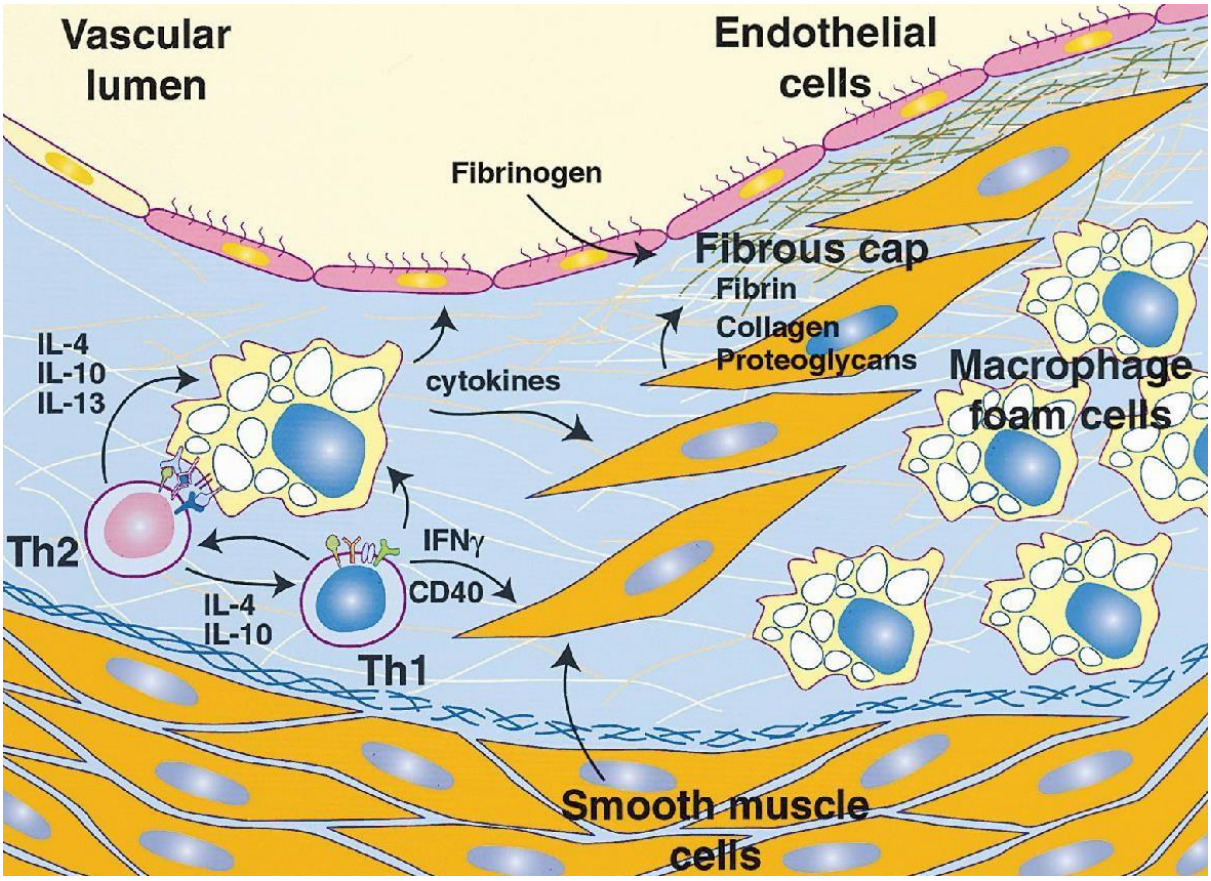


Figure 2.5 Chronic inflammation as a result of interaction between the macrophages, foam cells Th1 and Th2. Macrophages and lymphocytes release cytokines that influence both the anti and pro-atherosclerotic effects on the endothelial wall. Smooth muscles cells move from the medial portion to the arterial wall then they proliferate and release extracellular matrix proteins which are the constitutes of the plaque that is formed.(28) Th1 = T helper type 1 Th2 = T helper type 2 IL-4 = Interleukin 4 IL-10 = Interleukin 10 IL-13 = Interleukin 13 IFN-γ = Interferon gamma CD40 = Cluster of differentiation 40

Oxidative stress is an imbalance between the production of ROS and the elimination by antioxidants which are protective mechanisms.(66) This imbalance results in chronic inflammation which is acute inflammation that persists for a longer period than normal.(67) Various transcription factors are activated by oxidative stress, some of these factors lead to the expression of genes that play a role in inflammation.(67) Oxidative stress increases due to MG which accumulates in T2DM causing an increase in inflammation.(12, 67)

Inflammation is a protective mechanism that is activated in response to tissue injury.(68) The distinguishing feature of inflammation is the dysregulation of proinflammatory molecules that are found in circulation and these molecules have a pathological effect on the haematological system.(69) These molecules include

erythrocytes, platelets and plasma proteins like fibrin and fibrinogen, the structure of these plasma proteins changes and this leads to abnormal clot formation.(69)

Type 2 Diabetes Mellitus is a proinflammatory state with increased levels of cytokines in circulation which causes inflammation.(70) The association between inflammation and T2DM results in vascular dysfunction.(48) In T2DM there are inflammatory markers such as nuclear factor alpha, nuclear factor kappa B, cyclooxygenase 2 and interleukin 6, these markers are not regulated which causes oxidative stress resulting in increased inflammation.(48) The increase in hyperglycaemia and insulin lead to endothelial dysfunction which results in inflammation leading to atherothrombosis as seen on figure 2.6.

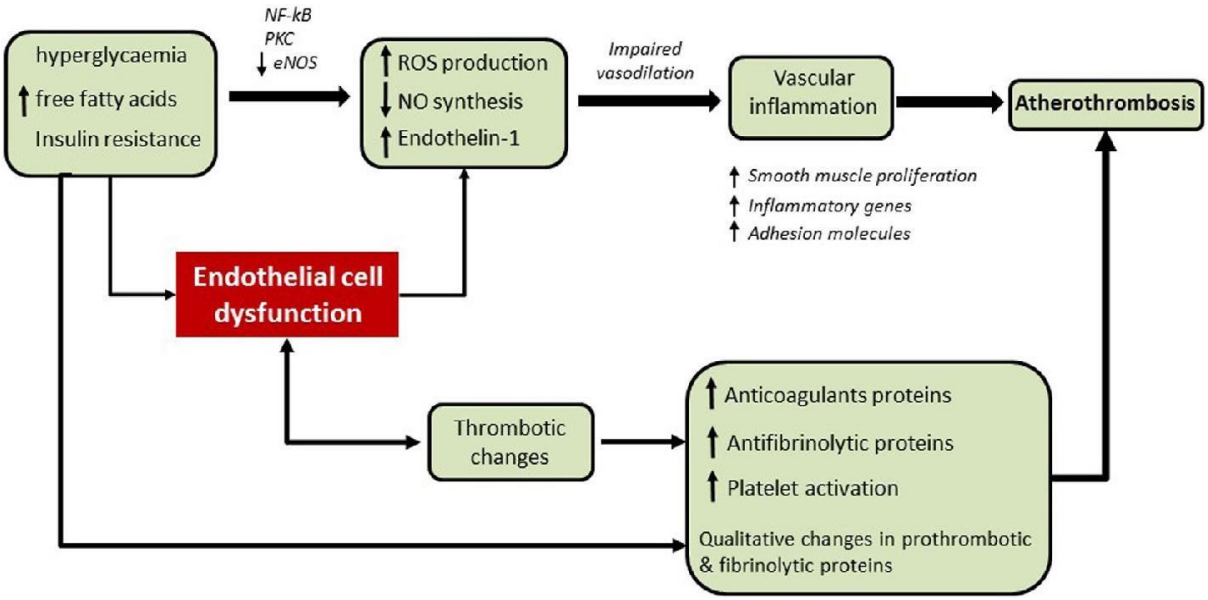


Figure 2.6 The endothelial dysfunction and formation of thrombosis in type 2 diabetes mellitus. The atherogenesis is influenced by endothelial cell dysfunction at all stages. During the initial stages of the disease there is insulin resistance, accumulation of glucose and free fatty acids which increase the production of reactive oxygen species (ROS) and decrease in the synthesis of nitric oxide (NO) which triggers the activation NF-κB and protein kinase C (PKC). Endothelial dysfunction also leads to the abnormal vasodilation, expression of adhesion molecules and inflammation. As the disease progress platelets are triggered, there is a decrease in fibrinolysis and the environment favours thrombosis which increase the formation of atherothrombosis.(53)

Haemostasis

Haemostasis is a regulated process that ensures maintenance of normal blood flow and reduces blood loss during tissue injury.(71) Haemostasis is a vital process and the mechanism needs to be controlled to prevent the formation of thrombosis.(71)

There needs to be a balance between prothrombotic and anticoagulant processes.(72) In healthy humans, haemostasis is linked to endothelial cells and coagulation factors to ensure an effective response to injury.(71)

The endothelium plays a vital role in separating blood components from structures in the subendothelial layer to prevent reactions, thus regulating the vascular tone and permeability.(72) Moreover, when there is no tissue injury, endothelial cells regulate the inhibition of coagulation and platelet accumulation.(72) In addition, endothelial cells also regulate the production of activators and inhibitors of fibrinolysis.(72) During tissue injury to prevent blood loss, there is immediate vasoconstriction due to collagen and tissue factors found in blood circulation.(71) The accumulation of collagen at the site of tissue injury induces the increase and activation of platelets which leads to the formation of a platelet plug and then a fibrin clot.(71) Fibrin clots need to be retracted to form a tight seal to prevent blood loss and needs to be drawn towards the endothelial wall to reduce obstruction of blood flow and promote wound healing.(73)

Haemostasis needs to be finely balanced to reduce the formation of pathological thrombosis.(74) The prevalence of pathological thrombosis differs with vascular sites and can also be as a result of other diseases.(72) In T2DM, there is an increase in thrombosis and a decrease in fibrinolysis which also contributes to the increased risk of plaque formation leading to CVD.(74)

Coagulation forms a platelet plug and a fibrin clot so that during injury, the blood vessel wall can be closed to reduce blood loss.(75) The coagulation process is not constantly activated to reduce the clots formed and to prevent the formation of thrombosis, this is controlled by several coagulation inhibitors. When there is an increase in the procoagulants or there are fewer inhibitors of coagulation it leads to an increase in clot formation.(76)

The coagulation cascade consists of the extrinsic pathway which is activated by factor XII (FXII) and the intrinsic pathway which is activated by FVIIa or a tissue factor (TF) that are expressed in the subendothelial wall as seen in figure 2.7.(75) The extrinsic pathway is the initial step to haemostasis. If there is any injury the TF binds to the FVIIa and calcium so that FX can be converted to FXa. In the intrinsic pathway thrombin is activated by FXII. The bond between FXII, High-molecular-weight (HMW) kininogen, prekallikrien and FXI lead to the activation FXI. After the activation of FXI, FXI acts as a cofactor and forms a phospholipid surface that results in the activation of FX.(76) The common pathway between the two is activated by FX as well as FV

which acts as a cofactor. The phospholipids and calcium lead to the formation of the prothrombinase complex that is responsible for the conversion of prothrombin to thrombin. In circulation, the fibrinogen is cleaved by thrombin into insoluble fibrin and this activates FXIII to form a platelet plug.(76)

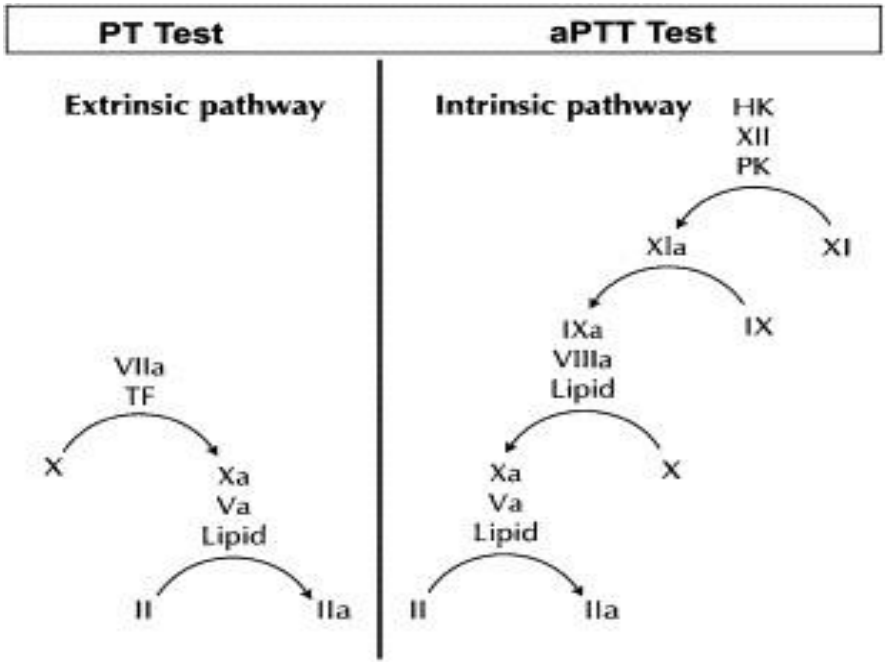


Figure 2.7 The extrinsic and intrinsic pathway in the coagulation cascade. The two pathways are responsible for the initiation of the formation of thrombin. A surface of phospholipids is required for the reaction to take place, hence, the presence of lipids. During blood analysis prothrombin time (PT) is used to assess the extrinsic pathway and the activated partial thromboplastin time (aPTT) is used for the intrinsic pathway. HK, high molecular weight kininogen; PK, prekalikrein.(75)

The analysis of the pathways is done using prothrombin time (PT) and activated partial thromboplastin time (aPTT).(75) In recent studies it has been shown that T2DM individuals have decreased PT and aPTT levels and have increased fibrinogen levels when compared to healthy individuals, which can result in increased formation of a clot.(77) It has been shown that there is a clear association between insulin resistance and the levels of FVII, FXII, FXIII and fibrinogen.(78)

Fibrin

Fibrinogen, a large fibrous glycoprotein is converted into fibrin in the presence of the enzyme thrombin, the formation of fibrin occurs at the last step of the coagulation cascade.(17) Fibrinogen is an acute plasma protein and has multiple functions that play a role in haemostasis as well as the coagulation cascade.(79) This molecule is a 340 kDa glycoprotein that is homodimeric and each of the subunits consists of 2Aα,

2Bβ and 2γ polypeptide chains with molecular masses of 66 500, 52 000 and 46 5000 respectively, linked by 29 disulphide bonds.(20) In human blood under normal physiological conditions, the concentration of fibrinogen is 1.5 mg/ml to 4 mg/ml, however, the fibrinogen levels can increase to more than 7 mg/mL during acute inflammation since fibrinogen is found in plasma.(80) Fibrinogen plays a role in haemostasis, cell adhesion, wound healing, inflammation, atherogenesis and fibrinolysis.(20, 79)

Post- translational modifications of fibrinogen are the reason why this molecule has a variety of forms in blood. There are about just over a million different forms of fibrinogen in normal physiology due to the several ways in which it can be synthesised as well as the genetic variations. However, some of the combined modifications that happen due to several other biochemical reactions can lead to pathophysiological conditions like ischemia or inflammation. The modifications on the fibrinogen can occur by phosphorylation, oxidation of methionine, modifications to the cysteine subunit and formation of carbonyl groups, etc.(20, 81) In T2DM, non-enzymatic reactions such as the glycation of lysine group on the fibrinogen occurs in the individuals. The chemical modifications produce fibrinogen and fibrin that have different structural and functional properties than those seen in normal physiology.(20)

Even though fibrinogen is soluble in plasma, this macromolecule forms fibrin which is insoluble forming a blood clot in response to vessel wall injury which occurs either in the blood vessel know as intravascular or extravascular in the spleen and liver.(18) A blood clot is vital to prevent the release of too much blood from the injured tissue and it also aids with wound healing.(20) During wound healing the fibrin binds to the other proteins and growth factors that are found in tissue or released in response to wound healing.(17) The interaction of proteins with fibrin makes it possible for specific receptor-mediator interactions to be activated in cells.(17)

The generation of fibrin is determined by how the clot forms, the structure, stability as well as the concentration of thrombin.(80) The structure of fibrin is defined by the thickness of fibrin fibres, the number of pores, the number of branching points as well as the permeability of the fibrin gel .(17) The type of structure produced is influenced by the salt concentration, pH, temperature and other proteins found in plasma such as fibronectin and albumin.(17) The level of prothrombin influences the size of fibrin fibre diameter, the diameter decreases as prothrombin increases.(82) In conditions where

thrombin levels are increased, the fibrin clots formed have small pores and the fibres are thin.(82)

Fibrin is a viscoelastic polymer meaning that fibrin has two properties namely elastic and viscous.(18) The elasticity is also characterised by how stiff fibrin is and if the there is a possibility to reverse any mechanical deformation.(18) The viscosity can be compared to plasticity because the mechanical deformation cannot be reversed.(18) The behavioural character of elasticity is strain, as the strain increases the elasticity is also increased, however, when the stress is removed the elasticity is recovered.(17) Fibrin fibres become more elastic as they are being stretched and not when bending, cross-linking is the determining factor of elasticity in fibrin.(83) The elasticity of a fibrin fibre shows how much mechanical damage has occurred and how well the blood clot will be removed so blood flow can be restored.(20)

A clot is not meant to be a permanent structure and it needs to be removed, therefore, the activation of fibrinolysis plays a role in the removal of clot to prevent the formation of thrombosis.(17) Plasmin is an enzyme that is derived from plasminogen and is the main enzyme involved in fibrinolysis, as shown in figure 2.8. Plasmin and plasminogen also have an important role in wound healing, inflammation, cells migration, angiogenesis and atherosclerosis.(20) The role of the fibrinolytic system is to trigger the removal of fibrin clots formed during wound healing. The endothelial cells release tissue plasminogen activator which is the trigger for fibrinolysis. This process converts plasminogen which is made in the liver, into plasmin.(17) After the activation this enzyme cleaves fibrinogen and fibrin into soluble molecules that are added to blood circulation.(17) However, this process is affected by the structure of the fibrin clot, the kinetic properties, and the homeostasis of fibrinolysis.(17) The production of dense fibrin networks with many branches due to high levels of thrombin makes the clot difficult to lyse, however, fibrin clots that are not dense and have few branches making it easier for the clot to lyse.(80)

