INVESTIGATING THE ULTRASTRUCTURAL AND VISCOELASTIC PROPERTIES OF WHOLE BLOOD, WITH SPECIFIC FOCUS ON ERYTHROCYTES, IN POORLY CONTROLLED TYPE 2 DIABETES

by

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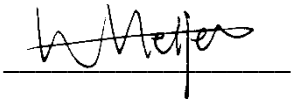
ABSTRACT

Type 2 diabetes mellitus is a metabolic disease associated with three main glycaemic disorders: Chronic hyperglycaemia, glycaemic variability and iatrogenic hypoglycaemia. Some comorbidities that often accompany type 2 diabetes mellitus are dyslipidemia and hypertension. There is no one particular cause of type 2 diabetes mellitus, but rather a series of risk factors that play a role in developing the disease. The most common risk factors include obesity, high blood pressure and a sedentary lifestyle. However, there are other factors such as age, polycystic ovarian syndrome, family history of type 2 diabetes and race, which may also contribute to the development of the disease. Type 2 diabetes mellitus can be diagnosed by measuring the glycated haemoglobin (HbA_1c) of a patient. When the HbA_1c is above a 6.5% the condition can be described as poorly controlled type 2 diabetes mellitus. There is evidence suggesting that diabetes is associated with chronic low-grade systemic inflammation. One of the signs of low-grade systemic inflammation is hypercoagulability of the blood. Hypercoagulability and the change in viscoelastic properties of the haemostatic system was the focus of this study, looking specifically in poorly controlled type 2 diabetes mellitus, by measuring erythrocyte sedimentation rate (ESR), thromboelastographic (TEG) parameters and viewing the ultrastructure of erythrocytes using scanning electron microscopy (SEM). Two groups, a healthy control group and poorly controlled type 2 diabetes mellitus group were compared in this study. ESR was used to measure inflammation and showed a significant increase in poorly controlled type 2 diabetes mellitus. Viscoelastic properties were measured with thromboelastography and displayed a decrease in clot formation time in poorly controlled type 2 diabetes mellitus as opposed to the control group. Scanning electron microscopy analysis of whole blood showed an increase in fibrin formation as well as an increase in the number of eryptotic cells in the poorly controlled type 2 diabetes mellitus group when compared to the control group. An increase of platelets was also observed in the full blood count. Results from this study showed a distinct difference between the two groups, indicating a significant change in the viscoelastic properties of individuals with poorly controlled type 2 diabetes mellitus but also an increase in the number of eryptotic cells.

Keywords: **Type 2 diabetes mellitus, hypercoagulability, inflammation, eryptosis, scanning electron microscopy, thromboelastography.**

DECLARATION

I, Wikus Meijer, hereby declare that this research dissertation is my own work and has not been presented for any degree at another university.

Signed: 

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LIST OF ABBREVIATIONS

Acronym	Definition
T2DM	Type 2 diabetes mellitus
ADA	American Diabetes Association
GV	Glycaemic variability
FFA	Free fatty acid
TEG	Thromboelastograph
SEM	Scanning electron microscopy
MCV	Mean corpuscular volume
HCT	Haematocrit
MCHC	Mean corpuscular haemoglobin concentration
TCT	Thrombocrit
MPV	Mean platelet volume
WBC	White blood cell
RBC	Red blood cell
WB	Whole blood
TTG	Total thrombus generation
TMG	Time to maximum thrombus generation
MRTG	Maximum rate of thrombus generation
R	Reaction time
MA	Maximum amplitude
MG	Elastic modulus
ESR	Erythrocyte Sedimentation Rate
OsO_4	Osmium tetroxide
PS	Phosphatidylserine
HbA_1c	Glycated haemoglobin
WHO	World Health Organization
CRP	C-reactive protein
IL	Interleukin

CHAPTER 1: INTRODUCTION

The World Health Organization (WHO) defines diabetes as a chronic disease where the pancreas does not produce enough insulin or where the body is unable to effectively use the insulin it produces (1). Before discussing the causes of diabetes, one must look at the different types of the disease.

Type 1 diabetes, also known as insulin-dependent, juvenile or childhood-onset diabetes, can be characterized by the deficiency of insulin production and its exact causes are unknown. Usually the insulin-producing cells in the pancreas are attacked by the immune system but genetics and exposure to viruses or other environmental factors may also play a role in the pathogenesis of this disease (2). To current knowledge, type 1 diabetes is an unpreventable disease and the treatment regimen for type 1 diabetes requires daily administration of insulin. Type 2 diabetes, also known as non-insulin-dependent or adult-onset diabetes, is a result of the body's ineffective use of insulin. The development of type 2 diabetes is largely a result of excess body weight and a sedentary lifestyle, making this disease preventable. Treatment regimen for type 2 diabetes depends on the severity of the condition. Metformin, a drug that increases insulin sensitivity of body tissues, is normally the first line of treatment. All treatment regimens for type 2 diabetes requires the affected individual to make changes to their lifestyle such as eating healthier and becoming more active.

According to a study by Xu *et al,* from 2016 to 2017, among adults in the United States, type 1 diabetes accounted for 5.6% of the total diabetic population where type 2 accounted for a staggering 91.2% (3). The remaining 3.2% of diabetics can be ascribed to women diagnosed with gestational diabetes, a condition of hyperglycaemia during pregnancy. Women with gestational diabetes have an increased risk of developing type 2 diabetes at a later stage during their life.

Once developed, diabetes is a lifelong burden and a condition that must be strictly controlled. Uncontrolled diabetes will result in hyperglycaemia that can lead to serious complications such as, damage to blood vessels and nerves in the body, increased risk of heart attack and stroke (two- to three-fold), nerve damage, atherosclerosis (which ultimately leads to limb amputation), diabetic retinopathy (which may lead to permanent blindness) and kidney failure. In 2016, diabetes was the direct cause of 1.6 million deaths worldwide (1), making it

the seventh leading cause of death. This is a staggering amount considering that for the majority of diabetics, it is a preventable disease.

All the above-mentioned complications develop as a combined result of chronic inflammation caused by diabetes (4) (5) (6) (7). The inflammatory response is triggered by hyperglycaemia and therefore, by chronically being hyperglycaemic, the body is exposed to chronic inflammation. The inflammatory response causes numerous inflammatory mediators to interact in an attempt to achieve homeostasis. These inflammatory mediators include increased platelet and white blood cell activation which, when activated chronically, will cause a state of hypercoagulability and vascular damage (8) (9).

One way of testing whether diabetes is controlled or not, is by testing glycated haemoglobin (HbA_1c), which gives an accurate estimation of the blood glucose concentrations over the last four months. HbA_1c forms when glucose binds to red blood cells (RBC's) and therefore, an increase of blood glucose concentration will lead to increased levels of HbA_1c. The binding of HbA_1c also leads to the formation of free radicals inside red blood cells which may alter membrane properties as well as blood viscosity (10).

Past studies have found that because of high levels of insulin, whole blood viscosity was increased and negative surface electric charge decreased in diabetics (11) (12). The negative surface electric charge creates a repulsion force between RBC's in order to prevent agglutination (13). The deformability of RBC's allows them to move through smaller capillaries (smaller than 8 µm) more easily. A decrease in RBC deformability will result in an increase of pressure needed to move RBC's through these capillaries and a decrease of microcirculation (14).

The aim of this study was to investigate the changes to RBC structure, morphology of RBC membranes as well as blood viscosity, caused by hyperglycaemia.

CHAPTER 2: LITERATURE REVIEW

According to a study by Wild *et al.* (15), in the year 2000, the global prevalence of diabetes over all age groups was estimated to be 2.8 % and the projected prevalence for the year 2030 is 4.4 %. However, more recent statistics from the World Health Organization (WHO) (1) as well as the American Diabetes Association®(ADA) (16) revealed that there is an even steeper increase in the prevalence of diabetes. According to a meta-analysis by Sarwar *et al.* (17), the global prevalence of diabetes in adults over the age of 18, has risen from 4.7 % in 1980, to 8.5 % in 2014, translating to an estimate of 422 million people affected by diabetes worldwide. The ADA reported a prevalence of 9.4 % of the American population to have diabetes (16) and a study by Pheiffer *et al.* (18) revealed that 9 % of South Africans over the age of 30 had diabetes. Across all of these studies it was clear that diabetes is more prevalent in males than in females, more prevalent in urban areas (where prevalence is expected to double by 2030) and more prevalent in people older than 65. This staggering increase can be attributed to population growth, ageing due to better medical care, increase in urbanization and an increase of obesity together with a decrease in physical activity (19) (20). Even though these studies do not distinguish between type 1 and type 2 diabetes, statistics from the WHO and ADA from 2012 showed that only 4.29 % of all diabetics were type 1 (21). This difference can be ascribed to the fact that type 1 diabetes is mainly caused by hereditary factors and is an idiopathic disease (22) (23). While the risk of developing type 2 diabetes can be increased by hereditary factors, the primary cause of this disease is due to lifestyle factors (24). (It is important to note that even though type 1 diabetes is considered to be idiopathic, the effect of the modern lifestyle on the prevalence of type 1 diabetics should not be ignored). Type 2 diabetes mellitus is currently the seventh leading cause of death in the United States, directly responsible for more than 79 000 deaths as well as being a contributing factor in more than 250 000 deaths annually (25). According to the WHO (1), diabetes is responsible for 3.8 million annual deaths worldwide.

Type 2 diabetes mellitus (T2DM) is a metabolic disease associated with three main glycaemic disorders: chronic hyperglycaemia, glycaemic variability and iatrogenic hypoglycaemia, but is also associated with comorbidities including dyslipidemia and hypertension (26).

The hyperglycaemia can be ascribed to a decrease of insulin sensitivity of cells, a decrease in insulin synthesis by the pancreas, increased glucose production or a combination of the above, which ultimately leads to improper utilization of glucose. Secondary pathophysiological changes in multiple organ systems arise due to the metabolic dysregulation associated with T2DM, which impose an enormous burden for the affected individual (26) (27) (28). In addition to ensure adequate delivery of glucose to tissues of the body, modern regimen for treating diabetes is currently aimed at preventing and controlling chronic hyperglycaemia in order to decrease the likelihood of tissues in the body being affected by hyperglycaemia. The effects of hyperglycaemia on the vascular tree is currently the largest source of morbidity and mortality in both type 1 and type 2 diabetes (29).

Glycaemic variability (GV) refers to acute fluctuations in blood glucose levels, including hypoglycaemic states as well as postprandial hyperglycaemia (30). Recent studies have indicated that intermittent hypoglycaemia, rather than chronic hyperglycaemia have deleterious effects regarding the cardiovascular system such as endothelial dysfunction (31) (32). According to Smith-Palmer *et al.* (33) *in vitro* studies have shown that high levels of fluctuations in GV lead to the production of reactive oxygen species (ROS), which suggest an increase in the development of microvascular complications. Other studies by Frontoni *et al.* (34) and Kota *et al.* (35) support this finding that GV is an important key determinant of microvascular damage.

Iatrogenic hypoglycaemia refers to hypoglycaemic states achieved by treating hyperglycaemia. As stated above, hypoglycaemia is known to cause severe microvascular complications and it is also the most feared adverse effect of glucose-lowering treatment, thus it is crucial to prevent this (36). Severe hypoglycaemia is not as common in the early state of T2DM, since there are physiological mechanisms to counter this. As the disease progress, and the body is exposed to more episodes of hypoglycaemia, these mechanisms become impaired, therefore increasing the threat of hypoglycaemic states (37). This means that every patient should receive tailored treatment instead of a ‘silver bullet’ therapy aimed at the whole diabetic population.

Increasing emerging evidence shows that diabetes is associated with low-grade systemic inflammation (26) (38) (39) (40) (41) (42). Inflammation is the local protective response to tissue injury, which results in redness, swelling, heat and pain in the area of injury. On a

microscopical level, this occurs due to vasodilation, accumulation of white blood cells, an increase in permeability of the capillaries, increase of interstitial fluid in the area and stimulation of nerve endings by the neuropeptide substance P (42). Inflammation is a hallmark reaction of innate immunity where activated macrophages release cytokines in order to attract more immune-related cells, increase capillary permeability and cause a rise in temperature. The immune response consists of two phases, acute and chronic (also referred to as systemic). Immediately after injury or exposure to a pathogen, inflammatory responses are responsible for the release of acute-phase proteins which includes opsonins, antiprotease molecules and C-reactive protein (CRP). Under normal conditions the concentrations of these cytokines will subside after a few hours to a few days as the immune response proceeds, however, in the case of chronic inflammation, these cytokine concentrations remain elevated and may have long term side effects on the body (43). The inflammatory state of chronic inflammation does not comply to the hallmark signs of acute phase inflammation as stated above, therefore its presence is not always as obvious (41). It is interesting to note that prolonged elevated levels of CRP are known to increase the risk of cardiovascular disease (44), diabetes (45) and are suspected to have numerous neurological side effects (from a PubMed search on 16 February 2017 containing the search parameters “CRP” and “inflammation”).

Extensive research by our research group has investigated inflammation and its involvement on the viscoelastic properties of the haemostatic system (46) (47) (48). Membrane changes on RBC’s directly reflect on the inflammatory state of the body since, during inflammation, the haematological system is constantly exposed to circulating inflammatory mediators. The plasma levels of many clotting factors including fibrinogen, factor VII, factor VIII, factor XI, factor XII, kallikrein, and von Willebrand factor have also been found to be elevated in patients with diabetes (49). In addition to these elevated factors, increased platelet count and platelet activation is also associated with inflammation (50) and *ipso facto* with type 2 diabetes (51). Activated platelets secrete clotting mediators which includes adenosine diphosphate (ADP) and thromboxane A2 (TXA2) which will in turn bind to their receptors on platelet surfaces both in an auto- and paracrine fashion (to itself as well as other platelets). This binding then results in a cascade of processes which ultimately leads to an increase of intracellular Ca^{2+}. The calcium increase then activates the calcium-dependent association of glycoprotein IIb

(GPIIb) and glycoprotein IIIa (GPIIIa) to form the activated membrane receptor complex GPIIb/GPIIIa. The formation of the GPIIb/GPIIIa complex is a necessary step in normal platelet aggregation and endothelial adherence (52) (53). This complex is a receptor for fibrinogen as well as von Willebrand factor which will bind to platelets and cause them to adhere together, ultimately resulting in a clot. The coagulation cascade will stabilise the clot as thrombin (factor IIa) will convert the soluble fibrinogen to insoluble fibrin strands. The insoluble strands are cross-linked by factor XIII in order to form a stabilised blood clot (54). Blood viscosity refers to the ability of blood to flow. Any changes, primary or secondary, in blood viscosity, such as those caused by chronic inflammation or diabetes, will have an effect on blood viscosity. This biophysical property is determined by haematocrit, RBC deformability, RBC aggregation and plasma viscosity (55) and is a critical determinant of how well tissues and organs are perfused with oxygen. The study of blood viscosity is also called hemorheology, the study the flow of blood, plasma and cellular elements in veins and arteries. The fact that RBC's are deformable and are able to aggregate causes blood to behave as a shear-thinning non-Newtonian fluid (55). This means that blood becomes less viscous at higher shear rates (such as those experienced at peak systole), and is more viscous at lower shear rates (such as with increased vessel diameter, downstream from an obstruction or during diastole). Increased blood viscosity may increase the predisposition of blood clots forming, even more so where a patient is in a hypercoagulable state. Some cardiovascular risks that are linked to an increased whole blood viscosity are:

- Hypertension
- Metabolic syndrome
- T2DM
- Obesity
- Smoking
- Male gender
- Age (viscosity goes up with age)

Severe anaemia can reduce blood viscosity to such an extent that it may lead to heart failure.

With type 2 diabetes where an individual is exposed to such a chronic state of inflammation, blood becomes hypercoagulable, meaning that there is an increased tendency towards blood clotting (56). This can be ascribed to the increased levels of platelets as well as platelet activation (57), but also the endothelial abnormalities caused by diabetes. As a result of this

hypercoagulable state, a method called erythrocyte sedimentation rate (ESR) can be used to detect the presence of inflammation in an individual. This method was thus used in this study as a marker of inflammation. ESR makes use of the rate that red blood cells settle in a tube filled with a whole blood sample. Red blood cells from a healthy individual will settle at the bottom of a tube after a set amount of time. In an individual with inflammation, fibrinogen in the blood are causing red blood cells to clump together which will cause red blood cells to settle at a faster rate. While the C-reactive protein test also confirms the presence of inflammation, it is able to measure the extent of inflammation. The aim of this study is to test for the presence of inflammation, rather than the extent of it, thus the ESR is the method of choice for this study (58) (59).

One thing that has become apparent with the research done by our group was the presence of eryptosis in inflammatory diseases. Eryptosis, the programmed death of RBC's, similar to apoptosis in nucleated cells, occur when the cells are exposed to stressors such as inflammatory mediators and ROS (47) (60). It is characterized by cell shrinking, membrane blebbing and membrane phospholipid scrambling. Similar to nucleated cells, RBC's have a transmembrane protein called phosphatidylserine (PS) which flips inside out in the event of eryptosis. This can be quantified with Annexin V analysis using flow cytometry and viewed with confocal microscopy, using Annexin V as a stain. Their studies have confirmed eryptotic RBC's in Parkinson's and Alzheimer's, both inflammatory diseases where IL-8 is known to be unregulated, however, it could not be established whether RBC's have a receptor for IL-8 and it has been proposed that further studies need to be done to confirm or refute this. It is however, clear that RBC health status can be utilized as an indicator of the inflammatory status, disease progression and treatment monitoring (47) (60) (61). A study done by Calderón-Salinas *et al.* (62) found an increase in eryptosis and PS-flip of RBC's as well as an increase of RBC fragility in T2DM patients however, their study was focused on renal damage and did not go into detail about the ultrastructural changes of the RBC's of T2DM patients. According to a PubMed search on 29 November 2018, using the search criteria "eryptosis" and "diabetes" little research has been done in order to explore the physiology behind this link (63).

The inflammatory state of T2DM has been linked to obesity and has also been labelled "obesity-induced inflammation". The inflammation in obese patients is metabolic and is

caused by an excess consumption of nutrients. As an individual becomes more obese, adipocytes enlarge and undergo molecular and cellular changes which affect their metabolism. Free fatty acid (FFA) and glycerol release are constantly increased in obese patients, resulting in an increase of insulin secretion (64). An increase of FFA are known to promote insulin resistance in muscle tissue (65). This already draws a direct line to the development of T2DM. As adipocytes get larger as an individual becomes more and more obese, more macrophages are attracted to them, binding to the adipocytes, resulting in an increase expression of pro-inflammatory cytokines. These adipose-bound macrophages are responsible for nearly all TNF-α expression in adipose tissue, as well as significant amounts of interleukin-6 and NOS production (66). It is not just adipocytes that show an increase of cytokine expression in the case of obesity, as previous studies show that cytokine expression similar to that found in adipose tissue, can be seen in liver (67), pancreas (68), brain (69) and possibly muscle cells (70) as well. Studies examining the upstream activity of inflammatory cytokine expression identified the kinases c-jun N-terminal kinase (JNK), inhibitor of κ kinase (IKK), and more recently, protein kinase R (PKR) as contributors to the initiation of inflammation in metabolic tissues. When compared to control samples, obese tissues showed a considerable increase in activity of these kinases and subsequently, their downstream signalling cascades (71) (72). In animal studies, upon deletion of the genes coding for these kinases, it was also found that they played an important role in mediating the inflammatory response found in obese individuals (67) (72) (73).