It has been seen that in T2DM the clots formed are more dense and are resistant to fibrinolysis.(48) The structure of the clots formed seems to be determined by the concentration of glucose in the blood.(81) In T2DM, the concentration of glucose is not regulated which results in rapid clot formation.(81) In previous studies it has been shown that individuals with T2DM have abnormal fibrin fibres than those seen in healthy controls where the fibrin fibres look more dense under scanning election microscope (SEM).(84) The change in morphology seen in the pathological state was

proposed to be due to the changes that occur in the structure of the fibrin or fibrinogen.(85)

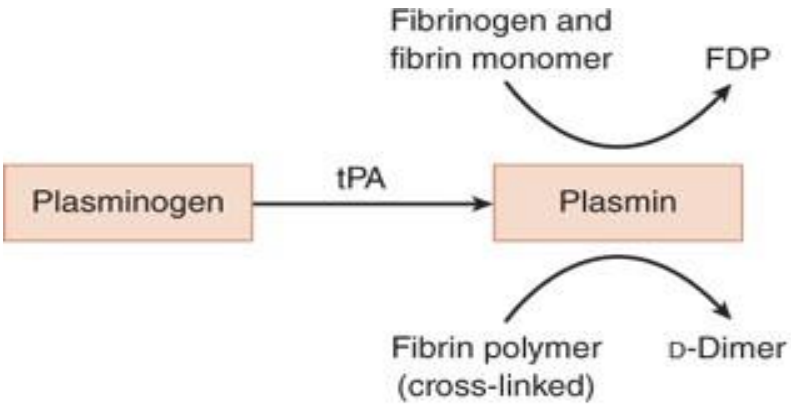


Figure 2.8 The fibrinolytic system responsible for the breakdown of fibrinogen and fibrin monomers by tissue plasminogen activator (tPA).(20)

The prevalence of CVD is increasing; hence it is important to identify the key factors involved in the pathogenic mechanisms as targets for new drug treatments.(19) Cardiovascular disease risks in T2DM may be due to the abnormal metabolic activities such as insulin resistance that occur in these individuals.(81) Over many years atherosclerotic plaques form and develop without causing any symptoms, but over decades of activation of coagulation and cross-linked fibrin clot formation, it leads to the development of thrombosis.(81) In previous studies, it has been shown that with increased levels of fibrinogen, there is an increased risk of developing CVD.(81) Fibrin clots are resistant to the fibrinolysis process due to their structure. The fibrin fibres determine how well the clot will lyse, dense clots have thin fibres and small pores which are more difficult to lyse.(86) The increased risk of developing thrombosis is due to the production of abnormal clots by fibrinogen glycation.(82) A study by Bochenek et al. (2013) also showed that T2DM is a modifier of fibrin clot properties in patients with coronary artery disease. (87)

The literature review above shows that oxidative stress and methylglyoxal is linked to increased inflammation that leads to vascular damage. We need to discover novel drug treatments that will help reduce the risk of CAD and high mortality rates in individuals with T2DM. This study will investigate the role of MG on clot kinetics and fibrin clot structure in CAD patients with T2DM in order to provide insight into mechanism of pathogenesis of atherosclerosis in T2DM which results in the development of CAD. A summary of the literature review is highlighted in figure 2.9.

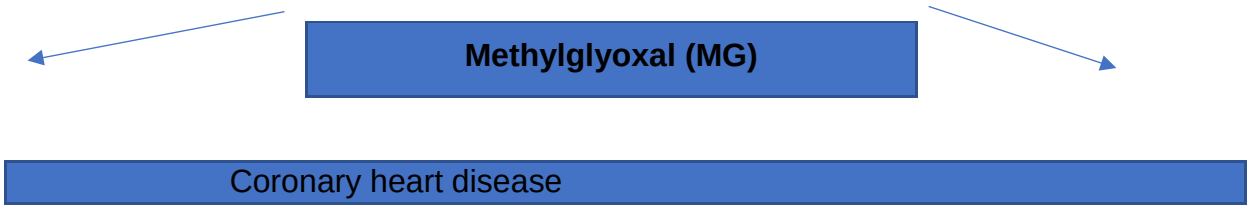


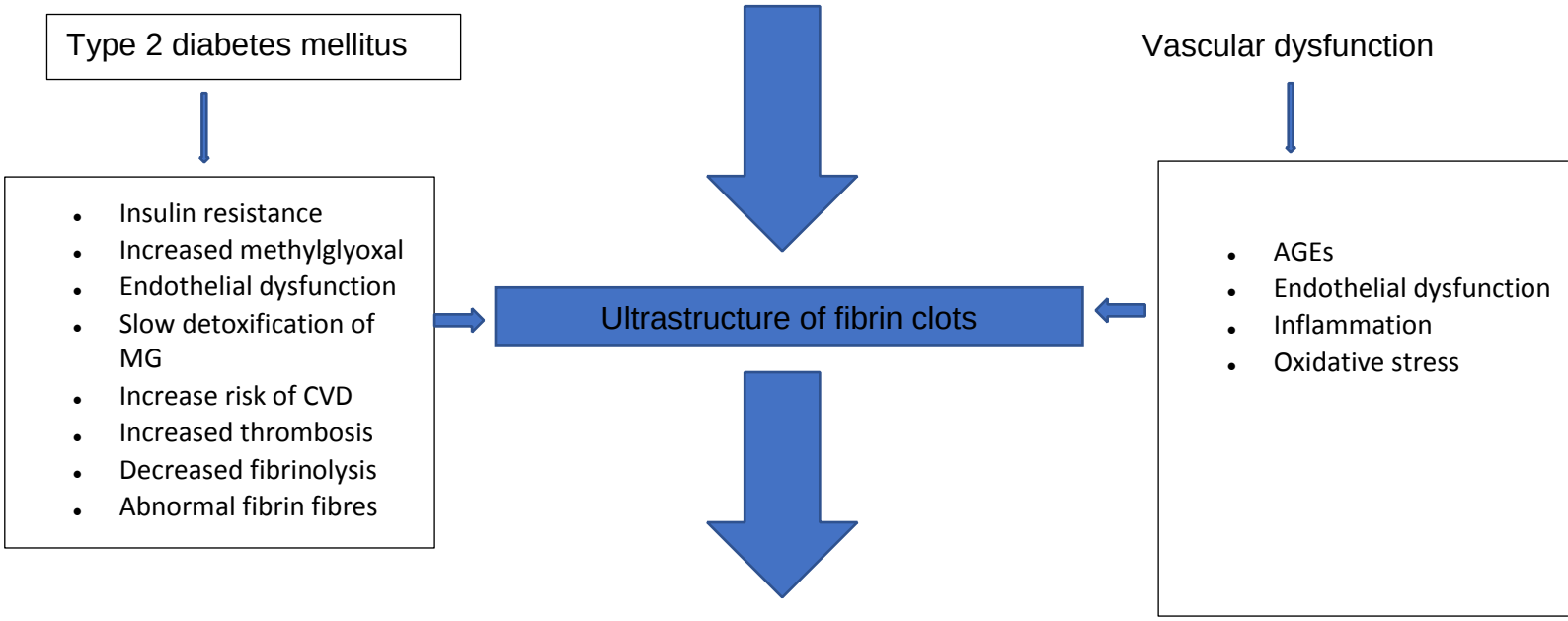
Figure 2.9: Schematic representation of the literature review

Study aims and objectives

The aim of the study was to determine the role of MG on clot kinetics and fibrin clot structure in CAD patients with and without T2DM

Objective 1: To evaluate if there is a difference in the morphology of fibrin clots in CAD patients with and without T2DM using SEM

Objective 2: To determine the physiological clot properties (kinetics) in CAD patients



with and without T2DM using TEG

Objective 3: To determine the levels of methylglyoxal-adducts in each group using an ELISA

CHAPTER 3 MATERIALS AND METHODS

Ethics

The study was granted ethics approval by the Faculty of Health Sciences Research Ethics Committee on 2019-08-28 (Ethical approval no.: 484/2019).

Study design

The design adopted for this study is a cross sectional, observational study as shown in figure 3.1. Patients admitted to the thoracic surgery ward at Steve Biko Academic Hospital for coronary artery bypass grafting (CABG) were recruited. Whole blood was obtained in citrate tubes via venipuncture following signed informed consent.

Setting

To determine the ultrastructure of fibrin network, the physiological clot properties, and the methylglyoxal influence on clot morphology, 10 control participants were recruited from the general population in Gauteng and 28 coronary artery bypass grafting (CABG) patients, 12 coronary heart disease with Type 2 diabetes mellitus patients and 10 Type 2 diabetes mellitus patients were recruited from Steve Biko Academic Hospital in Gauteng. These patients were generally recruited from public health institutions which are government funded. This means that this cohort was predominantly of a low-socio-economic population dependent on public health care.

Patient selection

Sample size and selection

Following consultation with a statistician (appendix 6), review of the protocol at the MSc committee (appendix 4) as well as the ethics committee (Ethical approval no.: 484/2019, appendix 5), 28 individuals with CAD, and 12 CAD individuals with T2DM and 10 T2DM patients were recruited from Steve Biko Academic Hospital, cardiology ward and in the Thoracic surgery ward. The 40 healthy staff and student volunteers were recruited from the University of Pretoria. Following completion of the informed consent forms (appendix 2), 5 ml of blood was collected in a citrate tube via venipuncture.

Inclusion Criteria for T2DM participants with CAD

- Individuals 18 years and older
- Individuals willing to sign inform consent
- Individuals with a diagnosis of T2DM for 3 or > months

- Documented diagnosis of CAD, either previous myocardial infarct, or undergoing CABG or admitted for percutaneous coronary intervention (PCI).

Inclusion and exclusion criteria for individuals with CABG/CAD

- Individuals 18 years and older
- Individuals willing to sign inform consent
- Documented diagnosis of CAD, either previous myocardial infarct, or undergoing CABG or admitted for percutaneous coronary intervention (PCI).

Inclusion and exclusion criteria for healthy volunteers:

- Individuals 18 years and older
- Individuals willing to sign inform consent
- They should not be smokers, on any chronic medication or oral contraceptives.

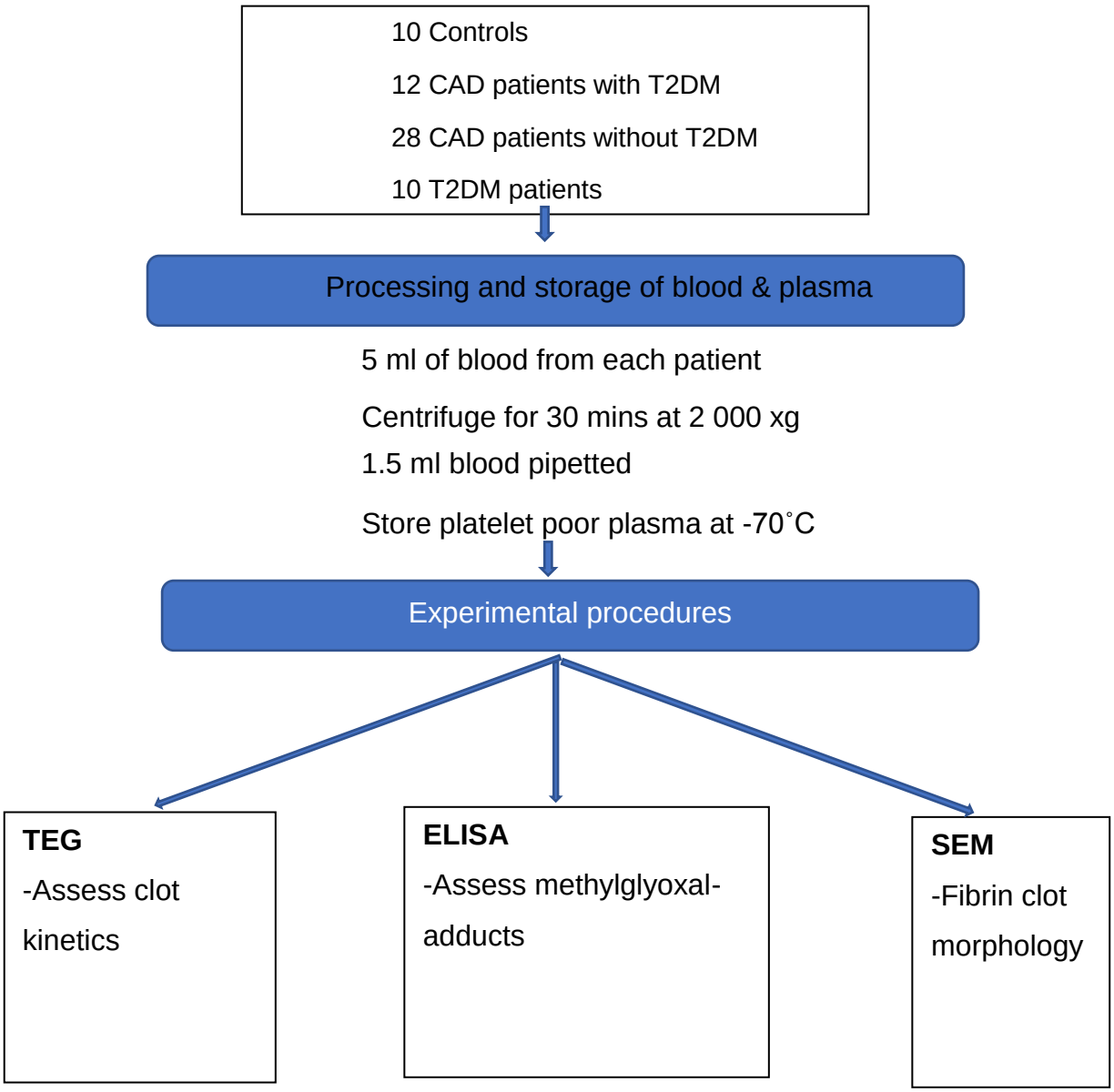


Figure 3.1 Schematic representation of the experimental design of the study

Blood collection:

Following informed consent (appendix 3), a 5 ml blood sample was collected in a citrate tube by venipuncture from all study participants.

Methods

Preparation of fibrin fibres for scanning electron microscope

The fibrin fibres were prepared by adding 10 µl of platelet poor plasma (PPP) and 5 µl of thrombin (20 U/ml in 0.2% human serum albumin - provided by the South African National Blood Service (SANBS)) was added in the middle of the PPP on a 10 mm round coverslip and then mixed using a pipette tip. The mixture was allowed to dry forming a gel like layer on the coverslip at room temperature for 1 to 2 minutes, (this allows time for the fibrin fibres to form) followed by placing the coverslip in an 18 well plate. Phosphate-buffered saline (PBS) (0.075M at a pH of 7.4) was added to the wells

and incubated for 15 minutes. The process allows for the removal of any proteins that may still be present in the formed fibrin fibres. The PBS was discarded and 4% formaldehyde was added and the sample was covered for 30 minutes. The fixative was discarded, and the coverslips were washed with PBS 3 times for 3 minutes. Third PBS wash was discarded and then 4-5 drops of osmium tetra-oxide (OT) were added onto the coverslip together with the same number of drops of ddH_2O. and kept in the fume cupboard for 15 minutes. The OT and ddH_2O mixture were discarded and the coverslips were washed with PBS thrice for 3 minutes.

For the dehydration series the samples were washed in 30%, 50%, 70%, 90% and 100% ethanol for 3 minutes each. The 100% ethanol wash was repeated twice. In the fume cupboard the ethanol was discarded and the coverslips were covered with hexamethyldisilazane (HMDS) and allowed to dry overnight.

Dried samples were mounted on metal plates and carbon coated. The carbon coated fibrin fibres were viewed and analysed using the Zeiss ULTRA plus SEM (Zeiss, Germany) and the micrographs taken were 1 kV. For the analysis, two samples were viewed per patient and the images were taken at a magnification of 10 000x and 20 000x respectively. The software: ImageJ was used to analyse the images to get the quantitative density of the fibrin fibres.

Thromboelastography (TEG)

The TEG is a method that is used to test the efficiency of blood coagulation.(88) This is a qualitative technique used to demonstrate the viscoelastic characteristics of clot formation (table 3.1).(89) This technique was developed in 1948 by Dr. Hellmut Hartert and he used it to determine the rate of coagulation in white blood cells.(88)

Table 3. 1 TEG parameters with the characteristics

TEG PARAMETERS	CHARACTERISTICS
Reaction time (R value; sec)	The reaction time it takes for a clot to form measured in minutes
Clot kinetics (K; sec)	The time taken to reach a level of clot strength with an amplitude of 20 mm.

α-angle (º)	The rate at which fibrin accumulates and formation of cross links.
Maximum amplitude (MA; mm)	It represents the strength or stiffness of the clot.
Coagulation index (CI; dynes/sec)	It represents the overall stability of the clot.
Lys 30 (% of the lysed clot)	The percentage of clot lysis in 30 minutes.
Clot firmness (G; dynes/cm²)	it represents the overall stability of the clot.
Maximum rate of thrombus generation (MRTG; Dyn cm−2 s−1)	The maximum velocity at which the clot grows
Time to maximum rate of thrombus generation (TMRTG; min)	It is the time that is measured before the maximum speed of the clot growth measures the total clot resistance.
Total thrombus generation (TTG; Dyn.cm−2)	It is the strength of the clot were the total amount of resistance is measured during clot formation. On the graph this is represented by the total area that is under the velocity curve while the clot is growing.