Apart from being associated with obesity, an individual with T2DM is at risk of developing multiple comorbidities related to the disease. The most common comorbidities of T2DM are listed in table 2.1 below, together with their estimated occurrence. Statistics were obtained from 2009-2012 from patients 18 years and older. Statistics from blindness and eye problems were obtained from patients 40 years and older (16).

Table 2.1: Comorbidities of T2DM.

The most common comorbidities in 2009-2012 according to the American Diabetes Association.

Comorbidity	**Occurrence**
Hypoglycaemia	1 % of diabetics visited the ER due to HG

Hypertension	71 % of diabetics had blood pressure greater than 140/90 mmHg or was on prescription medication for high blood pressure
Dyslipidaemia	65 % had blood LDL cholesterol of 100 mg/dL or higher. (Below 100 mg/dL is optimal(20)
Heart attack	1.8 times higher in patients with diagnosed diabetes than undiagnosed patients
Stroke	1.5 times higher in patients with diagnosed diabetes than undiagnosed patients
CVD death rates	1.7 times higher in patients with diagnosed diabetes than undiagnosed patients
Blindness and eye problems	28.5 % of patients 40 years and older had some case of diabetic retinopathy
Kidney disease	1 % of diabetics live on chronic dialysis due to kidney failure. 44 % of kidney failure cases was due to diabetes
Amputations	0.25 % of diabetics had non-traumatic lower limb amputations

CVD = Cardiovascular disease

The abovementioned comorbidities are just the most frequent occurring ones; there are numerous other known comorbidities as well as some that are intensively being investigated. It is important to note that obesity was not included in the list even though it is considered to be a comorbidity of diabetes. This is because obesity is not a result of diabetes but rather a major risk factor. Other known comorbidities include non-alcoholic fatty liver disease (74), obstructive sleep apnoea (75), cancer (76) and fractures (77). Search results from PubMed on 2 December 2018 using the search criteria "*diabetes*" and "*comorbidities*" showed papers investigating the prevalence of cognitive decline and dementia with diabetes, as well as hearing impairment, lower testosterone levels in men, more severe periodontal disease, schizophrenia and depression.

According to the WHO, the onset of T2DM can be delayed or even prevented by being physically active, maintaining a healthy body weight, eating a healthy diet and avoiding tobacco use (78). Treatment for T2DM focuses mainly on lifestyle-based changes, like the aforementioned methods, in order to manage the disease. It has been proven that in some cases, lifestyle changes were significantly more effective than metformin therapy, a drug commonly used to treat T2DM (25). The aim of treatment is to keep the glycated haemoglobin (HbA_1c) below 6.5% or 126 mg/dL, as suggested by the ADA, after which the patient is considered to have controlled T2DM. When glucose is processed in the body, it attaches to haemoglobin in order to glycate it, hence the term "glycated haemoglobin". Because RBC's

have a lifespan of 100 – 120 days, measuring the proportion of glycated haemoglobin will give an average blood glucose level over that period (79) (80). A reading above 6.5% indicates that the condition is poorly controlled and the likelihood of comorbidities will increase. Reducing the HbA_1c below 6.5% greatly reduces the incidence of complications and comorbidities due to the disease (81). The threshold of HbA_1c concentration where a patient is considered to be poorly controlled ranges from 6.5% to 8.5% depending on which source is used. The difference is due to the fact that this value is a guideline to which patients should be working towards in order for their condition to be considered controlled. For the purpose of this study, any HbA_1c value above 6.5% will be considered to be poorly controlled. Controlled type 2 diabetes can be achieved by complying to lifestyle changes which aim to increase weight loss, decrease fat intake, restrict calorie intake and increase physical activity (25).

According to the ADA, Mayo Clinic, the International diabetes federation and the National Institute of Diabetes and Digestive and Kidney Diseases (21) (82) (83) (84), each of the following factors will increase a patient's risk for T2DM:

- If the patient is above 45 years of age
- If the patient's BMI is equal to or above 25
- If the patient has a direct blood-relative with diabetes
- If the patient is from any one of the following races: African American, Hispanic/Latino, American Indian, Asian American or Pacific Islander
- If the patient had gestational diabetes or gave birth to a baby weighing more than 4.5 kg
- If the patient's blood pressure is equal to or above 140/90 mmHg
- If the patient has abnormal blood cholesterol levels (HDL lower than 35 and LDL higher than 250 mg/dL
- If the patient lives a sedentary lifestyle
- If the patient has polycystic ovarian syndrome

It is important to note that T2DM is not a genetically inherited disease, as some might deduce from the list above, but rather the many risk factors, of which obesity is the greatest.

The aim of this research was therefore to provide a greater understanding on the ultrastructural changes and viscoelastic properties of whole blood of individuals with poorly controlled type 2 diabetes. The current study is focused more on red blood cells due to the

fact that it has not been researched yet, however, changes in leukocytes and platelets will not be ignored and may result in further future studies.

CHAPTER 3: SAMPLE COLLECTION

3.1 Participant information

3.1.1 Research subject selection

All sample analysis was done on blood *ex vivo*. Blood was obtained from an age and sex matched control group of healthy individuals as well as a group of poorly controlled T2DM individuals from the Diabetic Clinic, Steve Biko Academic Hospital. Blood was collected between 9am and 12pm from non-fasting individuals. Healthy volunteers were identified and recruited by the principal investigator. Healthy volunteers consisted of university staff, students, co-workers, friends and family of the principal investigator. Poorly controlled T2DM individuals were identified by the principal investigator with the help of staff and hospital records at the Steve Biko Academic Hospital. Hospital records were used to confirm HbA1c levels of the diabetic group and to ensure they complied to the inclusion and exclusion criteria. All participants were asked to complete a questionnaire prior to sample collection in order to confirm that they comply with the inclusion and exclusion criteria of the study. Blood was drawn by the principal investigator after informed consent had been acquired. All participant information was handled anonymously.

As agreed with a statistician from the South African Medical Research Council (SAMRC), the sample size was to be 40 participants per group, 80 participants in total. The two groups included in this study were the healthy control group and the poorly controlled diabetic group. By the end of an 18-month recruitment period, samples were collected from 40 participants, 20 from each group. The statistician was satisfied with the final sample size of 20 participants per group. A total of 13 ml of blood was drawn from each participant in two citrate tubes and one EDTA tube. All research was done on these three tubes.

3.1.2 Control subjects

In order for participants to be classified as ‘healthy’, they had to comply with the following inclusion and exclusion criteria:

Inclusion criteria:

- Males and females

- Individuals aged 18 to 55 years

Exclusion criteria:

- Individuals that use any sort of chronic medication (including females on oral contraceptives).
- Individuals with any type of chronic disease including diabetes.
- Individuals who are smoking, “vaping”, or using any type of tobacco substance.
- Pregnant females.
- Individuals who are known to be HIV positive.
- Individuals who are taking warfarin.
- Individuals with any previous thrombotic events.

3.1.3 Poorly controlled T2DM participants

In order for participants to be part of the diabetic group, they had to comply to the following inclusion and exclusion criteria:

Inclusion criteria:

- Males and females
- Only individuals with poorly controlled type 2 diabetes (HbA1c >6.5%)
- Individuals aged 18 to 55 years

Exclusion criteria:

- Individuals that use any sort of chronic medication apart from that to treat their condition (including females taking oral contraceptives as well as all individuals taking anti-coagulants)
- Individuals who have any type of chronic disease other than type 2 diabetes
- Individuals who are smoking, “vaping” or using any type of tobacco substance
- Pregnant females
- Individuals who are known to be HIV positive
- Individuals with any previous thrombotic events

All participant information was handled anonymously. Participants were required to sign an informed consent form provided by the principal investigator (see 3.2.3). All participants were to be notified by the principal investigator (the medical practitioner in the case of participants from the diabetic group) if there was any concern about their medical wellbeing due to the results, however, none such cases occurred. There were no cost implications for any of the participants.

From here on out, the poorly controlled T2DM group will be referred to as the T2DM group.

3.2 Sample collection

All blood samples were collected by the principal investigator. Blood was drawn using a vacutainer in order to fill two citrate tubes and one EDTA tube. The blood in the tubes were used as follows:

Citrate tube 1: Scanning Electron Microscopy and thromboelastography

Citrate tube 2: Erythrocyte sedimentation rate

EDTA tube: Haematology analysis

3.2.1 Control group

All participants in the control group had to sign an informed consent form (see 3.2.3), approved by the Faculty of Health Sciences Ethics Committee, University of Pretoria, prior to any study related procedures and sample collection.

3.2.2 T2DM group

Participants from the T2DM group were contacted the day before their next appointment at the hospital to confirm their participation in the study. On the day of sample collection, participants were asked to complete an informed consent form (see 3.2.3). Participants who were recruited at sites, other than at the hospital were treated similarly to participants in the control group.

3.2.3 Consent form

INFORMATION LEAFLET AND INFORMED CONSENT FORM

TITLE OF STUDY: Investigating the ultrastructural and viscoelastic properties of whole blood, with specific focus on erythrocytes, in poorly controlled type 2 diabetes

Principal investigator: Wikus Meijer

Ethical clearance number: 363/2017

Department of Physiology

University of Pretoria

INTRODUCTION

You are invited to participate in a laboratory-based research study conducted by the Department of Physiology (School of Health Sciences) from the University of Pretoria. You are free to choose if you would like a family member to be present in the information session and with the decision making. This information leaflet is to help you to decide if you would like to participate. Before you agree to take part in this study you should fully understand what is involved. If you have any questions, which are not fully explained in this leaflet, do not hesitate to ask the investigator.

PURPOSE OF STUDY

The researcher is investigating the health complications in poorly controlled type 2 diabetes associated with erythrocyte normality, specifically focusing on how your blood clot, found in your blood (consisting of platelets and fibrin), as well as how red blood cells look like under a specialized microscope that can magnify up to 100 000x (Scanning Electron Microscope).

PROCEDURES

We will draw 3 tubes of 4.2 mL blood. Two tubes will be used for measuring haematological parameters. This includes test that determines blood count (the amount of white- and red blood cells as well as platelets) in a blood sample, as well as how fast the blood sample clots and settles down. Values outside of the normal ranges will affect the shape of red blood cells. The 3rd tube will be used for microscopic analysis in order to investigate any changes in the blood clotting mechanism. The 4th tube will be used for flow cytometry where we will determine the percentage of red blood cells that are dying.

The total amount of blood that will be drawn will be approximately 15 mL

HAS THE TRIAL RECEIVED ETHICAL APPROVAL?

This protocol was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria and written approval has been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki which deals with the recommendations guiding doctors in biomedical research involving human/subjects. A copy of the Declaration may be obtained from the investigator should you wish to review it.

WHAT ARE MY RIGHTS AS A PARTICIPANT IN THIS TRIAL?

Your participation in this study is entirely voluntary and you can refuse to participate or stop at any time without stating any reason. Your withdrawal will not affect your access to other medical care. The investigator retains the right to withdraw you from the study if it is considered to be in your best interest.

RESEARCH KNOWLEDGE OBTAINED IN THIS STUDY

The current study will have no benefit for the participant but will add to our knowledge of understanding the involvement of red blood cells in type 2 diabetes and may add to future research on this disease.

MAY ANY OF THESE TRIAL PROCEDURES RESULT IN DISCOMFORT OR INCONVENIENCE?

Venipunctures (i.e. drawing blood) are normally done as part of routine medical care and present a slight risk of discomfort. Drawing blood may result in a bruise at the puncture site, or less commonly fainting or swelling of the vein, infection and bleeding from the site. Your protection is that the procedures are performed under sterile conditions by experienced personnel. A total of three times 4.2 ml of blood will be collected during your single donation.

WHAT ARE THE RISKS INVOLVED IN THIS STUDY?

In previous studies, some participants have reported experiencing side effects, which included bruising, or swelling of the vein.

ARE THERE ANY WARNINGS OR RESTRICTIONS CONCERNING MY PARTICIPATION IN THIS STUDY?

None

INSURANCE AND FINANCIAL ARRANGEMENTS

Neither you nor your medical scheme will be expected to pay for the study. During a study-related injury (thrombophlebitis) the Department of Physiology assumes no obligation to pay

for the medical treatment of other injuries or illnesses, the medical practitioner performing the procedure will have insurance to cover any injury related to the procedure.

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. In connection with this study, it might be important for domestic and foreign regulatory health authorities and the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, as well as your personal doctor, to be able to review your medical records pertaining to this study.

Any information uncovered regarding your test results or state of health as a result of your participation in this study will be held in strict confidence. Your medical practitioner will be informed of any finding of importance to your health. Information will not be disclosed to any third party in addition to the ones mentioned above without your written permission.

INFORMED CONSENT

I hereby confirm that I have been informed by the investigator, Wikus Meijer about the nature, conduct, benefits and risks of the study: Investigating the ultrastructural and viscoelastic properties of whole blood, with specific focus on erythrocytes, in poorly controlled type 2 diabetes.

I have also received, read and understood the above written information (Participant Information Leaflet and Informed Consent) regarding the research study.

I am aware that the results of the study, including personal details 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.

Participant's name (Please print)

……

Participant's signature ……..Date……..

_____

Witness's name (Please print)

……

Witnesses's signature …… Date……

_____

I, Dr ……. herewith confirm that the above participant has been informed fully about the nature, conduct and risks of the above trial.

_____

Investigator's name

Wikus Meijer

Investigator's signature …… Date……

Contact details for Investigator: 0711312416

Contact details for ethics committee: 0123541677

INFORMATION FILLED BY MEDICAL PRACTITIONER/PRINCIPAL INVESTIGATOR

Age:	Gender:
Height:	Weight: BMI:
Diabetic status	Type 1 / Type 2 / Gestational / No history of diabetes
Any other chronic condition (including anaemia)?	Y / N
Do the participant smoke?	Y / N
If female, is the participant on an oral contraceptive?	Y / N
If female, is the participant pregnant?	Y/ N
Medication that the participant uses? If yes, please specify.	Y / N

CHAPTER 4: HAEMATOLOGY ANALYSIS

4.1 Chapter objectives

The chapter objective is to compare the full blood count parameters between healthy individuals and poorly controlled T2DM individuals, using the haematology analyser.

4.2 Introduction

Haematology analysis was the routine test of choice to gain an overall perspective of the blood profiles of the two groups. A full blood count is often used as a starting point for healthcare practitioners in order to determine the presence of a medical condition (85) (86). Although abnormalities in the results from the haematology analyser does not confirm a disease, differences in the components of whole blood may be associated with inflammation (87) (88) (89) (90). All parameters measured with the haematology analyser will be discussed briefly, specifying their meaning as well as the normal ranges.

WHITE BLOOD CELL (WBC): Total white blood cell count or the number of leukocytes includes all leukocytes but does not distinguish between them. Normal values range from 4 x 10^9 to 11 x 10^9 cells per litre (91). A normal-high to high value may indicate a current or recent infection that the immune system is trying to eliminate. It may also be a result of physical or emotional stress. A low value can most commonly be attributed to acute viral infections such as influenza but can also be associated with chemotherapy, radiation therapy and conditions such as aplastic anaemia, systemic lupus erythematosus or HIV, to name a few. Since we are using healthy individuals in the control group, we are expecting to see WBC count in the normal range whereas for the diabetic group, a higher WBC count is expected since they are in a constant state of low-grade inflammation (43) (92).

RBC: Red blood cell count refers to the number of red blood cells (RBC's) or erythrocytes in circulation. Normal values range from 4.5 x 10^9 – 6.5 x 10^9 cells per litre (91). A value above this range may be a result of compensation for low oxygen levels. The body will produce more erythrocytes to ensure adequate oxygen distribution throughout the body. Another cause of a high RBC count is performance enhancing drugs that stimulate erythropoiesis. Dehydration will also increase RBC count since the concentration of RBC's will increase with a decrease in plasma volume. A low RBC count can be the result of several underlying factors including, but

not limited to, anaemia, bone marrow failure, leukaemia, malnutrition and thyroid disorders. For this study, RBC values of the control group are expected to fall within normal ranges while the diabetic group are expected to be normal to low due to possible anaemia caused by diabetes-related complications.

Haemoglobin concentration (Hb): This reading refers to the amount of circulating haemoglobin that carries oxygen throughout the body. The normal range for this is 11 – 17 g/dL (91). Low haemoglobin is clinically used to determine the presence of anaemia. Possible causes of this include nutritional deficiencies, anaemia due to chronic disease and blood loss. A high value may be indicative of polycythaemia due to a decrease in plasma volume such as in the case of dehydration.

Mean corpuscular volume (MCV): This refers to the average volume of a red blood cell. Normal values range from 75 fL to 100 fL (91). A high value may be due to Vitamin B9 (folic acid) and B12 deficiencies but may also be associated with alcoholism. The most common causes for a low reading (microcytic anaemia) are iron deficiency (due to inadequate intake or chronic blood loss), thalassaemia (a genetic blood disorder characterized by abnormal haemoglobin production) or chronic disease.

Haematocrit (HCT): This refers to the ratio of the volume of RBC's to the total volume of blood. Normal values for HCT range from 34.9 to 44.5 % for women and 38.8 to 50 % for men (93). Causes of increased HCT may be due to dehydration or hypoxia while a low reading may indicate anaemia, loss of RBC's due to chronic bleeding or RBC destruction, bone marrow suppression, malnutrition, infection or overhydration.

Mean corpuscular haemoglobin (MCH): This parameter refers to the average mass of haemoglobin contained in each red blood cell. The normal range for this is 24 to 30 pg (91). A low MCH reading can be caused by microcytic anaemia (red blood cells are abnormally small and cannot contain the necessary amount of haemoglobin) and low blood concentrations of iron, something that is more prevalent in vegetarians or malnourished individuals. A high MCH reading is a typical sign of macrocytic anaemia (red blood cells are abnormally big) but may also be because of liver disease, overactive thyroid gland, regular consumption of alcohol, excessive administration of oestrogen and complications from infection.

Mean corpuscular haemoglobin concentration (MCHC): Although similar to MCH, MCHC refers to the concentration of haemoglobin in a given volume of packed red blood cells and is reported in g/dL. The normal MCHC range is 31 – 36g/dL (91). When MCHC is increased it can indicate hereditary spherocytosis or autoimmune haemolytic anaemia. It is also elevated in homozygous sickle cell anaemia (94).