Blood in a citrate tube was centrifuged at 2000 xg for 30 minutes to obtain PPP. Platelet poor plasma is used to measure how the coagulation functions in the absence of platelets and erythrocytes since they play a role in clot formation. The PPP was stored in a -70˚C freezer in aliquots of 500 µl in each microcentrifuge tube. The TEG experiment starts by aliquoting 340 µl into the oscillating cup and pin that is mounted into the TEG instrument as seen in figure 3.2 (TEG®5S, Haemonetics, Switzerland).

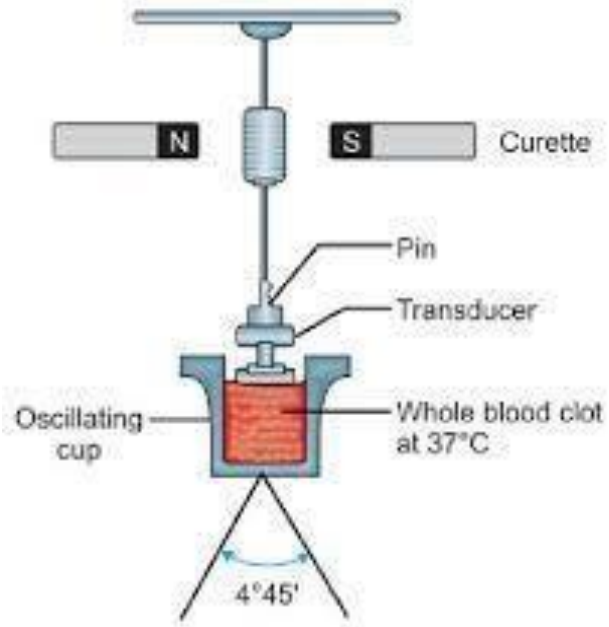


Figure 3.2 The components of the TEG that are used to monitor clot kinetics and coagulation. (90)

After the addition of PPP, 20 µl calcium chloride (0.2 M, Barker Medical, South Africa) was added so that the coagulation process can be activated. The coagulation process was measured by a detector (pin) as the sample was oscillated in the cup. A specialized TEG program (TEG® Manager Software, Switzerland) was used to monitor the clot kinetics and coagulation of the sample as seen in figure 3.3.

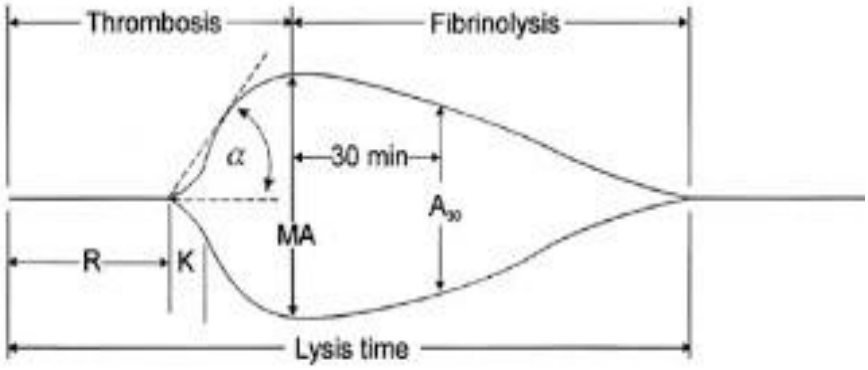


Figure 3.3 The characteristics of a typical waveform generated from thromboelastography. (90)

Determination of methylglyoxal-adducts

An enzyme-linked immunosorbent assay (ELISA) was used to determine the levels of methylglyoxal-adducts in plasma which is used as a measure of glycated proteins.(91)

The ab238543 Methylglyoxal (MG) ELISA kit develops rapid detection and quantification of the MG-H1 protein adducts, it is an enzyme immunoassay. The absorbance of a standard curve known as MG-BSA and the absorbance of MG adduct protein were compared to quantify of MG adduct in patient plasma samples.

MG Conjugate Coated Plate

To coat the plate the conjugated diluent was prepared by diluting the conjugate in PBS, MG conjugate was diluted in 1.0 mg/mL MG Conjugate in the 1X Conjugate Diluent and it was done in two step dilution. In the plate 100 µL of the 500 ng/mL MG conjugate was added to each well on the plate and it was incubated overnight at 4˚C. The MG conjugate was then removed, and the plate was washed twice with 1X PBS followed by blotting the plate on the paper towel to remove all that fluid. To block each well 200 µL of the assay diluent was added to the plate and incubated for an hour at room temperature.

Assay procedure

To make up the 1X wash buffer, the 10X wash buffer was diluted with 1X deionized water and it was stirred until there was homogeneity. The Anti-MG antibody and the secondary antibody were prepared by diluting both of them at a ratio of 1:1000 using the assay diluent.

On the ELISA plate 50 µL unknown plasma sample and MG-BSA standards of known concentration was added to the wells of the MG Conjugate coated plate. The unknown samples were diluted in 1X PBS that contained 0.1% BSA before adding and was incubated at room temperature for 10 minutes on an orbital shaker. Then 50 µL of diluted anti-MG antibody was added into each well and it was incubated at room temperature for 1 hour on an orbital shaker.

The plate was washed 3 times with 250 µL of 1X wash buffer including thorough aspiration between each wash. Following the last wash, the wells were emptied and the microwell strips were tapped on a paper towel to remove the excess 1X water buffer.

In the microwell strips 100 µL diluted secondary antibody-HRP conjugate was added to all wells and incubated for 1 hour at room temperature. The plate was washed 5 times with 250 µL of 1X wash buffer including thorough aspiration between each wash. Following the last wash, the wells were emptied and the microwell strips were tapped on a paper towel to remove the excess 1X water buffer.

The substrate was warmed to room temperature and then 100 µL was added to each well and was incubated at room temperature for 2 minutes. Then the reaction was stopped by adding 100 µL of the stop solution to each well and the results were read immediately. The absorbance of each well on the microplate reader using 450 nm as the primary wavelength was measured.

Statistical analysis

The primary objective of the study was to investigate the ultrastructure of fibrin network, the physiological clot properties, and the MG influence on clot morphology and in CAD patients with and without T2DM.

Analysis of differences between T2DM and controls for TEG parameters were determined by using a two-tailed non-parametric t-test (Mann Whitney test), one-way ANOVA (Kruskal-Wallis test) followed by Dunn's Multiple Comparison Test. Correlation analyses was done using Prism Graphpad Prism software (v 6.0 San Diego, California). The differences were considered to be significant when $p< 0.05$.

The quantity of MG-adduct in protein samples was determined by comparing its absorbance with that of a known MG-BSA standard curve.

Analysis of SEM was done by measuring the density of fibres using ImageJ (Image J is a public domain, Java-based image processing program developed at the National Institutes of Health: http://rsbweb.nih.gov/ij/).

CHAPTER 4 RESULTS

Cohort demographics

The mean age of the controls was 44 (24-65) years old, the CAD with T2DM group was 49 (40-79) years old, the CAD group was 68 (36-78) and T2DM group was 60 (22-65). The ratio of males to females in the control group was 1:0.5, in the CHD with T2DM group 1:0.09, in the CHD group 1:0.12 and in the group T2DM 0:10. The subjects included Caucasian South Africans 61.02%, Black South Africans 33.90% and Indian South Africans 5.08%. The subjects who had CHD were 67.79% and subjects with T2DM were 37.29%. Details of the demographics is shown in Table 4.1

Table 4.1: Cohort demographics

	Control n=10	**CHD and T2DM n=12**	**CHD n=28**	**T2DM n= 10**
Age (n) *Mean (range)*	44 (24-65)	49 (40-79)	68 (36-78)	60 (22-65)
Gender *(M/F)*	6/3	11/1	25/3	0/10
Ethnicity *(White: Black: Indian)*	5:4:0	8:3:1	21:5:2	0:10:0
Medication				
Statins	0	0	0	0
Anti-inflammatories	0	12	17	0
Insulin	0	0	0	0

Metformin	0	9	0	10

Scanning Electron Microscopy

The morphology of healthy controls was analysed using SEM. Figure 4.1 shows the representation of micrographs taken of healthy individuals and the micrographs show thick and thin fibres which create an organised mesh of fibrin fibres.

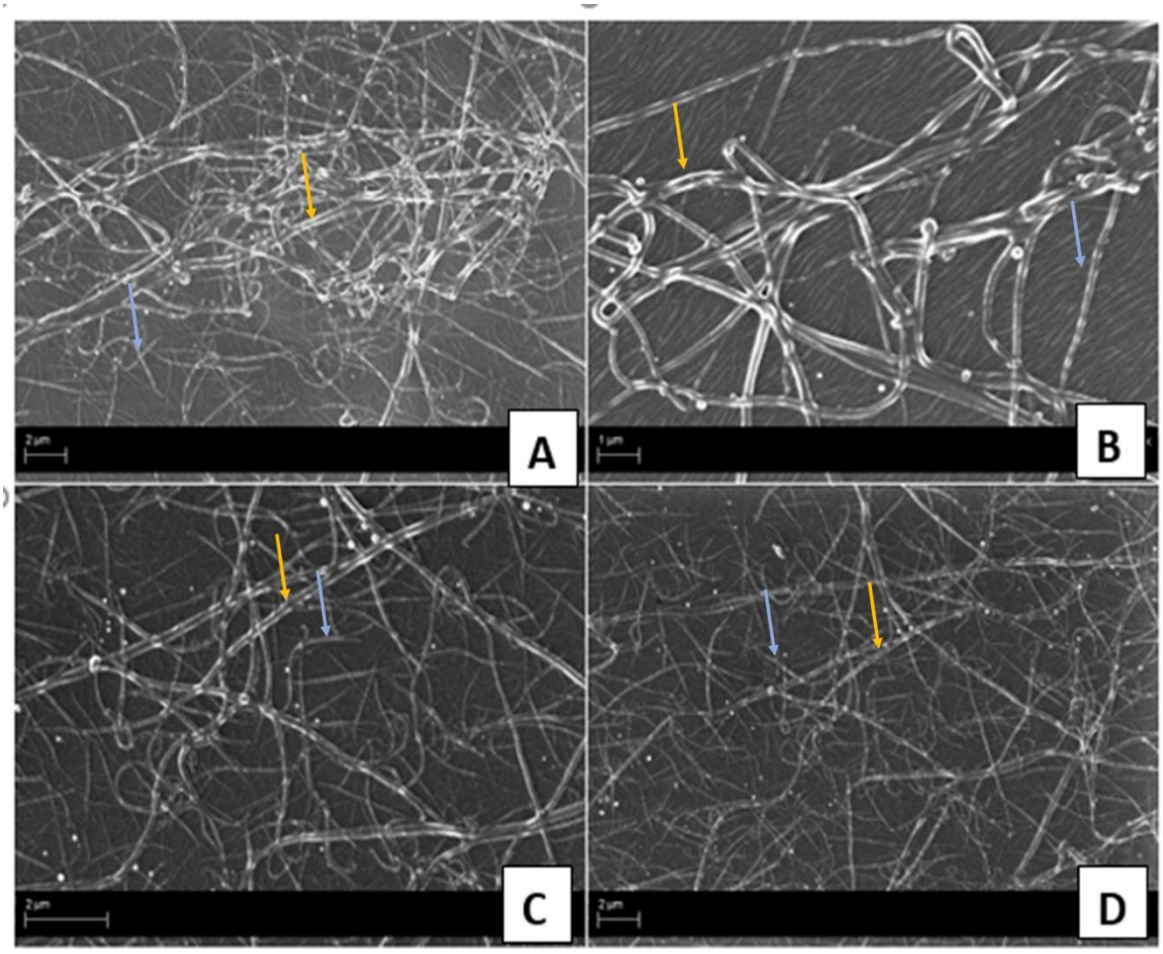


Figure 4.1 Micrographs of fibrin fibres of control subjects at (A, C, D) 10 000x and (B) 20 000x magnification. Normal fibrin fibres can be observed by the orange (thick fibres) and blue arrows (thin fibres) in A-D.

The morphology of fibrin fibers in T2DM, CHD and CHD with T2DM was analyzed using the SEM. The morphology of the fibrin fibers influences the clot formation resulting in decreased fibrinolysis.

The fibrin fibers were studied under high magnification to determine whether there were any alterations in the experimental groups. In T2DM, CHD with T2DM and CHD some alterations in the morphology were observed. The morphology observed in T2DM, CHD and CHD with T2DM patients showed that there were alterations, in particular, the fibrin fibers were not organized as seen in figure 4.2.

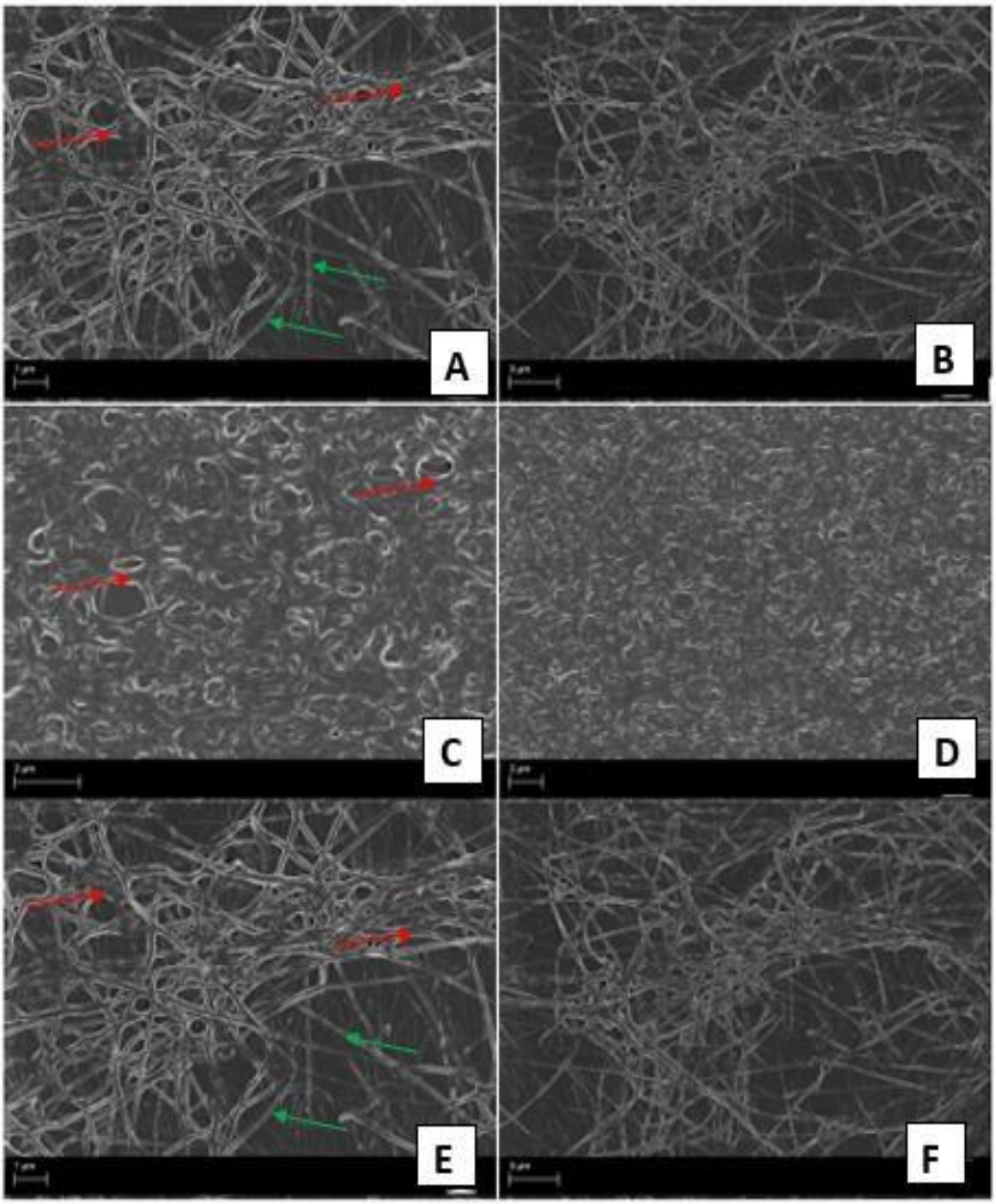


Figure 4.2 Micrographs of fibrin fibres of three T2DM subjects with CHD using the Scanning electron microscopy at 10 000x and 20 000x magnification: Red arrow: thick fibrin fibres in abnormal matted network, Green: thin fibrin fibre.

The ultrastructure shows that some of the fibrin fibres formed have individual fibres with both thick and thin fibres as well as a thick mass of fibres with a net-like structure that forms dense-matted deposits as seen in figure 4.2, 4.3 and 4.4. The fibrin fibres are not organised, and this was seen in patients with CHD.