Red cell distribution width (RDWc): RDW is a measure of the range of variation of RBC volume. It is presented as a coefficient of variation and is therefore reported as a % and not as a unit of length. The following formula explains how it is calculated: RDWc = (Standard deviation of MCV ÷ MCV) x 100. The normal range is 11.5 – 14.5% (91). Elevated RDWc helps to distinguish between different types of anaemia but may also be due to chronic liver disease, vitamin B12 and folate deficiency and cytotoxic chemotherapy. It is uncommon for RDWc to be below normal.

Platelet distribution width (PDW): PDW, like RDWc, measures the range of variation of platelet size. The normal range for this is 10 – 17.9% (91). PDW is increased during platelet activation.

Platelet count: Platelet count is suggestive of the blood's ability to clot. The normal range for this is 150^9 – 450^9 platelets per litre (91). A low platelet count is referred to as thrombocytopenia. This can be because platelets get trapped in the spleen, or there is decreased production of platelets or platelets are being destroyed faster than they are produced. On the other hand, a higher than normal reading (thrombocythemia) can cause blood clots to develop spontaneously, however this is a rare condition.

Mean platelet volume (MPV): MPV refers to the average size of platelets in blood. The normal MPV range is 8 – 15 fL (91). Higher than normal MPV is indicative of excessive platelet destruction, something that is typically seen inflammatory bowel syndrome. Lower than normal MPV readings correlates with thrombocytopenia when it is due to impaired platelet production.

Thrombocrit (PCT): PCT refers to the volume of platelets in whole blood and is expressed in %. The normal PCT range is 0.1 – 0.4% (91). PCT is increased in some inflammatory diseases including diabetes, viral infections, tuberculosis, hyperthyroidism and necrosis. A low PCT

reading, although uncommon, may be because of aplastic or megaloblastic anaemia, systemic lupus erythematosus, leukaemia and chronic liver and kidney failure.

White blood cell 3-differential: The WBC 3-differential counts the different types of WBC's, lymphocytes, monocytes and granulocytes in the blood. Normal ranges for these are (95):

Lymphocytes: 1.00 x 10^9 – 4.00 x 10^9 cells per litre

Monocytes: 0.00 x 10^9 – 1.00 x 10^9 cells per litre

Granulocytes: 1.50 x 10^9 – 8.00 x 10^9 cells per litre

4.3 Materials and methods

After blood sample collection, the filled EDTA tube was inverted 5 times to allow for proper mixing of the contents after which the cap was removed and it was inserted into a SAMSUNG™ LABGEO HC 10 haematology analyser. Twenty-five microliters of the sample were then aspirated into a sampling needle and mixed with 4mL of diluent (Samsung LABGEO HC Diluent) after which it was stored in the needle. A lysing reagent (Samsung LABGEO HC 10 Lyse) was then added to the mix dilution in order to perform a 3-part white blood cell differential analysis. Five millilitres of diluent were then added to 35 µL of mix dilution to perform the red blood cell and platelet count. After this process, white blood cell, red blood cell, platelet counting and haemoglobin readings were performed. A washing process cleaned and prepared the unit for the next analysis. Everything from insertion of the EDTA tube into the instrument is automated.

The SAMSUNG™ LABGEO HC 10 haematology analyser measures impedance by using the Coulter Principle for counting cells passing through an aperture and optically measures the haemoglobin content of red blood cells. A constant DC current flows between the external and internal electrodes, while each cell passing through the aperture causes some change in the impedance of the conductive cell suspension. Such changes are recorded as increases in the voltage between electrodes. The number of pulses is proportional to the number of particles and the intensity of each pulse to the volume of the particle. The volume distribution of the cells is displayed as WBC, RBC and PLT histograms on the screen. Refer to figure 4.3.1 below that illustrates this concept and process:

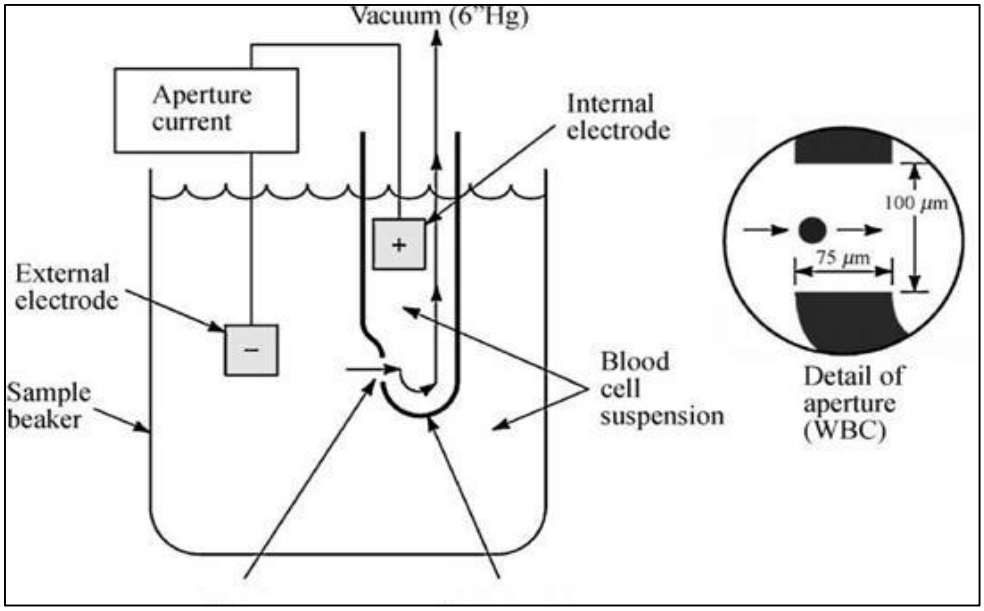


Figure 4.3.1. Concept of Haematology analysis.
The concept and process of haematology analysis done by the SAMSUNG™ LABGEO HC 10 haematology analyser.

The lysed sample dilution can be measured by a photometric method. The reagent lyses the red blood cells which release haemoglobin. The chemical process forms a stable form of methaemoglobin, which is then measured with a photodetector in the chamber. Haematology analysis was the routine test of choice for this study due to the fact that it is able to give an adequate overview of the blood profile of the participant. This will help to explain results in subsequent methods.

Table 4.3.1. Parameters measured in Haematology analysis.
Parameters with descriptions thereof measured by the hematology analyzer.

Parameters	
White Blood Cells: WBC (cells/L, cells/µL)	Number of leukocytes WBC = WBCcal X counted WBC (cells/L, cells/µL)
Red Blood Cells: RBC (cells/l, cells/µL)	Number of erythrocytes RBC = RBCcal X counted RBC (cells/L, cells/µL)
Haemoglobin concentration: Hb (g/dL, g/L, mmol/L)	Measured photometrically at 540 nm: in each cycle blank measurement is performed on diluent. Hb = Hbcal X (Hb measured–Hb blank)
Mean Corpuscular Volume: MCV (fL)	Average volume of individual erythrocytes derived from the RBC histogram
Haematocrit: HCT (percentage, absolute)	Calculated from the RBC and MCV values HCT percentage = RBC X MCV X 100 HCT absolute = RBC X MCV

Mean Corpuscular Haemoglobin: MCH (pg, fmol)	Average haemoglobin content of erythrocytes, calculated from RBC and Hb values MCH = Hb/RBC
Mean Corpuscular Haemoglobin Concentration: MCHC (g/dL, g/L, mmol/L)	Calculated from the Hb and HCT values MCHC = Hb/HCT absolute Unit of measurement is displayed according to the one chosen for Hb result (g/dL, g/L or mmol/L)
Red Cell Distribution Width: RDW-SD (fL) Platelet Distribution Width: PDW-SD (fL) Red cell Distribution Width: RDW-CV (absolute) Platelet Distribution Width: PDW-CV (absolute)	The distribution width of the erythrocyte or platelet population derived from the histogram at 20 % of peak 100%200P1P2RBC60%20% xDW-SD = RDWcal X (P2-P1) (fL), xDW-CV = RDWcal X 0.56 X (P2-P1)/(P2+P1) by the factor of 0.56 CV is corrected to the 60 % cut
Platelet: PLT (cells/L, cells/µL)	Number of thrombocytes (platelets) PLT = PLTcal X counted PLT (cells/L, cells/µL)
Mean Platelet Volume: MPV (fL)	Average volume of individual platelets derived from the PLT histogram
Thrombocrit: PCT (percentage, absolute)	Calculated from the PLT and MPV values PCT (percentage) = PLT X MPV X 100 PCT (absolute) = PLT X MPV
White blood cell 3-diff: LYM, LYM %: lymphocytes MON, MON %: monocytes and some eosinophils GRA, GRA %: neutrophil granulocytes	Absolute values counted in the channels determined by the three WBC discriminators: LYM, GRA, MON Percentages calculated from the absolute WBC value

4.4 Results

The parameter means from the haematology analysis will be described using the Mann-Whitney *U* test in GraphPad Prism 7® for Windows. All test results were evaluated at a 5% level of significance.

The two groups are denoted as “Control” for the control group, and “Experimental” for the T2DM group.

4.4.1 White blood cell count

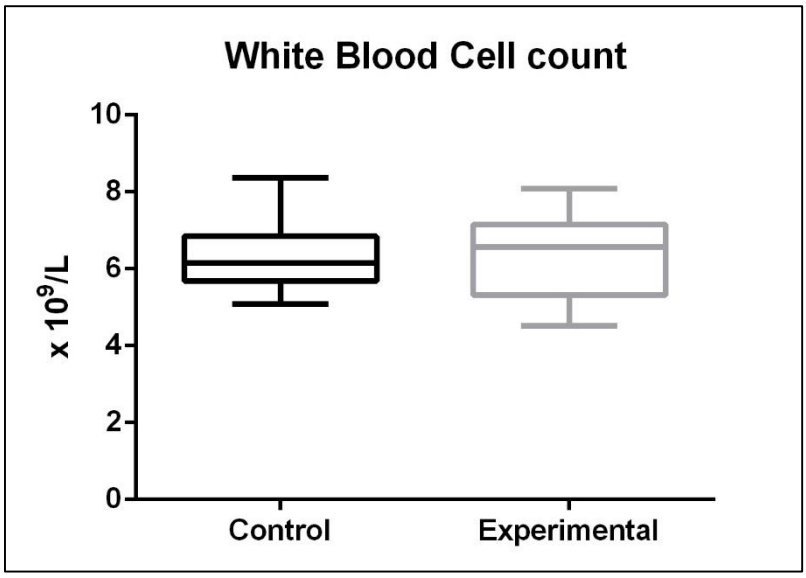


Figure 4.4.1. Box-and-whisker plot of white blood cell counts.

Comparison between the control and T2DM groups.

Table 4.4.1. Statistical analysis of the white blood cell count.

	Mean	Median	Standard Deviation	P-value	(P < 0.05)
Control	6.32	6.15	0.97	0.7493	No
T2DM	6.35	6.57	1.20		

Contrary to what was expected, results from the white blood cell count showed no statistically significant differences between the two groups however, the white blood cell count for the diabetic group is slightly increased when compared to that of the control group. Under a state of chronic inflammation, it would be expected that the white blood cell count should be higher than normal since they are the cells that initiate the inflammatory process. This minor difference as opposed to the expected higher difference can be attributed to the fact that all of the participants from the diabetic group were taking aspirin to treat the secondary diseases caused by their condition. Aspirin is known to have an effect on the bone marrow, the site where, among others, white blood cells are produced (96). Another explanation for this is that it may be due to the type of lifestyle that poorly controlled diabetics lead, however, this cannot be confirmed by the information obtained in this study alone. Alcohol abuse is frequently seen in individuals living a sedentary lifestyle and chronic alcohol abuse is known to decrease white blood cell count by a significant amount (97). This is only a speculation and it cannot be concluded by results from this study alone. The similarities in the white blood cell

counts between the two groups can also be seen in the lymphocyte, monocyte and granulocyte counts.

4.4.2 Lymphocyte count

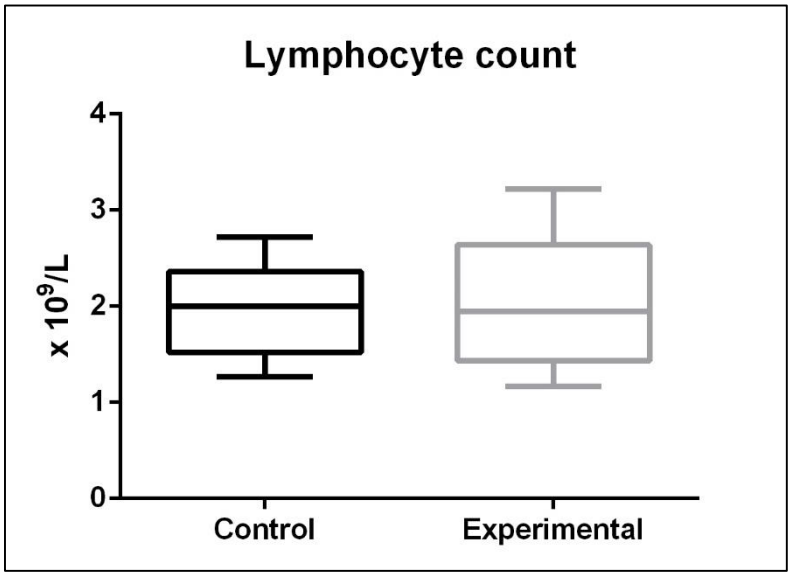


Figure 4.4.2. Box-and-whisker plot of the lymphocyte counts.

Comparison between the control and T2DM groups.

Table 4.4.2. Statistical analysis of the lymphocyte count.

	Mean	Median	Standard Deviation	P-value	(P < 0.05)
Control	1.93	0.68	0.51	0.9839	No
T2DM	2.02	1.95	0.71		

Analysis of the lymphocyte count showed no statistically significant difference between the two groups. The lymphocyte count for the diabetic group had a lower mean but a greater spread due to one outlier.

4.4.3 Monocyte count

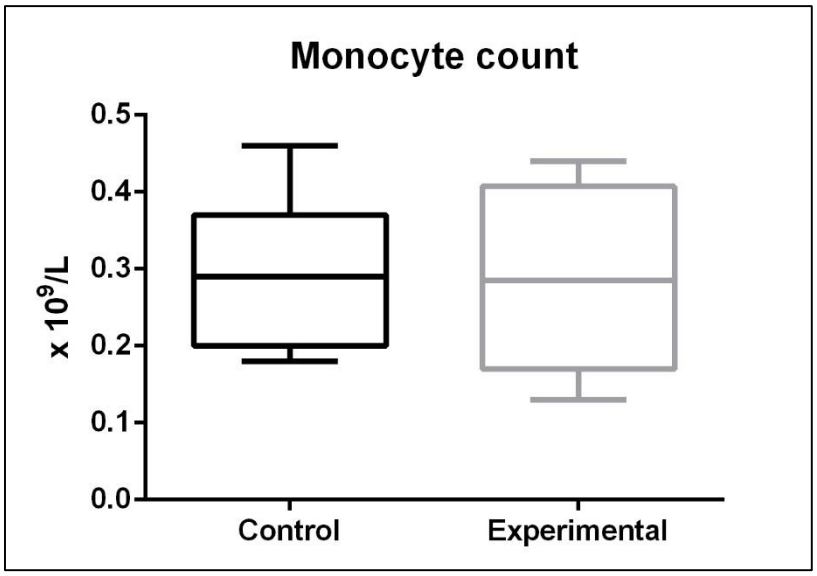


Figure 4.4.3. Box-and-whisker plot of the monocyte counts.

Comparison between of the control and T2DM groups.

Table 4.4.3. Statistical analysis of the monocyte count.

	Mean	Median	Standard Deviation	P-value	(P < 0.05)
Control	0.29	0.29	0.10	0.6997	No
T2DM	0.28	0.29	0.12		

Analysis of the monocyte count showed no significant difference between the two groups.

4.4.4 Granulocyte count

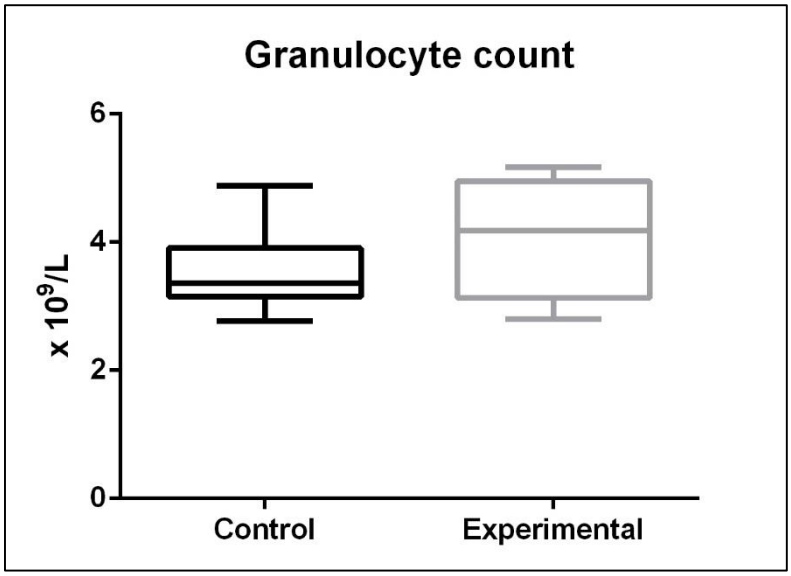


Figure 4.4.4. Box-and-whisker plot of the granulocyte counts.

Comparison between the control and T2DM groups.

Table 4.4.4. Statistical analysis of the granulocyte count.

	Mean	Median	Standard Deviation	P-value	(P < 0.05)
Control	3.75	3.42	0.89	0.3950	No
T2DM	4.05	4.18	0.90		

Results from the granulocyte count showed similar results between the two groups with no statistically significant difference.

4.4.5 Red blood cell count

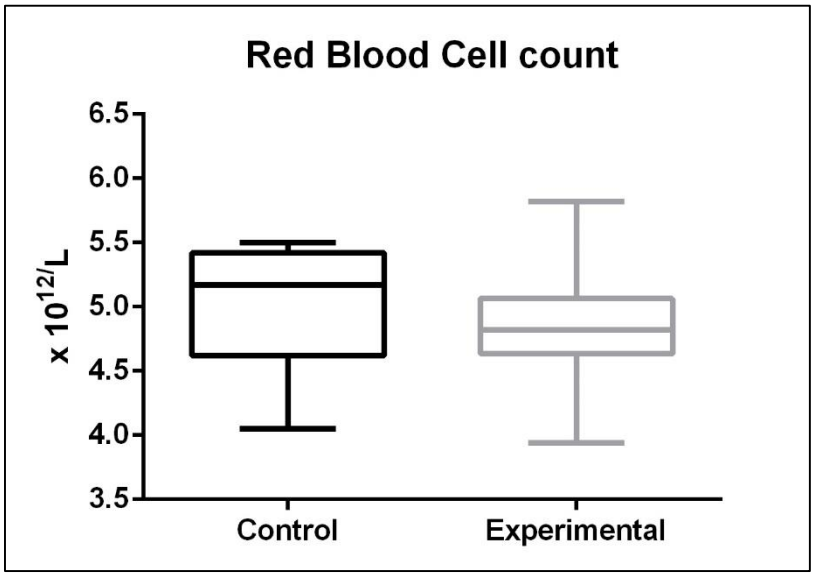


Figure 4.4.5. Box-and-whisker plot of the red blood cell counts.