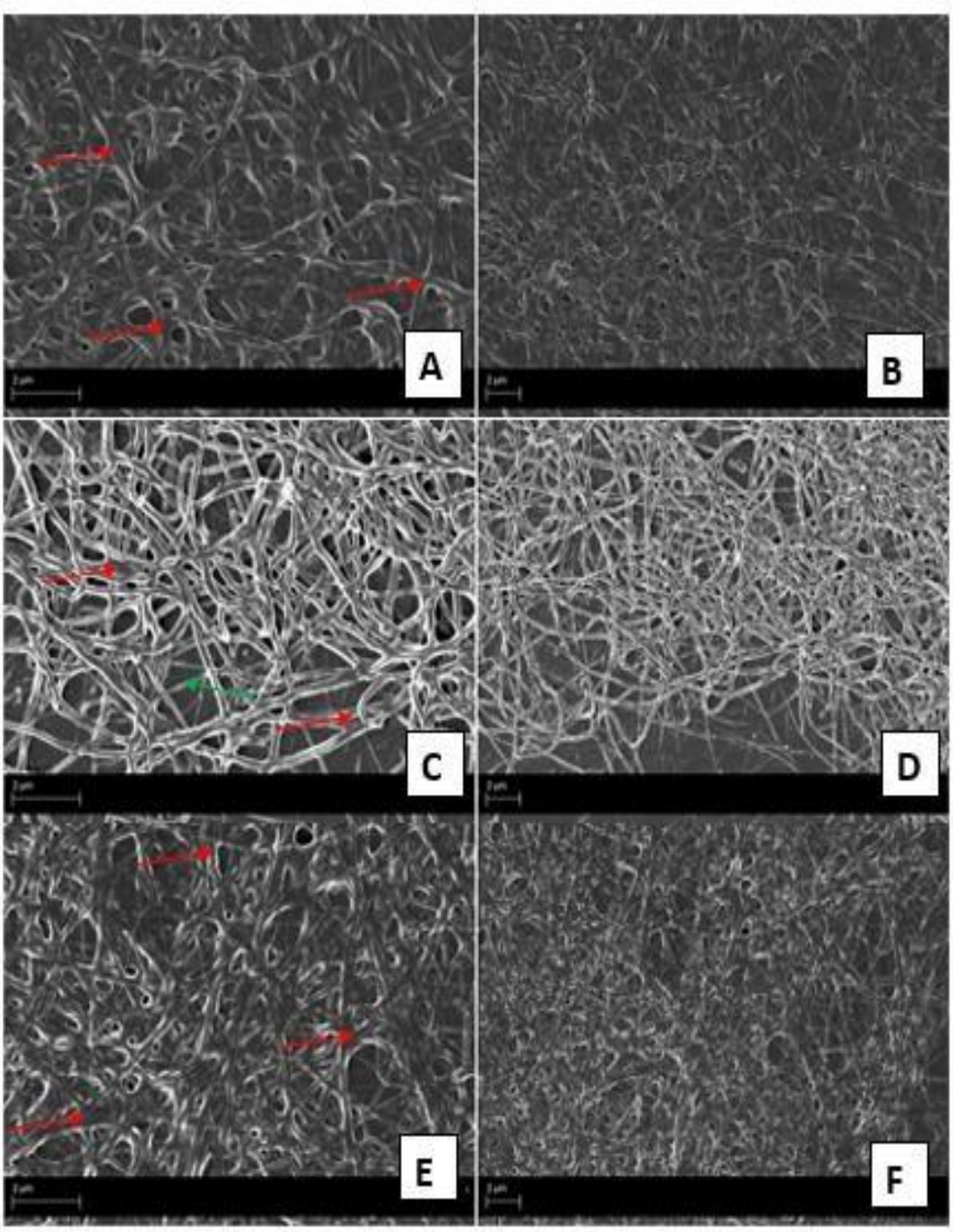


Figure 4.3 Micrograph of fibrin fibres of CHD subjects without T2DM at 10 000x and 20 000x magnification: Red arrow: thick fibrin fibres in matted network, Green: Thin fibrin fibre.

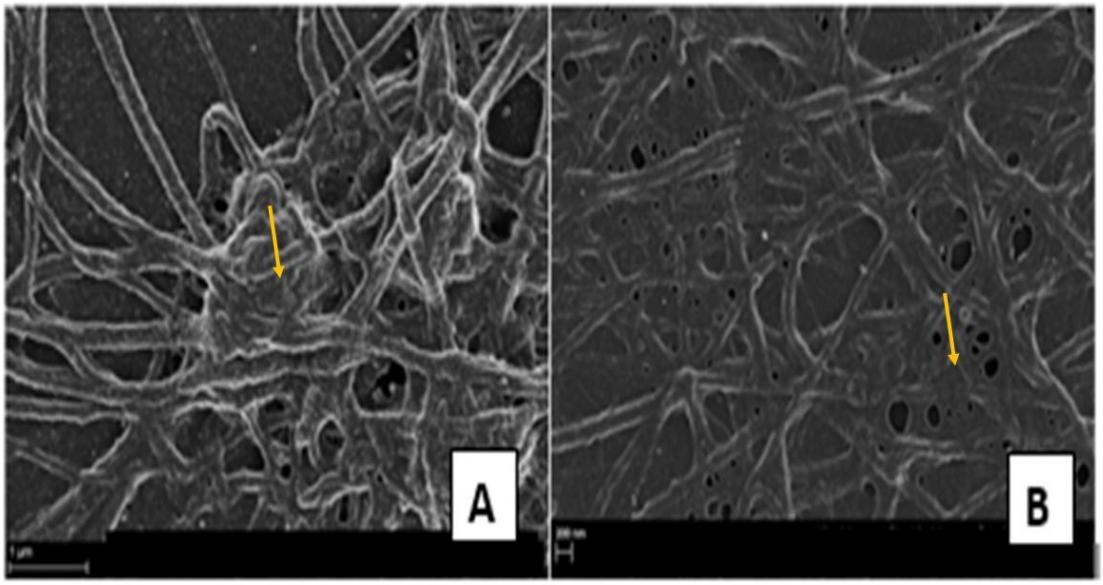


Figure 4.4 Micrographs of fibrin fibres from T2DM subjects at 10 000x and 20 000x magnification. The clumping and abnormal matted network of fibrin fibres can be seen by the orange arrows.

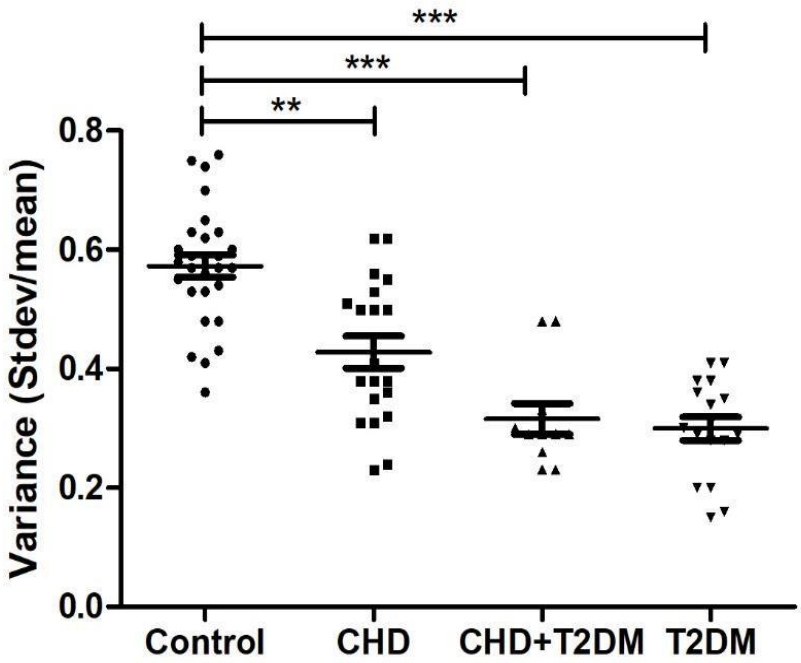


Figure 4.5 Densitometry analysis of scanning electron micrograph images of fibrin clots in controls, CHD, CHD and T2DM and T2DM patients. Where **p<0.001 and ***p<0.0001.

The densitometry analysis between controls and patient groups' (CHD: mean (standard deviation) 0.42±0.11; CHD+T2DM: 0.31±0.08 and T2DM: 0.29±0.08) was found to be significantly lower in all groups compared to the controls which had a mean

of 0.57±0.1, p<0.0001 (Figure 4.5). Density of fibrin networks was analysed to calculate variance. The variation and colour gradient are lower in clots that are denser, compared to control clots where there is a more pronounce colour gradient between the individual fibrin fibres and the darker background as seen in figure 4.3.

Thromboelastography

Clot kinetics

The clot kinetic analyses done on T2DM, CHD with T2DM and CHD using thromboelastography indicated that there was a difference in the clotting process compared to healthy controls.

Table 4.2 Summary of TEG parameters of controls and on T2DM, CHD with T2DM and CHD patients.

TEG Parameters	Control		T2DM with CHD		CHD		T2DM		p value
	Median	Mean (SD)	Median	Mean (SD)	Median	Mean (SD)	Median	Mean (SD)	
R time (mins)	7.15	8.32 (7.092)	13.28*	32.58 (21.94)	7.85	21.29 (19.21)	11.10	23.92 (16.99)	P<0.0001
K (min)	2.50	2.84 (1.585)	2.60	5.12 (6.552)	2.80	3.76 (2.949)	3.20	3.46 (2.56)	ns
Alpha angle (˚)	68.30	66.47 (9.816)	62.90	59.09 (13.88)	64.05	60.88 (16.41)	62.45	60.81 (13.91)	ns
MA (mm)	32.70	33.60 (11.41)	35.00	33.12 (14.67)	30.75	34.10 (15.04)	39.95*	40.51 (11.16)	P=0.0102
MRTG (dyn cm^{-2} s^{-1})	5.440	6.54 (4.830)	4.02*	5.05 (4.248)	5.25	10.74 (23.58)	3.67*	4.27 (4.419)	P=0.0096
TMRTG (min)	9.50	10.10 (5.513)	17.46*	20.46 (16.00)	11.75	14.14 (9.104)	21.04*	23.91 (15.23)	P<0.0001
TTG (dyn cm^{-2})	244.50	287.70 (155.5)	253.00	258.5 (174.8)	221.40	343.70 (508.5)	278.20	231.30 (183.6)	ns

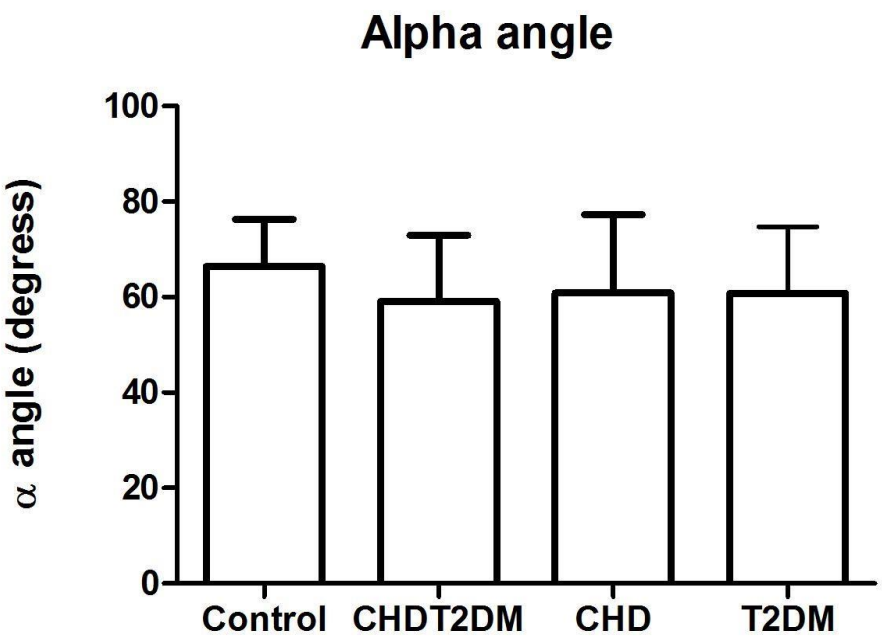


Figure 4.5 Alpha angle of clots from T2DM, CHD with T2DM, CHD and control subjects.

No significant differences between CHD, T2DM, CHD with T2DM and controls was found (60.88 ± 2.321˚ vs. 60.81 ± 2.385˚ vs. 59.09 ± 3.185˚ vs. 66.47 ± 1.300˚, p=0.0711).

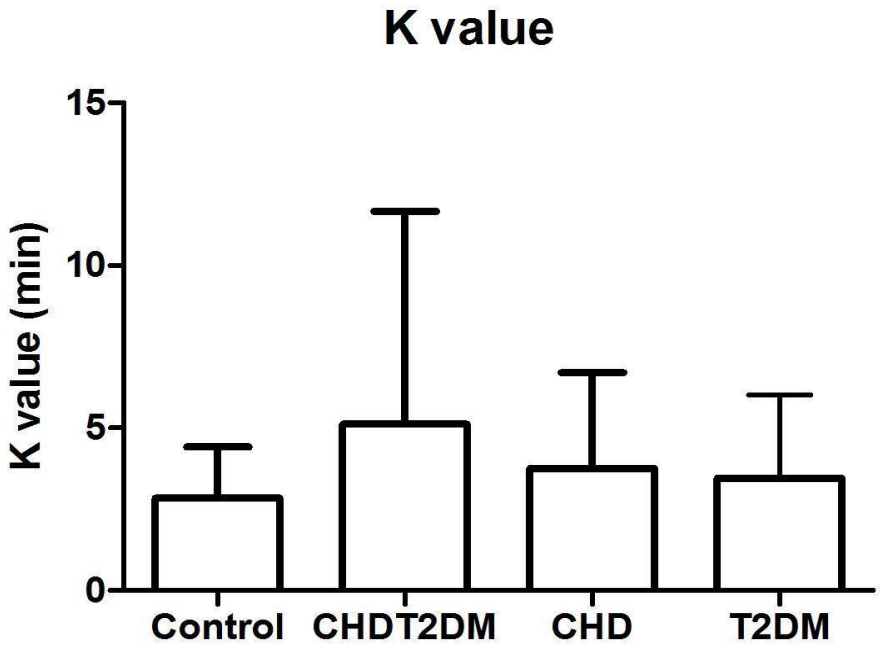


Figure 4.6 Clot kinetics (K-value) of T2DM, CHD with T2DM, CHD and control subjects.

The K-value between T2DM, CHD with T2DM, CHD and control subject was 3.458 ± 0.446mins vs. 5.118 ± 1.589mins vs. 3.758 ± 0.450mins vs. 2.839 ± 0.2156mins, respectively. The time taken to reach a level of clot strength with an amplitude of 20 mm (K-value) and the rate at which fibrin accumulates and forms cross links (α-angle) had no significant difference (Figure 4.5, p=0.5279 and Figure 4.6, p=0.0102, respectively).

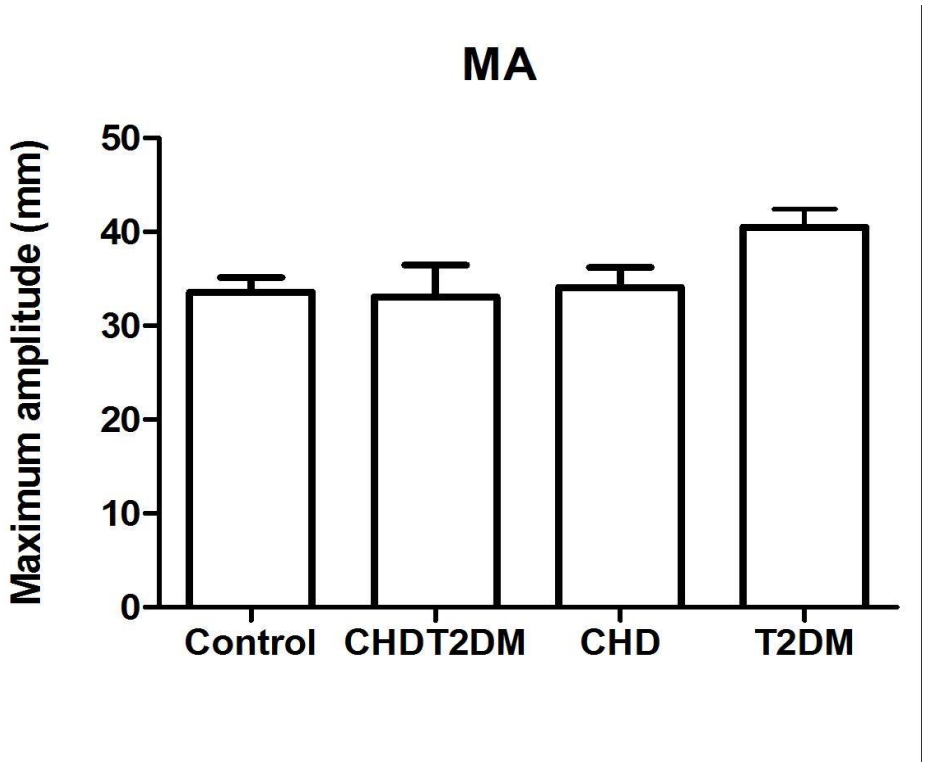


Figure 4.7 The maximum amplitude of clots from T2DM, CHD with T2DM, CHD and control subjects.

The maximum amplitude was higher in T2DM patients compared to CHD, CHD with T2DM and controls (40.51 ± 1.914mm vs. 34.10 ± 2.127mm vs. 33.12 ± 3.365mm vs. 33.60 ± 1.525mm, respectively). The clot strength (MA) was significantly different in T2DM patients compared to controls and there was a significant difference in CHD compared to T2DM patients (Figure 4.7, p=0.0102).

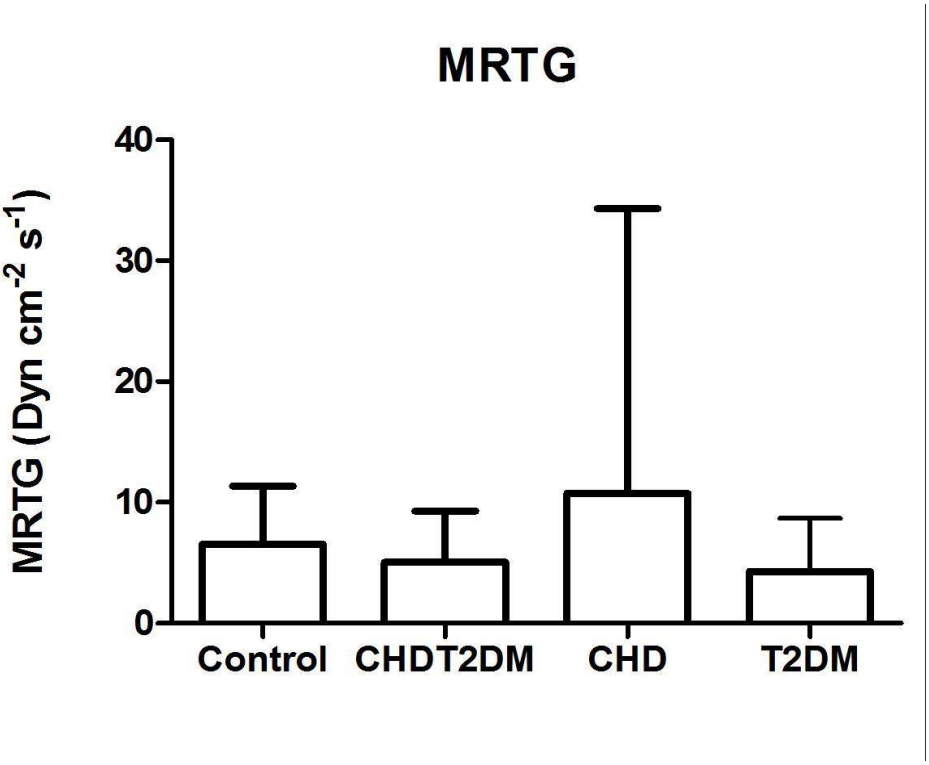


Figure 4.8 Maximum speed of the clot growth (MRTG) between T2DM, CHD with T2DM, CHD and control subjects.