Comparison between the control and T2DM groups.

Table 4.4.5. Statistical analysis of the red blood cell count.

	Mean	Median	Standard Deviation	P-value	(P < 0.05)
Control	5.02	5.17	0.48	0.3400	No
T2DM	4.85	4.82	0.53		

Analysis of the red blood cell count showed no statistically significant difference between the two groups, however, the diabetic group did have a slightly lower mean and median but a greater spread with both higher and lower values than the control group. Slightly lower red blood cell counts in the diabetic group can be a result of the abnormal ultrastructure of the red blood cell as can be seen in the results of chapter 6 of this study. Poor red blood cell quality may ultimately lead to a shorter lifespan of the cell. The slightly lower red blood cell count in the diabetic group can also be attributed to the chronic use of aspirin since it has an effect on erythropoiesis (98). Combined with the hypothesized shorter lifespan of red blood

cells, this will lead to a net loss of cells. A low red blood cell count (anaemia) is a typical secondary condition in diabetics and it may be ascribed to a multitude of factors (99). The fact that this was not observed in this study can be attributed to the small sample size but should be a point of future investigation.

4.4.6 Haemoglobin

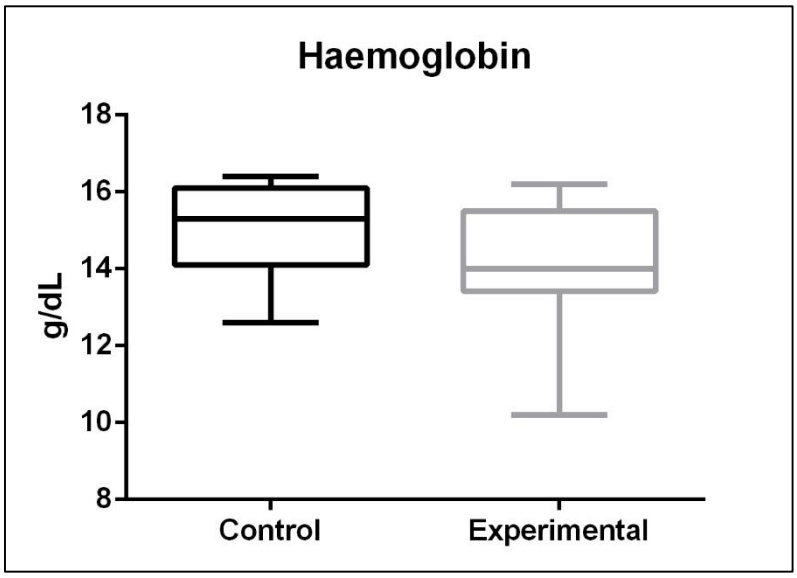


Figure 4.4.6. Box-and-whisker plot of the haemoglobin counts.

Comparison between the control and T2DM groups.

Table 4.4.6. Statistical analysis of the haemoglobin count.

	Mean	Median	Standard Deviation	P-value	(P < 0.05)
Control	15.07	15.30	1.21	0.1128	No
T2DM	13.96	14.00	1.83		

Results of the haemoglobin concentrations between the two groups showed no statistically significant difference. The spread for the diabetic group was more than that of the control group with a slightly lower mean and median. Again, this can be ascribed to the possible influence of aspirin use by the T2DM group that has an effect on haemoglobin synthesis in the bone marrow.

4.4.7 Haematocrit

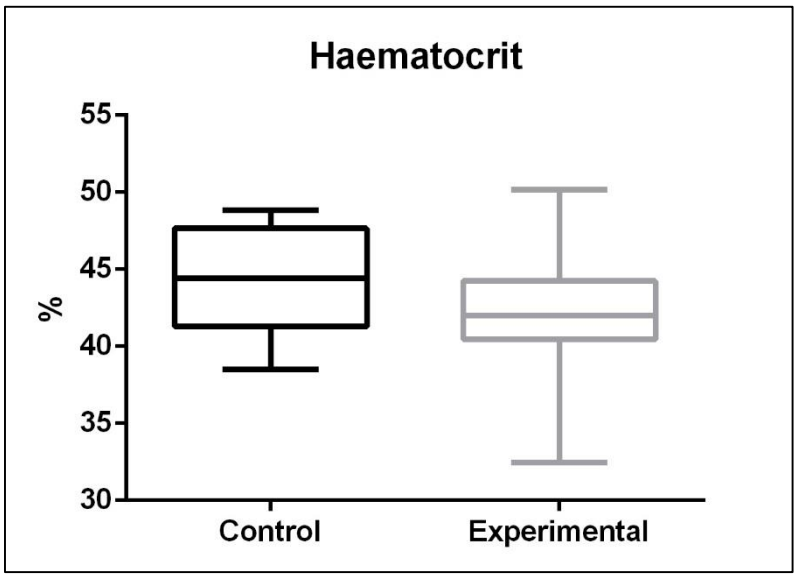


Figure 4.4.7. Box-and-whisker plot of haematocrit.

Comparison between the control and T2DM groups.

Table 4.4.7. Statistical analysis of haematocrit.

	Mean	Median	Standard Deviation	P-value	(P < 0.05)
Control	44.26	44.42	3.52	0.3413	No
T2DM	41.97	42.02	4.94		

Analysis of the results of haematocrit showed no statistically significant difference across the two groups, however, the diabetic group did have a greater spread than the control group. The effect of diabetes on haematocrit has previously been studied and it was found that haematocrit percentages returned to normal ranges after insulin levels have been adjusted (100).

4.4.8 Mean corpuscular volume

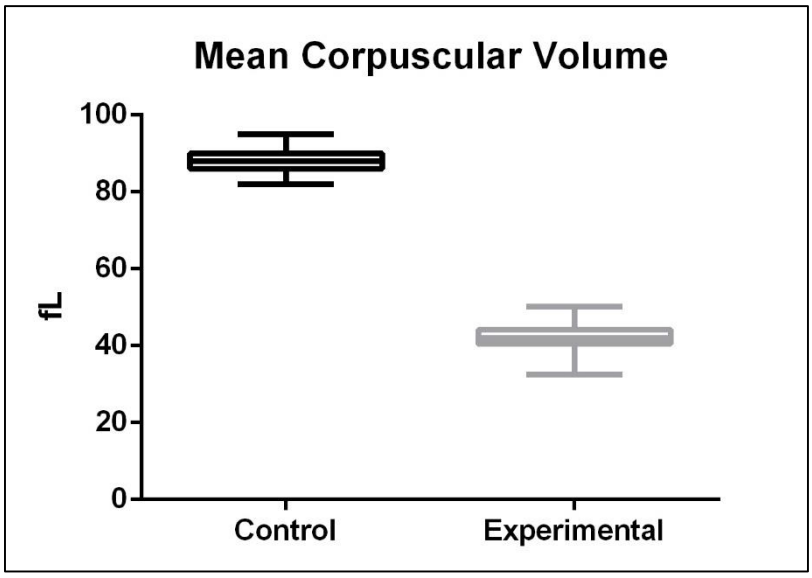


Figure 4.4.8. Box-and-whisker plot of the mean corpuscular volumes.

Comparison between the control and T2DM groups.

Table 4.4.8. Statistical analysis of the mean corpuscular volume.

	Mean	Median	Standard Deviation	P-value	(P < 0.05)
Control	88.27	88.00	3.61	<0.0001	Yes
T2DM	86.38	86.50	2.26		

Mean corpuscular volume refers to the average volume of red blood cells. Analysis of the mean corpuscular volumes of the two groups showed a statistically significant difference where the MCV for the diabetic group was much lower than that of the control group. The spread for both of the groups were similar in size, however, for the diabetic group, all of the values were significantly lower than that of the control group with a p-value of <0.0001. The MCV for the diabetic group is still considered to fall within normal ranges (80-100 fL), albeit on the low side (101). Although a low MCV can be used to diagnose iron deficiency anaemia, it can also be ascribed to chronic use of aspirin by participants from the T2DM group (102) (103).

4.4.9 Mean corpuscular haemoglobin

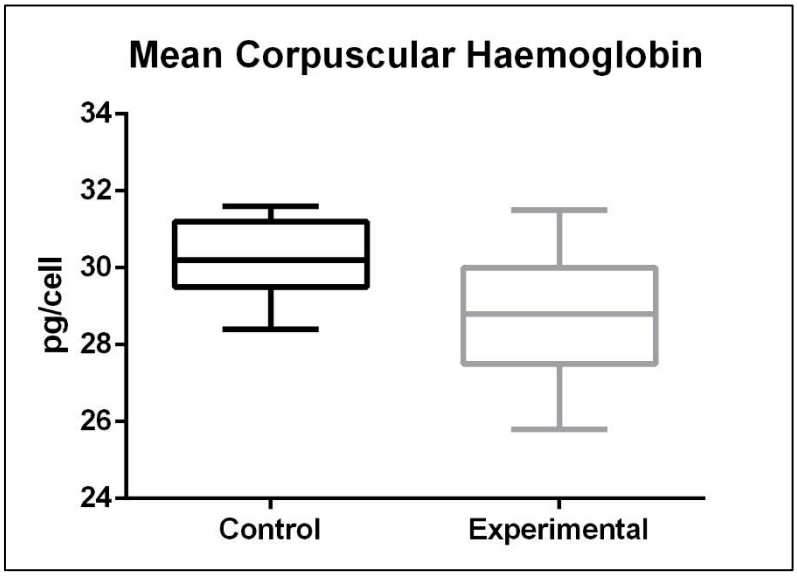


Figure 4.4.9 Box-and-whisker plot of the corpuscular haemoglobin.