The MRTG was higher in CHD patients compared to T2DM, CHD with T2DM and controls (10.74 ± 3.335 dyn cm^{-2} s^{-1} vs. 4.268 ± 0.690 dyn cm^{-2} s^{-1} vs. 5.046 ± 0.927 dyn cm^{-2} s^{-1} vs. 6.535 ± 0.664 dyn cm^{-2} s^{-1}, respectively). The maximum speed of clot growth (MRTG) was significantly different in CHD compared to T2DM patients (Figure 4.8, p=0.0096).

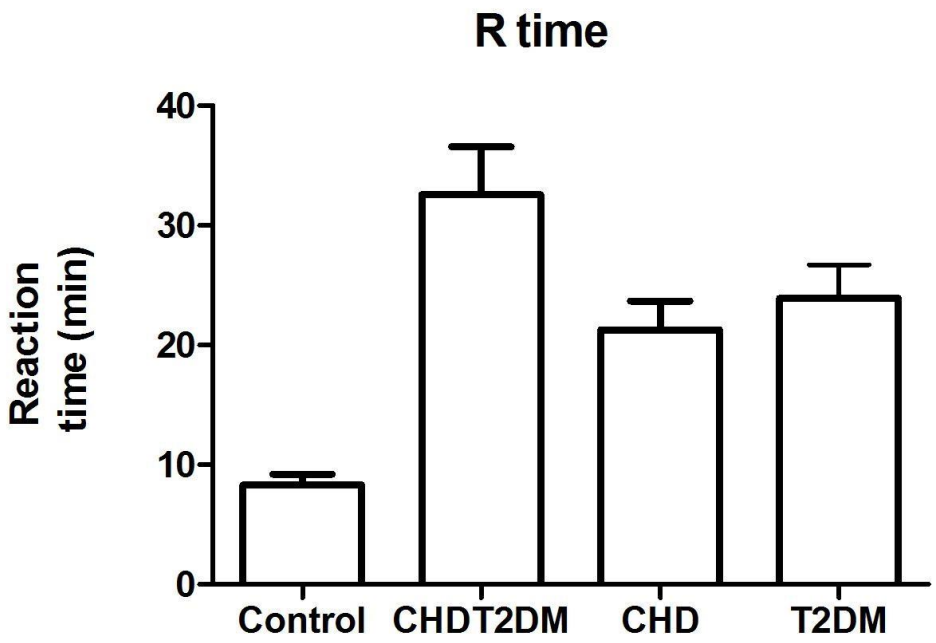


Figure 4.9 The reaction time for clotting to begin in samples from T2DM, CHD with T2DM, CHD and control subjects.

The reaction time was higher in CHD with T2DM patients compared to T2DM, CHD and controls (32.58 ± 4.005min vs. 23.92 ± 2.793min vs. 21.29 ± 2.383min vs. 8.322 ± 0.886min, $p<0.0001$). The reaction time it takes for the first measurable clot to form (R-time) was significantly longer in T2DM, CHD and CHD with T2DM patients compared to controls (Figure 4.9, $p<0.0001$). In addition, the R-time was significantly longer in CHD with T2DM compared to CHD patients.

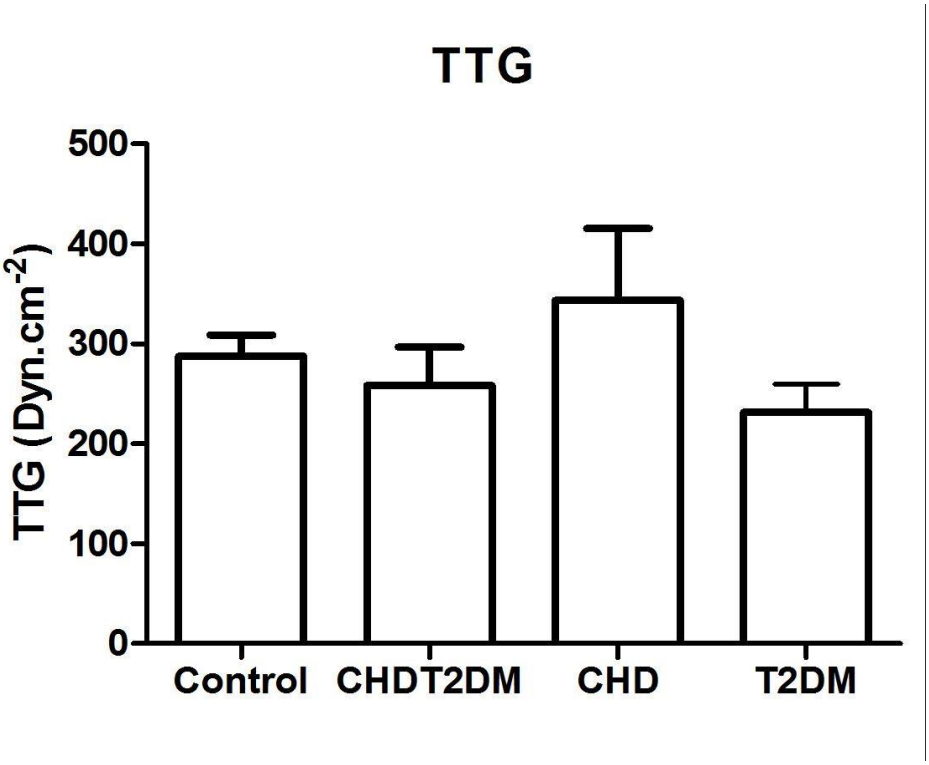


Figure 4.10 The total clot resistance (TTG) between T2DM, CHD with T2DM, CHD and control subjects.

There was no significant difference found in the TTG between T2DM, CHD with T2DM, CHD and control subjects (231.3 ± 28.68 dyn cm^{-2} vs. 258.5 ± 38.15 dyn cm^{-2} vs. 343.7 ± 71.92 dyn cm^{-2} vs. 287.7 ± 21.37 dyn cm^{-2}, p=0.8421).

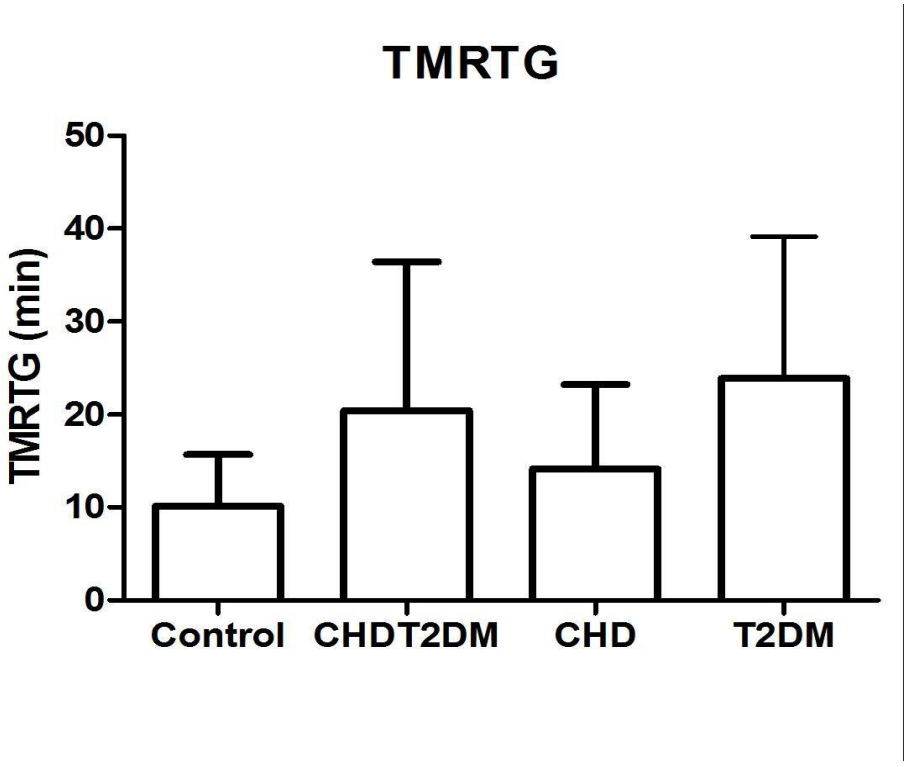


Figure 4.11 The time taken before maximum speed of the clot growth (TMRTG) was reached in samples from T2DM, CHD with T2DM, CHD and control subjects.

The TMRTG was higher in T2DM patients compared to T2DM, CHD with T2DM, CHD and controls (23.91 ± 2.409mins vs. 20.46 ± 3.411mins vs. 14.14 ± 1.287mins vs. 10.16 ± 0.751mins, p<0.0001).

There was no significant difference in the total clot resistance (TTG) between the subjects (Figure 4.10, p=0.8421). The time before the maximum speed of the clot growth (TMRTG) was significantly increased in T2DM and CHD with T2DM patients compared to controls (Figure 4.11, p‹0.0001). In addition, the TMRTG was significantly increased in T2DM compared to CHD patients.

Methylglyoxal-adduct ELISA

The quantification of methylglyoxal-adducts in CHD, T2DM, CHD with T2DM and control subjects was determined by extrapolating the concentration of adducts in plasma from each of the subjects using an equation derived from standard curve using MG-BSA adducts of known concentration.

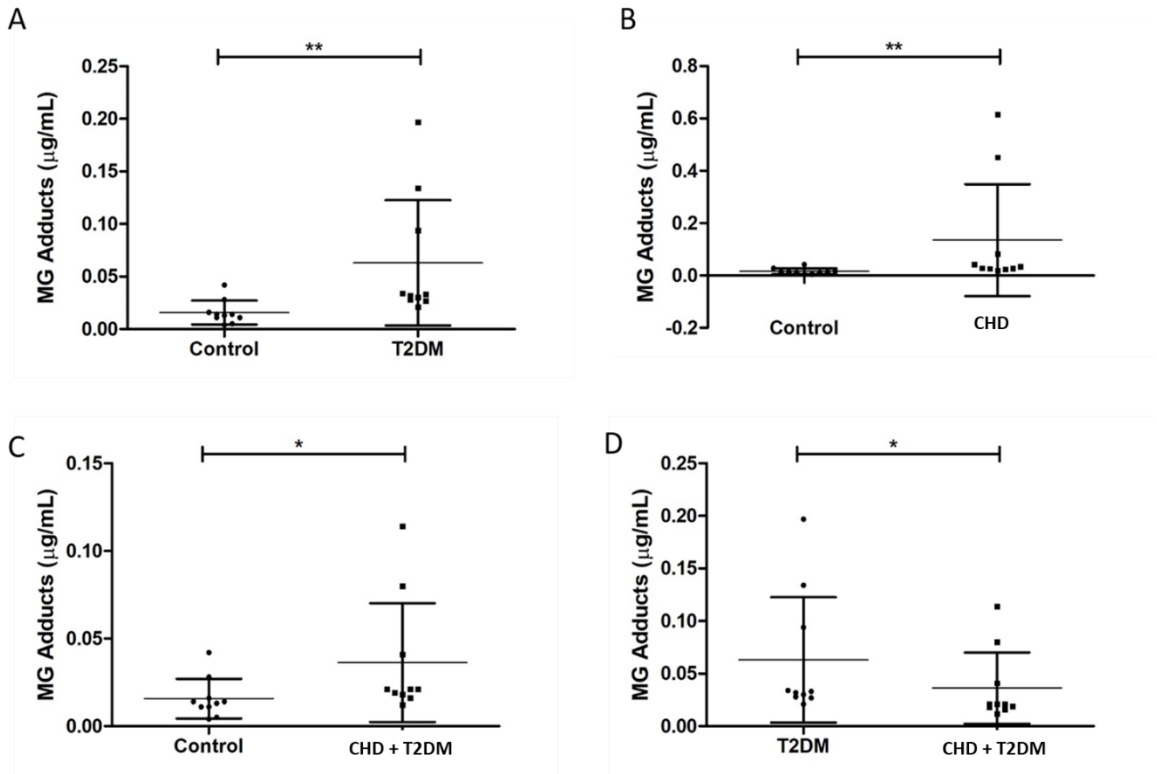


Figure 4.12 Dot plots illustrate the MG-adducts between T2DM, CHD with T2DM, CHD and control subjects. *p<0.05, **p<0.001

The levels of MG-adducts were significantly higher in the T2DM, CHD, CHD with T2DM compared to controls ($p<0.05$).

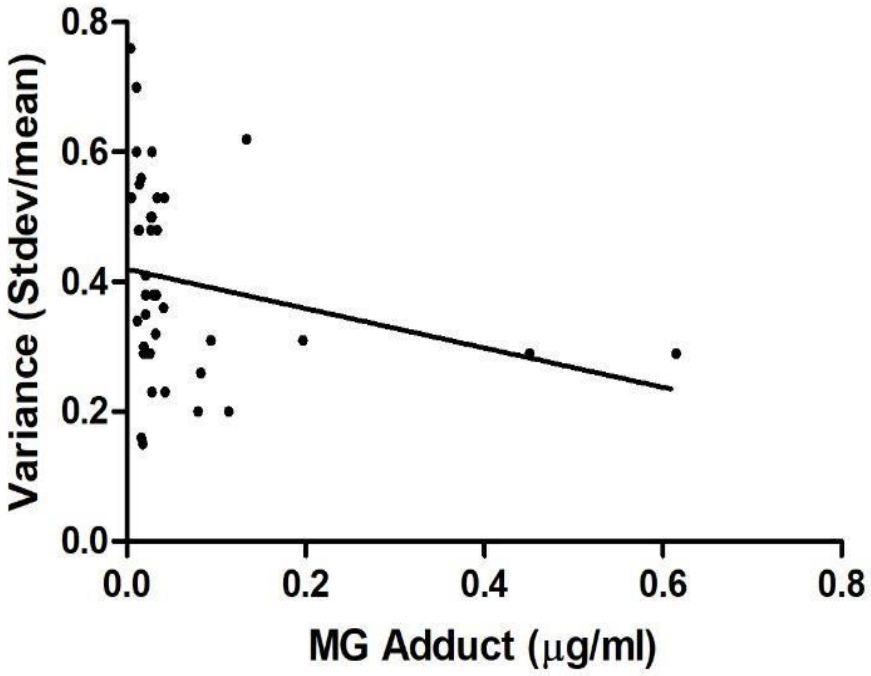


Figure 4.13 Dot plot showing a weak but significant correlation between MG-adducts and Variance between matched samples.

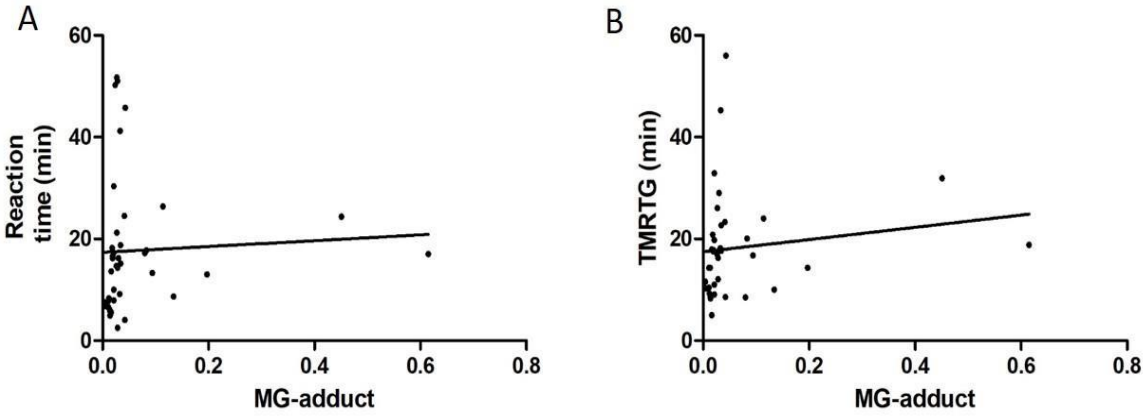


Figure 4.14 Dot plots indicating significant correlations between MG-adducts and clot kinetics, specifically, (A) reaction time and (B) time taken before maximum speed of the clot growth was achieved.

The Spearman r analysis of MG-adducts with the variance derived from the analysis of SEM micrographs showed a slight significant correlation (p=0.0198, 95% confidence interval: -0.615 to -0.053, Figure 4.13).

To assess if an association between MG-adducts and clot kinetics exists, the Spearman r correlation was completed for each clot parameter. The reaction time (p=0.0047, 95% CI: 0.138 to 0.665, Figure 4.14A) and time taken before maximum speed of the clot growth to be achieved (p=0.3958, 95% CI: 0.072 to 0.644, Figure 4.14B) was significant. This indicates the relationship between the parameters i.e. the higher the level of MG-adducts present, the longer it takes for clotting to begin and reach maximum speed of formation.

CHAPTER 5 DISCUSSION

Type 2 diabetes mellitus is associated with irregular circulation of inflammatory molecules which has prompted the interest to investigate the pathways of coagulation and glycation even more.(91) Insulin resistance has been linked to T2DM, cardiovascular diseases and endothelial dysfunction.(92) In T2DM, the risk of having vascular diseases is increased due to the increase in the activation of clotting factors.(93) The increased glucose in T2DM leads to irregular activation of inflammatory molecules that affect metabolism and the activation of the hypercoagulable state, with increased formation of fibrinogen which triggers coagulation.(94)

In coagulation, there must be a balance between clot formation and lysis, and the structure of fibrinogen, which plays a critical role in the maintenance of haemostasis.(29) In T2DM, the formation of MG is greater than the detoxification. The increased levels of MG leads to the glycation of proteins and this results in the formation of different protein structures leading to biochemical dysfunctions. These biochemical dysfunctions increase AGEs leading to the abnormal NO signalling and increased oxidative stress which results in the formation of atherosclerotic plaque.(38)

Fibrin plays an important role in the initiation and causation of hypercoagulation resulting in vascular injury which leads to increased risk of cardiovascular diseases.(91) In addition, it is known that fibrin is increased in T2DM which leads to the common occurrence of CHD in patients with T2DM.(91) The fibrin that is formed in T2DM is denser and resistant to fibrinolysis.(94) The change in structure of the fibrin clots and increased permeability is attributed to the glycated fibrinogen.(94) These abnormal morphological characteristics in fibrin clots are associated with the increased occurrence of cardiovascular diseases in T2DM.(94)

The structure of a clot is determined by the type of fibrin fibres mainly because they are the core components in fibrin clots.(95) In previous studies, fibrin fibres were investigated using SEM in T2DM and there were visible ultrastructural differences when compared to controls, this makes SEM the ideal technique to assess the morphological differences.(96) The fibrin fibres in the T2DM were thicker, disorganized, dense and matted in structure whereas in the controls, the images showed organized thin fibres.(96) The change in the clot structure and properties plays

a role in the risk of thrombosis in patients that have T2DM.(95) The structure of the thrombus is also influenced by damaged or activated endothelium, the manner in which the fibrin is formed and the contractile forces of fibrin fibres and type of cells. These effects resulted in a higher rate of thrombin growth which has an effect on fibrin network and the formation of the fibrin clot bundles.(97)

In this study, patients with T2DM, CHD with T2DM, and CHD without T2DM; and controls, ultrastructure properties were investigated using SEM. The ultrastructure properties in T2DM and controls were aligned with previous studies conducted on fibrin clots. The images in this study show that CHD with T2DM form fibrin clots with thick, matted, dense and disorganized fibres. In CHD, the images show that the fibrin fibres at some parts are significantly thick and dense when compared to controls. The structure of the fibrin fibres influences the rate of clot formation and fibrinolysis.(98)

Over the years the ability to quantify the rate of fibrinolysis, coagulability and the effects of anticoagulation treatment, has been of great interest.(99) The TEG can provide information on coagulation status, which makes it easy to determine the problem and subsequent treatment of patients.(99) With the use of TEG, the initiation of the clot, clot formation and how stable the clot can be determined.(100) The rate at which a clot forms is dependent on the ultrastructure of fibrin which can be altered when a patient has a disease.(99)

Studies have shown that fibrin is a protein and that its elasticity determines the type of fibrin clots formed.(99) The mechanical properties of fibrin have been studied using samples with different fibrin morphologies and it has been shown that single fibres determine the behaviour of the fibrin clot formed.(99) In addition, the stiffness of the clot formed in patients with T2DM is increased significantly when compared to controls. Furthermore, it has been shown that there is a correlation between the concentration of fibrinogen and the stiffness of the clot formed. It has been previously shown that there is an increase in CHD due to the stiffness of the clot formed. In addition, fibrinolysis is decreased in T2DM patients and this is as a result of the alterations that occur in the clot structure and the irregular fibrinolysis mechanism.(101)

In CHD, it has been shown that there is increased fibrinogen in plasma when compared to controls. The clot strength is used to determine the viscoelastic properties of the

thrombus formed. In a state were the fibrinogen levels are high the clots formed are rigid.(102)

In this study, the morphology of fibrin fibres in the CHD with T2DM, CHD without T2DM and T2DM groups were significantly different, which led to the investigation of coagulation to assess if the viscoelastic properties were also altered, using TEG. In previous studies using TEG in PPP, significant variations in viscoelastic parameters were seen in individuals with T2DM when compared to controls. In T2DM, TEG showed a significant decrease in the reaction time it took for the clot to form and therefore the clot formed at a slower rate. There was also a decrease in clot kinetics (K-value), increased initial rate of thrombus generation and an increased clot amplification.(96) In addition, there was an increase in the rate of accumulation of fibrin and crosslinking in T2DM. A significant increase was observed in the maximum clot amplitude (MA) and maximal rate of thrombus generation (MRTG) of T2DM individuals confirming that the clot formed has increased rigidity and is more stable. Furthermore, the significant variation in the total thrombus generation (TTG) shows that there is increased stability and strength in CHD when compared with controls.(96) The data found is aligned with previous studies proving a hypercoagulable state in T2DM.(96)

In this study, the findings in some of TEG parameters were aligned with previous studies but others were not. Using TEG to measure viscoelastic parameters in PPP for T2DM group, CHD with T2DM group, CHD without T2DM group and healthy controls; showed there were significant differences between the four groups. In T2DM compared to controls, there were significant differences in the clot strength (MA), maximum speed of clot growth (MRTG) and in the time from clot initiation till the maximum speed of clot formation (TMRTG). In T2DM compared to CHD, there were significant differences in MA, MRTG, R-time and TMRTG. In CHD with T2DM compared to control, there were significant differences in R-time and TMRTG. In CHD compared to control, there was a significant difference in the R-time. In CHD compared to CHD with T2DM, there was a significant difference in the TMRTG. There was no significant difference found in the clot kinetics (K-value), in the total thrombus generation (TTG) and the alpha angle. The CHD with T2DM patients showed altered clot kinetics. The time it took for a clot to formed in CHD/T2DM was 32.58 min compared to control which was 8.322 min. Therefore, the rate at which clot formed for CHD/T2DM was slower.. This shows that haemostasis is altered, and this has resulted

in a reduction in fibrinolysis. This means that in the experimental groups it takes longer for the clot to form (a slower reaction rate), but once the clot has formed it is harder to break down the clot.

Glycation occurs in proteins post-translation which results in the formation of AGEs.(37) When there is a multiplication of glycated proteins it leads to modification and permanent loss of function.(100) It has been seen that the accumulation of AGE is not only caused by glucose but compounds like MG and glyoxal which has led to the interest in investigating mechanisms that lead to increased AGE formation.(100)

The presence of MG in humans has been used as an indicator of glycation. When MG is in the body at high concentrations it becomes toxic due to the formation of AGEs. The detoxification of the MG which is known as glyoxalase, where MG is converted to D-lactate using glutathione and is not toxic, however, the activity of glyoxalase can be decreased in diseased patients.(101) It has been shown previously that MG is associated with late T2DM complications, therefore, confirming the role that MG plays in the development of pathologies, which are high, thus requiring methods that are reliable and fast need to be developed.(103) In previous research it has been shown that an increase in MG results in the increased formation of AGEs using immunoblot analyses on macrophages; yet there were no structural changes seen but the function of the macrophages changed significantly.(100)

Studies have shown the change in structure of fibrin clots in T2DM, however, the data on the effect of glycation on clot properties is limited.(102) Studies have shown that fibrin clots in T2DM were glycated by 35% more when compared to controls and an earlier study showed diabetic rats had a clotting velocity that was slower than normal by 50%.(102) In another study, human plasma fibrin clots were analysed where glucose treated fibrin clots were compared with untreated fibrin clots and it was found that the samples treated with glucose had a lower maximal velocity.(102)

The accumulation of MG has been shown to cause tissue damage and proliferation in the smooth muscle cells leading to atherosclerosis.(16) Type 2 diabetes mellitus is responsible for the increase in prevalence of many complications. Studies have shown that MG plays a role in the increase in the formation of AGEs.(104) In this study, the levels of methylglyoxal-adducts in patients with T2DM, CHD with T2DM, CHD without

T2DM and controls were analyzed. This assay has been used for rapid detection by using specific antibody-antigen interactions.(105) The MG adducts between T2DM group, CHD with T2DM group, CHD group and control subjects were significantly different. There was a significant increase of MG adducts in both T2DM group and CHD group when compared to the controls. In addition, there was a significant increase in the CHD with T2DM group compared to controls and there was a significant increase in the T2DM group compared to CHD with T2DM group.

In clinical studies it has been suggested that the accumulation of AGEs is the reason for endothelial dysfunction seen in T2DM. The damage caused by AGEs occurs due to the formation of cross-links in proteins which changes the structure and the ability for degradation.(104) The present study shows that patients with T2DM and CHD have significant morphological changes in fibrin clots which is seen in SEM images. This gives insight on the effect that the levels of methylglyoxal have on morphology. Furthermore, there were significant differences when comparing controls to CHD group, controls to CHD with T2DM group, controls to T2DM group and T2DM group to CHD with T2DM group.

CHAPTER 6 CONCLUSION

This study has shown that there are ultrastructural differences in fibrin fibres formed from glycated fibrinogen contributing to the pathogenesis of CHD in individual with T2DM. Key structural differences seen in CHD with T2DM, CHD without T2DM and T2DM is the matting of fibres as opposed to regular sized strands which could be seen in controls.

The viscoelastic parameters indicated that haemostasis was altered in CHD and T2DM. The clot parameters measured using TEG showed alterations in the clot strength, maximum speed of clot growth and in the time from clot initiation till the maximum speed of clot formation.

The MG adducts between T2DM, CHD with T2DM, CHD and control subjects were significantly different. The alteration in the coagulation may be as a result of the presence of MG-adducts which are not only found in albumin but also in other serum proteins such as fibrin.

Study limitations

Due to the COVID-19 pandemic and announcement of lockdown, the recruitment of study participants was significantly affected. A larger sample size would have yielded better results. The initial sample size was supposed to be 40 healthy individuals, 40 individuals with CAD, and 40 CAD individuals with T2DM. The pandemic affected the sample size influencing the reliability of the results and the accuracy.

REFERENCE

1. Khan MAB, Hashim MJ, King JK, Govender RD, Mustafa H, Al Kaabi J. Epidemiology of type 2 diabetes–global burden of disease and forecasted trends. Journal of epidemiology and global health. 2020;10(1):107.
2. Godman B, Basu D, Pillay Y, Mwita JC, Rwegerera GM, Paramadhas BDA, et al. Review of ongoing activities and challenges to improve the care of patients with type 2 diabetes across Africa and the implications for the future. Frontiers in pharmacology. 2020;11.
3. Bain SC, Mosenzon O, Arechavaleta R, Bogdański P, Comlekci A, Consoli A, et al. Cardiovascular safety of oral semaglutide in patients with type 2 diabetes: rationale, design and patient baseline characteristics for the PIONEER 6 trial. Diabetes, Obesity and Metabolism. 2019;21(3):499-508.

4. Oguntibeju OO. Type 2 diabetes mellitus, oxidative stress and inflammation: examining the links. International journal of physiology, pathophysiology and pharmacology. 2019;11(3):45.
5. Yi S, Allen SD, Liu Y-G, Ouyang BZ, Li X, Augsornworawat P, et al. Tailoring nanostructure morphology for enhanced targeting of dendritic cells in atherosclerosis. ACS nano. 2016;10(12):11290-303.
6. Anand M, Rajagopal K, Rajagopal KR. A Model for the Formation and Lysis of Blood Clots. Pathophysiology of Haemostasis and Thrombosis. 2005;34(2-3):109-20. 7. Behl T, Velpandian T, Kotwani A. Role of altered coagulation-fibrinolytic system in the pathophysiology of diabetic retinopathy. Vascular pharmacology.
2017;92:1-5.
8. Tangvarasittichai S. Oxidative stress, insulin resistance, dyslipidemia and type 2 diabetes mellitus. World journal of diabetes. 2015;6(3):456.
9. Pretorius E. Mechanisms of hypercoagulation and aberrant clot lyses in type 2 diabetes. Mechanisms of Vascular Defects in Diabetes Mellitus: Springer; 2017. p. 377-93.
10. Kosmopoulos M, Drekolias D, Zavras PD, Piperi C, Papavassiliou AG. Impact of advanced glycation end products (AGEs) signaling in coronary artery disease. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease. 2019.
11. Van den Eynde MD, Geleijnse JM, Scheijen JL, Hanssen NM, Dower JI, Afman LA, et al. Quercetin, but Not Epicatechin, Decreases Plasma Concentrations of Methylglyoxal in Adults in a Randomized, Double-Blind, Placebo-Controlled, Crossover Trial with Pure Flavonoids. The Journal of nutrition. 2018;148(12):1911-6.
12. Dorenkamp M, Müller JP, Shanmuganathan KS, Schulten H, Müller N, Löffler I, et al. Hyperglycaemia-induced methylglyoxal accumulation potentiates VEGF resistance of diabetic monocytes through the aberrant activation of tyrosine phosphatase SHP-2/SRC kinase signalling axis. Scientific reports. 2018;8(1):14684.
13. Acharya UR, Fujita H, Adam M, Lih OS, Sudarshan VK, Hong TJ, et al. Automated characterization and classification of coronary artery disease and myocardial infarction by decomposition of ECG signals: A comparative study. Information Sciences. 2017;377:17-29.
14. Wirtz PH, von Känel R. Psychological stress, inflammation, and coronary heart disease. Current cardiology reports. 2017;19(11):111.
15. Brings S, Fleming T, De Buhr S, Beijer B, Lindner T, Wischnjow A, et al. A scavenger peptide prevents methylglyoxal induced pain in mice. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease. 2017;1863(3):654-62.
16. Deshmukh AB, Bai S, Aarthy T, Kazi RS, Banarjee R, Rathore R, et al. Methylglyoxal attenuates insulin signaling and downregulates the enzymes involved in cholesterol biosynthesis. Molecular BioSystems. 2017;13(11):2338-49.
17. Noori A, Ashrafi SJ, Vaez-Ghaemi R, Hatamian-Zaremi A, Webster TJ. A review of fibrin and fibrin composites for bone tissue engineering. International journal of nanomedicine. 2017;12:4937.
18. Litvinov RI, Weisel JW. Fibrin mechanical properties and their structural origins. Matrix Biology. 2017;60:110-23.
19. Ferrannini E, DeFronzo RA. Impact of glucose-lowering drugs on cardiovascular disease in type 2 diabetes. European heart journal. 2015;36(34):2288-96.
20. Weisel JW, Litvinov RI. Fibrin formation, structure and properties. Fibrous Proteins: Structures and Mechanisms: Springer; 2017. p. 405-56.

21. Bhat LR, Vedantham S, Krishnan UM, Rayappan JBB. Methylglyoxal–an emerging biomarker for diabetes mellitus diagnosis and its detection methods. Biosensors and Bioelectronics. 2019.
22. Goedeke L, Perry RJ, Shulman GI. Emerging pharmacological targets for the treatment of nonalcoholic fatty liver disease, insulin resistance, and type 2 diabetes. Annual review of pharmacology and toxicology. 2019;59:65-87.
23. Lv Q, Meng X-F, He F-F, Chen S, Su H, Xiong J, et al. High serum uric acid and increased risk of type 2 diabetes: a systemic review and meta-analysis of prospective cohort studies. PloS one. 2013;8(2):e56864.
24. Zaccardi F, Webb DR, Yates T, Davies MJ. Pathophysiology of type 1 and type 2 diabetes mellitus: a 90-year perspective. Postgraduate medical journal. 2016;92(1084):63-9.
25. Sah SP, Singh B, Choudhary S, Kumar A. Animal models of insulin resistance: A review. Pharmacological Reports. 2016;68(6):1165-77.
26. Daniel MK, James SH, Han KC, Elfas RN. Health Status and Permanent Loss to Follow up of Ellisras Longitudinal Study Subjects: Rural South African Context. Nutrition in Health and Disease: IntechOpen; 2019.
27. Antman EM, Loscalzo J. Ischemic Heart Disease. In: Jameson JL, Fauci AS, Kasper DL, Hauser SL, Longo DL, Loscalzo J, editors. Harrison's Principles of Internal Medicine, 20e. New York, NY: McGraw-Hill Education; 2018.
28. Glass CK, Witztum JL. Atherosclerosis: the road ahead. Cell. 2001;104(4):503-16.
29. Kattoor AJ, Pothineni NVK, Palagiri D, Mehta JL. Oxidative stress in atherosclerosis. Current atherosclerosis reports. 2017;19(11):42.
30. Lawal AO. Air particulate matter induced oxidative stress and inflammation in cardiovascular disease and atherosclerosis: the role of Nrf2 and AhR-mediated pathways. Toxicology letters. 2017;270:88-95.
31. Incalza MA, D'Oria R, Natalicchio A, Perrini S, Laviola L, Giorgino F. Oxidative stress and reactive oxygen species in endothelial dysfunction associated with cardiovascular and metabolic diseases. Vascular pharmacology. 2018;100:1-19.
32. Pothineni NVK, Subramany S, Kuriakose K, Shirazi LF, Romeo F, Shah PK, et al. Infections, atherosclerosis, and coronary heart disease. European heart journal. 2017;38(43):3195-201.
33. Robson R, Kundur AR, Singh I. Oxidative stress biomarkers in type 2 diabetes mellitus for assessment of cardiovascular disease risk. Diabetes & Metabolic Syndrome: Clinical Research & Reviews. 2018;12(3):455-62.
34. Gao S, Liu J. Association between circulating oxidized low-density lipoprotein and atherosclerotic cardiovascular disease. Chronic diseases and translational medicine. 2017;3(2):89-94.
35. Domingueti CP, Dusse LMSA, das Graças Carvalho M, de Sousa LP, Gomes KB, Fernandes AP. Diabetes mellitus: the linkage between oxidative stress, inflammation, hypercoagulability and vascular complications. Journal of Diabetes and its Complications. 2016;30(4):738-45.
36. Liu G, Xia Q, Lu Y, Zheng T, Sang S, Lv L. Influence of quercetin and its methylglyoxal adducts on the formation of α-dicarbonyl compounds in a lysine/glucose model system. Journal of agricultural and food chemistry. 2017;65(10):2233-9.

37. Dhananjayan K, Irrgang F, Raju R, Harman DG, Moran C, Srikanth V, et al. Determination of glyoxal and methylglyoxal in serum by UHPLC coupled with fluorescence detection. Analytical biochemistry. 2019;573:51-66.
38. Zhou Q, Cheng K-W, Gong J, Li ET, Wang M. Apigenin and its methylglyoxaladduct inhibit advanced glycation end products-induced oxidative stress and inflammation in endothelial cells. Biochemical pharmacology. 2019.
39. Pfaff DH, Fleming T, Nawroth P, Teleman AA. Evidence against a role for the Parkinsonism-associated protein DJ-1 in methylglyoxal detoxification. Journal of Biological Chemistry. 2017;292(2):685-90.
40. Nigro C, Leone A, Raciti G, Longo M, Mirra P, Formisano P, et al. Methylglyoxal-glyoxalase 1 balance: the root of vascular damage. International journal of molecular sciences. 2017;18(1):188.
41. Bellier J, Nokin M-J, Lardé E, Karoyan P, Peulen O, Castronovo V, et al. Methylglyoxal, a potent inducer of AGEs, connects between diabetes and cancer. Diabetes research and clinical practice. 2019.
42. Vulesevic B, McNeill B, Giacco F, Maeda K, Blackburn NJ, Brownlee M, et al. Methylglyoxal-induced endothelial cell loss and inflammation contribute to the development of diabetic cardiomyopathy. Diabetes. 2016;65(6):1699-713. 43. Groener JB, Oikonomou D, Cheko R, Kender Z, Zemva J, Kihm L, et al. Methylglyoxal and advanced glycation end products in patients with diabetes–what we know so far and the missing links. Experimental and Clinical Endocrinology & Diabetes. 2017.
44. Shen Y, Xu Z, Sheng Z. Ability of resveratrol to inhibit advanced glycation end product formation and carbohydrate-hydrolyzing enzyme activity, and to conjugate methylglyoxal. Food chemistry. 2017;216:153-60.
45. Lee JH, Parveen A, Do MH, Kang MC, Yumnam S, Kim SY. Molecular mechanisms of methylglyoxal-induced aortic endothelial dysfunction in human vascular endothelial cells. Cell Death & Disease. 2020;11(5):1-15.
46. Chu P, Han G, Ahsan A, Sun Z, Liu S, Zhang Z, et al. Phosphocreatine protects endothelial cells from methylglyoxal induced oxidative stress and apoptosis via the regulation of PI3K/Akt/eNOS and NF-κB pathway. Vascular pharmacology. 2017;91:26-35.
47. Duflot T, Moreau-Grangé L, Roche C, Iacob M, Wils J, Remy-Jouet I, et al. Altered bioavailability of epoxyeicosatrienoic acids is associated with conduit artery endothelial dysfunction in type 2 diabetic patients. Cardiovascular diabetology. 2019;18(1):35.
48. Pretorius E, Bester J, Vermeulen N, Alummoottil S, Soma P, Buys AV, et al. Poorly controlled type 2 diabetes is accompanied by significant morphological and ultrastructural changes in both erythrocytes and in thrombin-generated fibrin: implications for diagnostics. Cardiovascular diabetology. 2015;14(1):30.
49. Lee D-Y, Chiu J-J. Atherosclerosis and flow: roles of epigenetic modulation in vascular endothelium. Journal of biomedical science. 2019;26(1):1-17.
50. Souilhol C, Harmsen MC, Evans PC, Krenning G. Endothelial–mesenchymal transition in atherosclerosis. Cardiovascular research. 2018;114(4):565-77. 51. Park K-H, Park WJ. Endothelial dysfunction: clinical implications in cardiovascular disease and therapeutic approaches. Journal of Korean medical science. 2015;30(9):1213-25.
52. Saffi MAL, Furtado MV, Polanczyk CA, Montenegro MM, Ribeiro IWJ, Kampits C, et al. Relationship between vascular endothelium and periodontal disease in atherosclerotic lesions. World journal of cardiology. 2015;7(1):26.

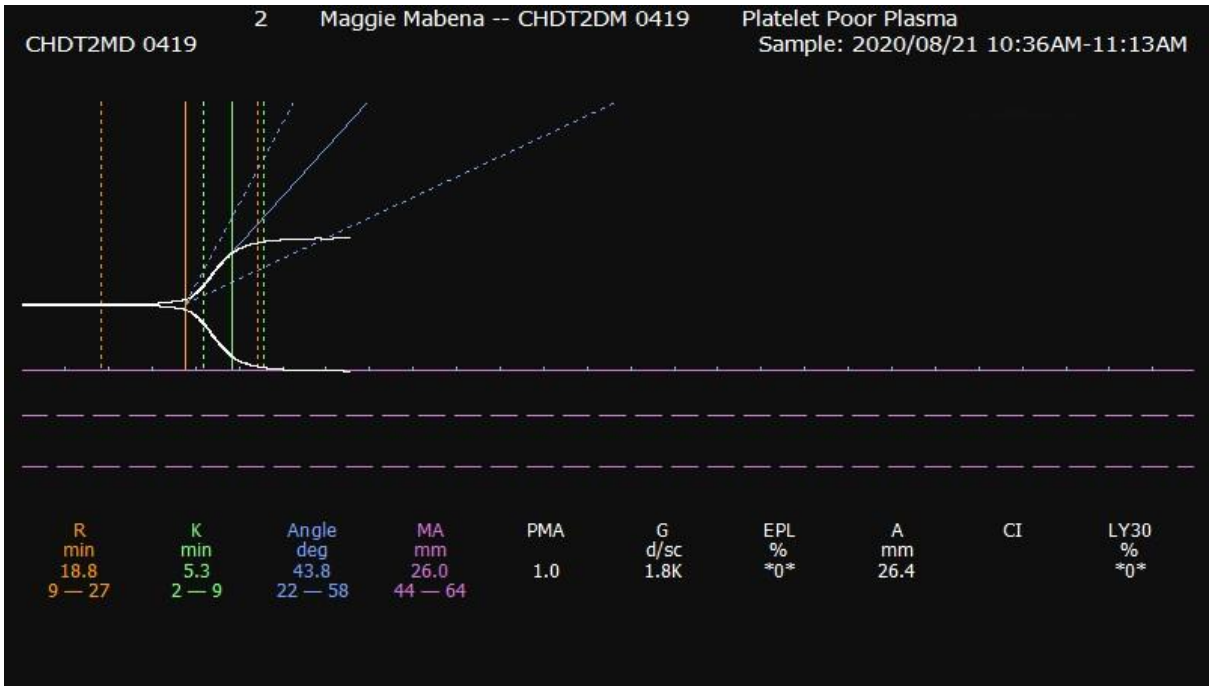
53. Pechlivani N, Ajjan RA. Thrombosis and vascular inflammation in diabetes: mechanisms and potential therapeutic targets. Frontiers in cardiovascular medicine.
2018;5:1.
54. Castellon X, Bogdanova V. Chronic inflammatory diseases and endothelial dysfunction. Aging and disease. 2016;7(1):81.
55. Christiansen MK, Larsen SB, Nyegaard M, Neergaard-Petersen S, Ajjan R, Würtz M, et al. Coronary artery disease-associated genetic variants and biomarkers of inflammation. PloS one. 2017;12(7):e0180365.
56. Grandl G, Wolfrum C, editors. Hemostasis, endothelial stress, inflammation, and the metabolic syndrome. Seminars in immunopathology; 2018: Springer.
57. Hartwig H, Roig CS, Daemen M, Lutgens E, Soehnlein O. Neutrophils in atherosclerosis. Hämostaseologie. 2015;35(02):121-7.
58. O'Neil LJ, Kaplan MJ, Carmona-Rivera C. The role of neutrophils and neutrophil extracellular traps in vascular damage in systemic lupus erythematosus. Journal of clinical medicine. 2019;8(9):1325.
59. Gaul DS, Stein S, Matter CM. Neutrophils in cardiovascular disease. Oxford University Press; 2017.
60. Bertin FR, Rys R, Mathieu C, Laurance S, Lemarié C, Blostein M. Natural killer cells induce neutrophil extracellular trap formation in venous thrombosis. Journal of Thrombosis and Haemostasis. 2019;17(2):403-14.
61. Bobryshev YV, Ivanova EA, Chistiakov DA, Nikiforov NG, Orekhov AN. Macrophages and their role in atherosclerosis: pathophysiology and transcriptome analysis. BioMed research international. 2016;2016.
62. Shao B-z, Han B-z, Zeng Y-x, Su D-f, Liu C. The roles of macrophage autophagy in atherosclerosis. Acta Pharmacologica Sinica. 2016;37(2):150-6.
63. Parisi L, Bassani B, Tremolati M, Gini E, Farronato G, Bruno A. Natural killer cells in the orchestration of chronic inflammatory diseases. Journal of immunology research. 2017;2017.
64. Rombouts M, Ammi R, Van Brussel I, Roth L, De Winter BY, Vercauteren SR, et al. Linking CD11b+ dendritic cells and natural killer T cells to plaque inflammation in atherosclerosis. Mediators of inflammation. 2016;2016.
65. Shah PK, Lecis D. Inflammation in atherosclerotic cardiovascular disease. F1000Research. 2019;8.
66. Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB. Oxidative stress, inflammation, and cancer: how are they linked? Free Radical Biology and Medicine.
2010;49(11):1603-16.
67. Hussain T, Tan B, Yin Y, Blachier F, Tossou MC, Rahu N. Oxidative stress and inflammation: what polyphenols can do for us? Oxidative Medicine and Cellular Longevity. 2016;2016.
68. Pickup JC. Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes. Diabetes care. 2004;27(3):813-23.
69. Pretorius E, Page MJ, Engelbrecht L, Ellis GC, Kell DB. Substantial fibrin amyloidogenesis in type 2 diabetes assessed using amyloid-selective fluorescent stains. Cardiovascular diabetology. 2017;16(1):141.
70. Banerjee M, Saxena M. Interleukin-1 (IL-1) family of cytokines: role in type 2 diabetes. Clinica chimica acta. 2012;413(15-16):1163-70.
71. Zaidi A, Green L. Physiology of haemostasis. Anaesthesia & Intensive Care Medicine. 2019;20(3):152-8.
72. Austin SK. Haemostasis. Medicine. 2017;45(4):204-8.

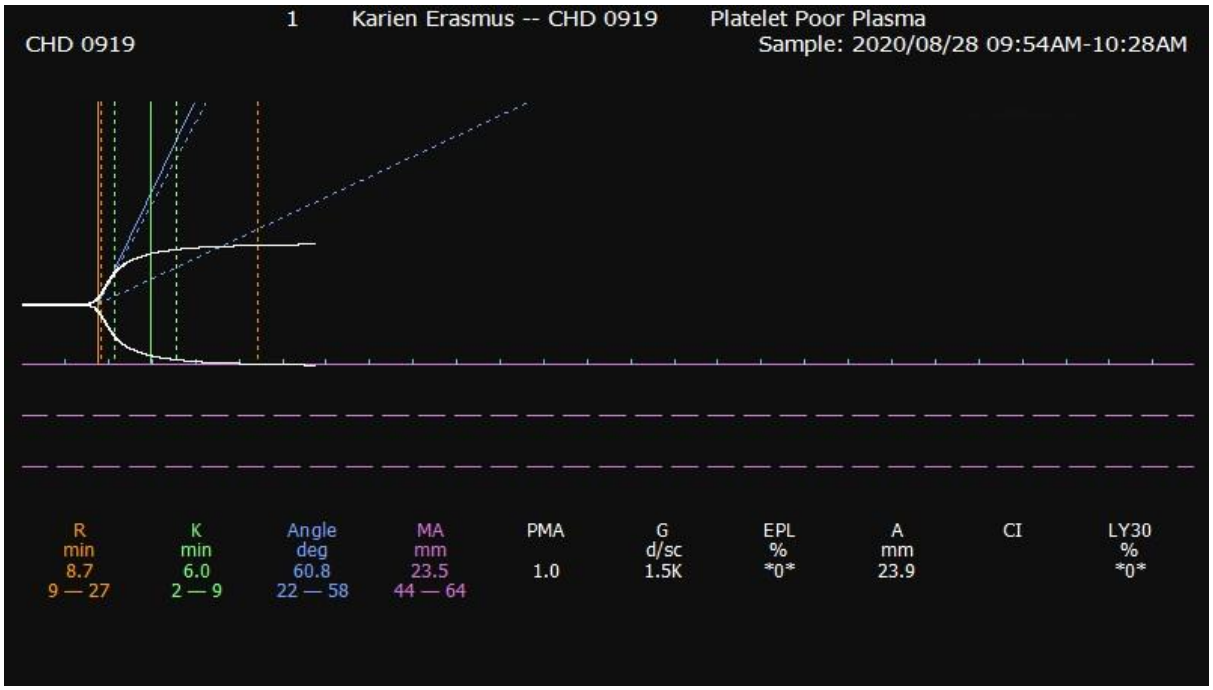
73. Litvinov RI, Weisel JW. Role of red blood cells in haemostasis and thrombosis. ISBT science series. 2017;12(1):176-83.
74. Soma P, Swanepoel AC, Bester J, Pretorius E. Tissue factor levels in type 2 diabetes mellitus. Inflammation Research. 2017;66(5):365-8.
75. Hoffman M, Monroe DM. Coagulation 2006: a modern view of hemostasis. Hematology/oncology clinics of North America. 2007;21(1):1-11.
76. Palta S, Saroa R, Palta A. Overview of the coagulation system. Indian journal of anaesthesia. 2014;58(5):515.
77. Agarwal C, Bansal K, Pujani M, Singh K, Chauhan V, Rana D, et al. Association of coagulation profile with microvascular complications and glycemic control in type 2 diabetes mellitus-a study at a tertiary care center in Delhi.
Hematology, transfusion and cell therapy. 2019;41(1):31-6.
78. Barillari G, Fabbro E, Pasca S, Bigotto E. Coagulation and oxidative stress plasmatic levels in a type 2 diabetes population. Blood coagulation & fibrinolysis.
2009;20(4):290-6.
79. Perween S, Abidi M, Faizy AF. Post-translational modifications on glycated plasma fibrinogen: A physicochemical insight. International journal of biological macromolecules. 2019;126:1201-12.
80. Kattula S, Byrnes JR, Wolberg AS. Fibrinogen and fibrin in hemostasis and thrombosis. Arteriosclerosis, thrombosis, and vascular biology. 2017;37(3):e13-e21.
81. Dunn E, Ariëns R, Grant P. The influence of type 2 diabetes on fibrin structure and function. Diabetologia. 2005;48(6):1198-206.
82. Undas A, Ariëns RA. Fibrin clot structure and function: a role in the pathophysiology of arterial and venous thromboembolic diseases. Arteriosclerosis, thrombosis, and vascular biology. 2011;31(12):e88-e99.
83. Van Rooy M-J, Duim W, Ehlers R, Buys AV, Pretorius E. Platelet hyperactivity and fibrin clot structure in transient ischemic attack individuals in the presence of metabolic syndrome: a microscopy and thromboelastography® study.
Cardiovascular diabetology. 2015;14(1):86.
84. Pretorius E, Lipinski B, Bester J, Vermeulen N, Soma P. Albumin stabilizes fibrin fiber ultrastructure in low serum albumin type 2 diabetes. Ultrastructural pathology. 2013;37(4):254-7.
85. Page MJ, Bester J, Pretorius E. Interleukin-12 and its procoagulant effect on erythrocytes, platelets and fibrin (ogen): the lesser known side of inflammation.
British journal of haematology. 2018;180(1):110-7.
86. Konings J, Hoving LR, Ariëns RS, Hethershaw EL, Ninivaggi M, Hardy LJ, et al. The role of activated coagulation factor XII in overall clot stability and fibrinolysis.
Thrombosis research. 2015;136(2):474-80.
87. Bochenek M, Zalewski J, Sadowski J, Undas A. Type 2 diabetes as a modifier of fibrin clot properties in patients with coronary artery disease. J Thromb Thrombolysis. 2013;35(2):264-70.
88. Thakur M, Ahmed AB. A review of thromboelastography. Int J Periop Ultrasound Appl Technol. 2012;1(1):25-9.
89. Ostrowsky J, Foes J, Warchol M, Tsarovsky G, Blay J. Plateletworks™
Platelet Function Test Compared to the Thromboelastograph™ for Prediction of Postoperative Outcomes. JOURNAL OF EXTRACORPOREAL TECHNOLOGY.
2004;36:149-52.

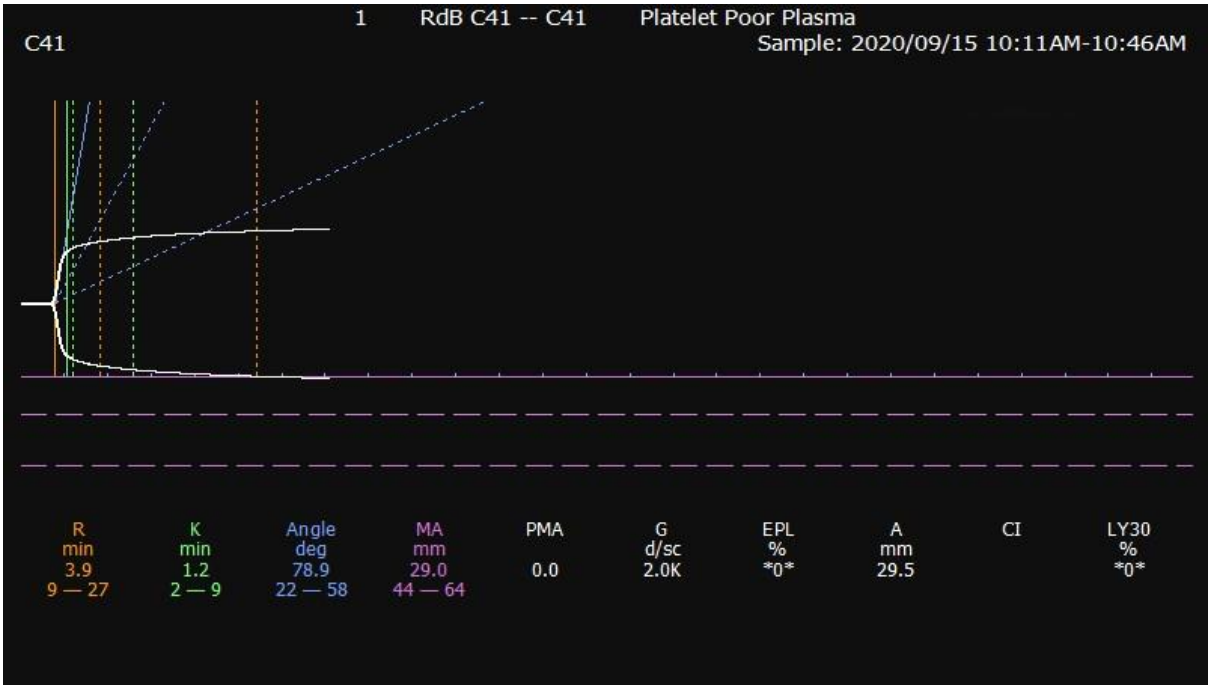
90. Verma A H. Thromboelastography as a novel viscoelastic method for hemostasis monitoring: Its methodology, applications, and constraints. Gjtm Online.
2017;2(1):10.
91. Pretorius E. Platelets as potent signaling entities in type 2 diabetes mellitus. Trends in Endocrinology & Metabolism. 2019;30(8):532-45.
92. Maamoun H, Abdelsalam SS, Zeidan A, Korashy HM, Agouni A. Endoplasmic reticulum stress: A critical molecular driver of endothelial dysfunction and cardiovascular disturbances associated with diabetes. International journal of molecular sciences. 2019;20(7):1658.
93. Di Pino A, DeFronzo RA. Insulin resistance and atherosclerosis: implications for insulin-sensitizing agents. Endocrine reviews. 2019;40(6):1447-67.
94. Vilar R, Fish RJ, Casini A, Neerman-Arbez M. Fibrin (ogen) in human disease: both friend and foe. haematologica. 2020;105(2):284-96.
95. Vries MJ, Macrae F, Nelemans PJ, Kuiper GJ, Wetzels RJ, Bowman P, et al. Assessment and determinants of whole blood and plasma fibrinolysis in patients with mild bleeding symptoms. Thrombosis Research. 2019;174:88-94.
96. Randeria SN, Thomson GJ, Nell TA, Roberts T, Pretorius E. Inflammatory cytokines in type 2 diabetes mellitus as facilitators of hypercoagulation and abnormal clot formation. Cardiovascular diabetology. 2019;18(1):72.
97. Hofer S, Ay C, Rejtö J, Wolberg AS, Haslacher H, Koder S, et al. Thrombingenerating potential, plasma clot formation, and clot lysis are impaired in patients with bleeding of unknown cause. Journal of Thrombosis and Haemostasis.
2019;17(9):1478-88.
98. Dias JD, Lopez-Espina CG, Bliden K, Gurbel P, Hartmann J, Achneck HE. TEG® 6s system measures the contributions of both platelet count and platelet function to clot formation at the site-of-care. Platelets. 2020;31(7):932-8.
99. Britton S, Kim O, Pancaldi F, Xu Z, Litvinov RI, Weisel JW, et al. Contribution of nascent cohesive fiber-fiber interactions to the non-linear elasticity of fibrin networks under tensile load. Acta biomaterialia. 2019;94:514-23.
100. Bezold V. Impact of glycation and advanced glycation end products (AGEs) on macrophage activation. 2020.
101. Schmoch T, Uhle F, Siegler BH, Fleming T, Morgenstern J, Nawroth PP, et al. The glyoxalase system and methylglyoxal-derived carbonyl stress in sepsis: glycotoxic aspects of sepsis pathophysiology. International journal of molecular sciences. 2017;18(3):657.
102. Luzak B, Boncler M, Kosmalski M, Mnich E, Stanczyk L, Przygodzki T, et al. Fibrinogen Glycation and Presence of Glucose Impair Fibrin Polymerization—An In Vitro Study of Isolated Fibrinogen and Plasma from Patients with Diabetes Mellitus. Biomolecules. 2020;10(6):877.
103. Kold-Christensen R, Johannsen M. Methylglyoxal metabolism and agingrelated disease: Moving from correlation toward causation. Trends in Endocrinology & Metabolism. 2020;31(2):81-92.
104. Khan MY, Alouffi S, Khan MS, Husain FM, Akhter F, Ahmad S. The neoepitopes on methylglyoxal (MG) glycated LDL create autoimmune response; autoimmunity detection in T2DM patients with varying disease duration. Cellular Immunology. 2020:104062.
105. Yan C, Teng J, Liu F, Yao B, Xu Z, Yao L, et al. Signal amplified enzymelinked immunosorbent assay with gold nanoparticles for sensitive detection of trace furaltadone metabolite. Microchemical Journal. 2020;159:105414.

APPENDICES

Appendix 1: Thromboelastography

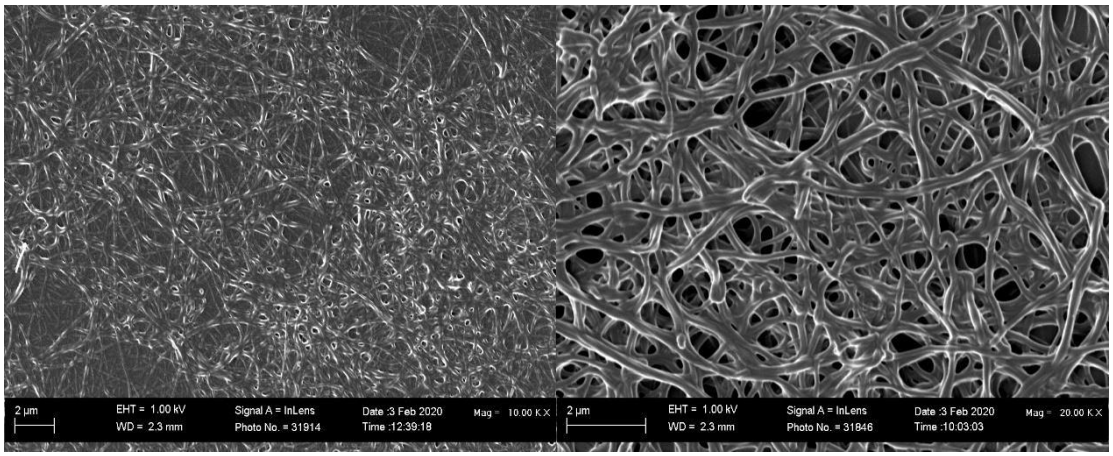




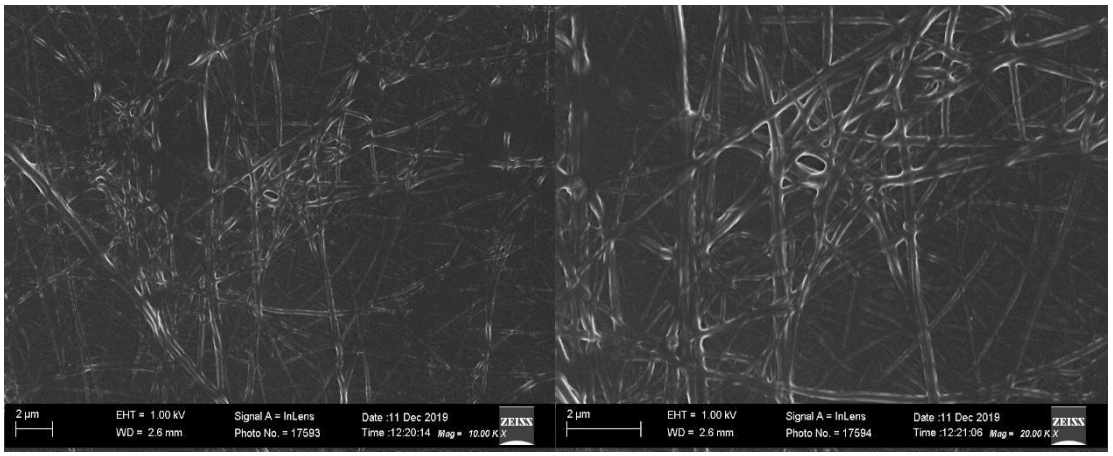


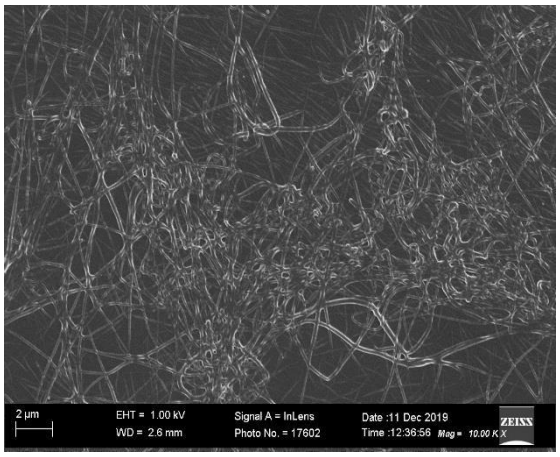
Appendix 2: SEM images

CHD

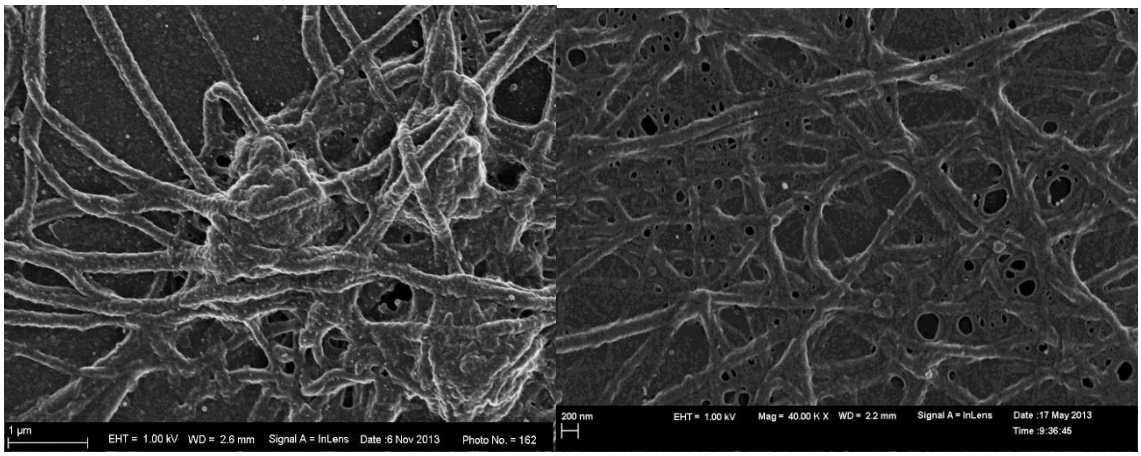


CHD with T2DM





T2DM



Appendix 3: Informed consent from

Annexures 1.

Participant's information and information leaflet and consent form (patient group)

Ethics No:

Project title: The association of methylglyoxal-adducts with kinetics and ultrastructure of fibrin clots in coronary artery disease patients with type 2 diabetes mellitus **Principal Investigator:** Mikateko Nxumalo, Department of Physiology, University of Pretoria (Under supervision of Prof A Phulukdaree, Prof P Soma and Dr Sajee Alummoottil)

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. This information leaflet is to help you understand what the study is about, should you like to participate in the study. The investigator will explain the reason for drawing blood. 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 measure the clot kinetics of fibrin clots, look at the ultrastructural differences in the fibrin clots and the level of methylglyoxal-adducts in coronary heart disease patients with and without type 2 diabetes mellitus. An amount of 5 ml (1 citrate tube) of blood will be drawn and centrifuged so that the platelet poor plasma (PPP) can be used for the study. The remaining amount will be stored at -80ºC for experimental repeats. The PPP will be stored for the duration of the study which is 2 years.

Experimental procedures

Once agreeing to participate, you will fill in a form that will ask you regarding your illness, weight, height and blood pressure will be measured. The blood will then be taken to the lab and processed by the investigator and the following procedure will be used to assess the blood: (1) Thromboelastography (TEG) will be used to assess clotting properties of blood, (2) Scanning electron microscope (SEM) will be used to assess the ultrastructural of fibrin clots and (3) ELISA will be used to look at the level methylglyoxal-adducts. **Who will draw the blood?**

Either your medical practitioner, Prof Soma or a qualified phlebotomist will draw the blood.

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 view 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

Research knowledge obtained in this study

The laboratory part of this study is not intended to benefit you but will add more to our knowledge on how the levels methylglyoxal-adducts influence clotting kinetics and the ultrastructure of fibrin clots and it 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 our medical practitioner, Prof Soma or a qualified phlebotomist. **Confidentiality**

All information obtained during the course of 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.

Informed consent

I hereby confirm that I have been informed by the investigator, M Nxumalo 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.....

ANNEXURES 1.2

Participant information (patient group) Ethics No:

Project title: The association of methylglyoxal-adducts with kinetics and ultrastructure of fibrin clots in coronary artery disease patients with type 2 diabetes mellitus

Principal Investigator: Mikateko Nxumalo, Department of Physiology, University of Pretoria (Under supervision of Prof A Phulukdaree, Prof P Soma and Dr Sajee Alummoottil)

Information filled in by the investigator Sample ID

Age

Sex

Weight

Hight

Blood pressure

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?

Appendix 4: MSc Committee Approval Letter

MSc Committee
20 June 2019

Prof A Phulukdaree
Department of Physiology
Faculty of Health Sciences

Dear Prof,

Ms M Nxumalo, Student no 14249996

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

Student name	Ms Mikateko Nxumalo	**Student number**	14249996
Name of study leader		Prof Alisa Phulukdaree	
Department		Physiology	
Title of MSc		The association of methylglyoxal-adducts with kinetics and ultrastructure of fibrin clots in coronary artery disease patients with type 2 diabetes mellitus	

Date of first submission	**May 2019**
Comments to study leader May 2019	• Please revise the literature review; expand on the aspects of inflammation, coagulation and methods used to study these aspects. Clarify the choice of methods, linkage and the outcomes of the study. • Please revise the timeline. • Please revise the reference list; include all authors' names. • Include how each method will be analysed; be more descriptive. • Expand the ethics section; include all considerations applicable for this study. • Please correct typographical and grammatical errors. • Please include the manufacturer's details for instruments and reagents used. • Expand the data analysis and statistical section.
June 2019	□ Thank you for submitted a revised protocol and new MSc form.
Decision	This protocol has been provisionally approved. Please submit the revised protocol to ethics, and supply the MSc committee with proof of acceptance. The internal and external examiners can be nominated and submitted to the MSc Committee six months prior to submission of the dissertation. Please ensure that the CV of the examiners includes: supervision, examination and publication records.

Appendix 5: Ethics Approval Letter

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through February 28, 2022 and Expires: 03/04/2023.

27 August 2020

Approval Certificate
Annual Renewal

Ethics Reference No.: 484/2019
Title: The association of methylglyoxal-adducts with kinetics and ultrastructure of fibrin clots in coronary artery disease patients with type 2 diabetes mellitus

Dear Mikateko Nxumalo

The **Annual Renewal** as supported by documents received between 2020-07-09 and 2020-08-26 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2020-08-26 as resolved by its quorate meeting.

Please note the following about your ethics approval:
Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2021-08-27.
Please remember to use your protocol number (484/2019) 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, monitor the conduct of your research, or suspend or withdraw ethics approval. **Ethicss approval is subject to the following:**

- 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) MPharmMed 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)

Announcement and instruction to all researchers from the Faculty of Health Sciences Research Ethics Committee

This is an update following previous instructions from the Research Ethics Committee, accounting for recent announcements by government in relation to COVID-19 on 24 May 2020.

All researchers must minimise the risk of transmission at research sites and in studies involving human participants approved by the Research Ethics Committee. To this end,

1) all non-therapeutic or non-interventional research data gathering involving contact with human participants remain suspended, with the exception of studies involving telephonic or other online/remote methods of data collection;

2) research that is entirely situated in a laboratory is permitted provided that COVID-19

precautionary measures are in place;research that is merely utilising existing records or data is

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Fakulteit Gesondheidsw
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permitted provided that COVID-19 precautionary measures are in place

3) emergency research related to COVID-19 is permitted after ethics approval;
4) everyone should endeavour protecting research participants, personnel and students in reducing the risk of transmission of COVID-19.

For therapeutic and clinical research trials:

1) each research study or study site must maintain a plan to minimise exposure to COVID19 risk for all parties involved in the study, including but not limited to research participants, researchers and student researchers;
2) Whenever feasible, in-person visits should be substituted with telephonic visits;
3) Principal investigators and study sites should maintain measures to ensure that there is no interruption of required medication/essential treatment and monitoring of adverse events;
4) Researchers and study sites should develop a 'COVID-19' template register in case retrospective contact tracing becomes necessary;
5) New enrolments into clinical trials remain suspended. Potential exceptions to this announcement should be discussed with the chair or a deputy chair of the REC;

Appendix 6: Letter of Statistical Clearance

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences
Department of Immunology

Letter of Statistical Clearance

Thursday, May 09, 2019

This letter is to confirm that the student with the Name, Mikateko Nxumalo, Student No: 14249996 studying at the University of Pretoria, discussed the project with the title; Investigating the role of methylglyoxal on kinetics and ultrastructure of fibrin clots in coronary artery disease patients with type 2 diabetes mellitus with me.

I hereby confirm that I am aware of the project and the statistical analysis of the data generated from the project.
The analytical tool, Graphpad Prism software that will be used will suffice to achieve the objective(s) of the study.

Yours sincerely

Prof Pieter WA Meyer

Ass. Professor and HoD

Biostatistician Stamp

Biostatistician Stamp