Comparison between the control and T2DM groups.

Table 4.4.9. Statistical analysis of corpuscular haemoglobin.

	Mean	Median	Standard Deviation	P-value	(P < 0.05)
Control	30.09	30.20	1.09	0.0709	No
T2DM	28.71	28.80	1.75		

Results of the mean corpuscular haemoglobin showed no statistically significant difference between the two groups however, the diabetic group did have a lower mean, median and a greater spread than the control group. This was also seen in similar studies however; some studies reported a lower MCH value among diabetic individuals but causality was not confirmed (104).

4.4.10 Mean corpuscular haemoglobin concentration

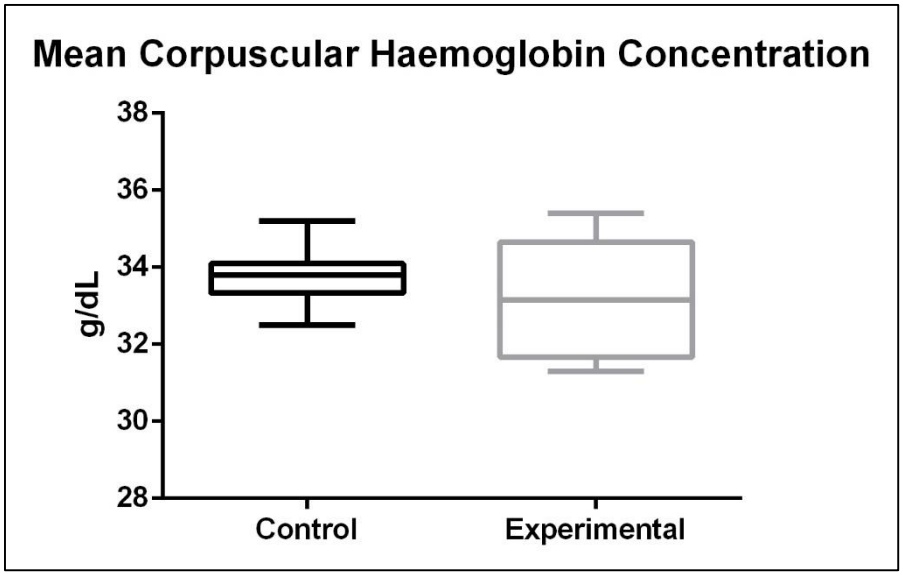


Figure 4.4.10. Box-and-whisker plot of the mean corpuscular haemoglobin concentration. Comparison between the control and T2DM groups.

Table 4.4.10. Statistical analysis of the mean corpuscular haemoglobin concentration.

	Mean	Median	Standard Deviation	P-value	(P < 0.05)
Control	33.75	33.80	0.76	0.4204	No
T2DM	33.19	33.15	1.54		

The analysis of the mean corpuscular haemoglobin concentration showed no statistically significant difference between the groups with the means and medians showing very similar results. The diabetic group did have a greater spread than that of the control group.

4.4.11 Platelet count

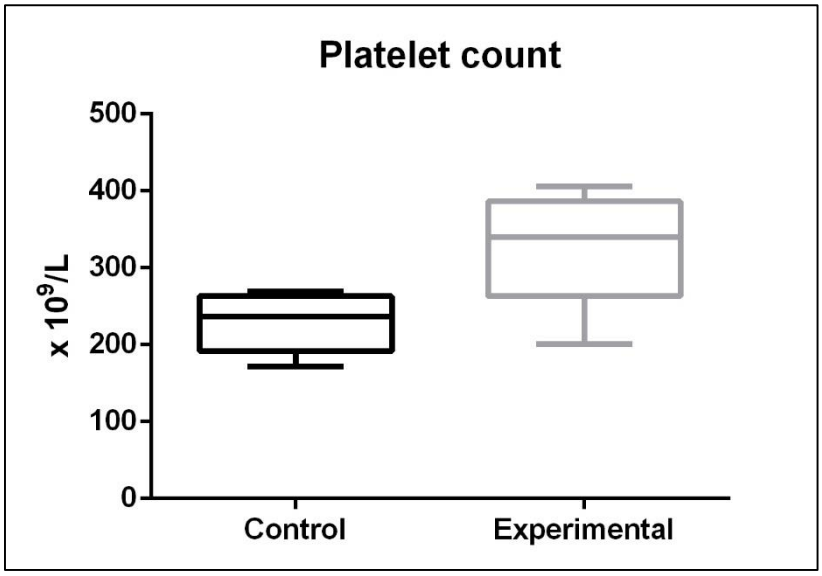


Figure 4.4.11. Box-and-whisker plot of the platelet count.

Comparison between the control and T2DM groups

Table 4.4.11. Statistical analysis of the platelet count.

	Mean	Median	Standard Deviation	P-value	(P < 0.05)
Control	228.91	237.00	36.84	0.0071	Yes
T2DM	284.13	310.00	98.29		

Results from the platelet count showed a statistically significant higher platelet count in the diabetic group as opposed to the control group with a p-value of 0.0071. This correlates to what was found in previous similar studies (105) (106) (107), and also reinforces the fact that participants from the diabetic group are in a state of chronic inflammation as well as a state of hypercoagulability.

4.5 Discussion

By reviewing the results obtained from the haematology analysis, it is clear that there were slight differences, albeit not statistically significant, between the control and diabetic groups. There was however, significant differences in mean corpuscular volume and in platelet count. The increased platelet count reinforces the fact that participants in the diabetic group are experiencing a constant state of inflammation as well as a state of hypercoagulability. For the other parameters, most values for the diabetic group were somewhat lower than that of the control group. This minor difference can be due to a multitude of reasons including the chronic use of aspirin, which has an effect on the growth of red- and white blood cells as well as the synthesis of haemoglobin (96). Consequently, a study needs to be done to confirm whether this is true for diabetics or not. Contrary to what was expected, the white blood cell count in the diabetic group was slightly lower than that of the T2DM group. In a state of inflammation, one would expect the white blood cell population to increase in order to initiate the inflammatory process, since it is considered to be a clinical marker of inflammation (108) (109). This was not seen in this study even though inflammation was detected. Alternative explanations in 4.5.1 are merely speculations and needs to be confirmed first. Even though it is not detrimental to this particular study, the minor difference in white blood cell count between the two groups needs to be revisited.

4.6 Conclusion

Of all the parameters measured, only the results of the MCV and platelet count had a statistically significant difference. MCV from the T2DM group were significantly lower and platelet count from the T2DM group were significantly higher than that of the control group. A low MCV indicates microcytic anaemia however, the reason for a low MCV cannot be narrowed down by result from haematology analysis alone. The significantly higher platelet count of the T2DM group reinforces the fact that participants from this group are in a state of inflammation. The increased platelet count may cause or exacerbate an already hypercoagulable state. Contrary to what was expected, there was no statistically significant difference in WBC count between the two groups. A high WBC count is normally expected in an inflammatory state however, this can be ascribed to the small sample size as well as the chronic use of aspirin among participants in the T2DM group.

CHAPTER 5: ERYTHROCYTE SEDIMENTATION RATE

5.1 Chapter objectives

The objective of this chapter was to measure the Erythrocyte Sedimentation Rate (ESR) as a marker of inflammation in both healthy volunteers and individuals with T2DM.

5.2 Introduction

ESR was used as non-specific marker for inflammation. A C-reactive protein (CRP) assay was considered since some sources argue that ESR is an outdated method. CRP is an acute phase protein produced by the liver during inflammation and would be elevated during the initial state of inflammation. A CRP assay is capable of testing the extent of inflammation whereas ESR is a non-specific test for inflammation, meaning that it tests for the presence of inflammation but not the severity or cause of it. Since the aim was not to test for the level of inflammation but rather only the presence of inflammation, ESR was the method of choice. ESR measures the rate at which red blood cells sediment in mm per hour. During inflammation, among others, plasma fibrinogen and pro-sedimentation factor is activated which cause RBC's to adhere to each other to form large aggregates known as 'rouleaux'. Rouleaux formation causes the red blood cells to settle faster, ultimately causing an increase in the ESR value. Bigger clots mean that the blood will settle at a faster rate than blood without activated fibrinogen (110) (111).

5.3 Materials and methods

For the test, a citrate tube, filled with whole blood, was inverted 5 times to ensure proper mixing of the contents, after which the cap was taken off and an ESR pipette was inserted until the blood reached the stopper at the top of the tube. The citrate tube with ESR pipette was then placed upright in an ESR tray for one hour after which a reading was done. Blood will settle in the ESR pipette, leaving plasma behind. The point that the blood reached after one hour is measured from the starting point (the stopper at the top). The normal ESR ranges that were used to compare results from this study is displayed in table 5.3.1 below (112):

Table 5.3.1. Normal ESR values according to age and sex (112).

Age	Male	Female
0-50	<15 mm/h	<20 mm/h
51-85	<20 mm/h	<30 mm/h
>85	<30 mm/h	<42 mm/h

5.4 Results

The parameter means from ESR will be described using the Mann-Whitney *U* test in GraphPad Prism 7® for Windows. All test results were evaluated at a 5% level of significance.

5.4.1 Erythrocyte Sedimentation Rate

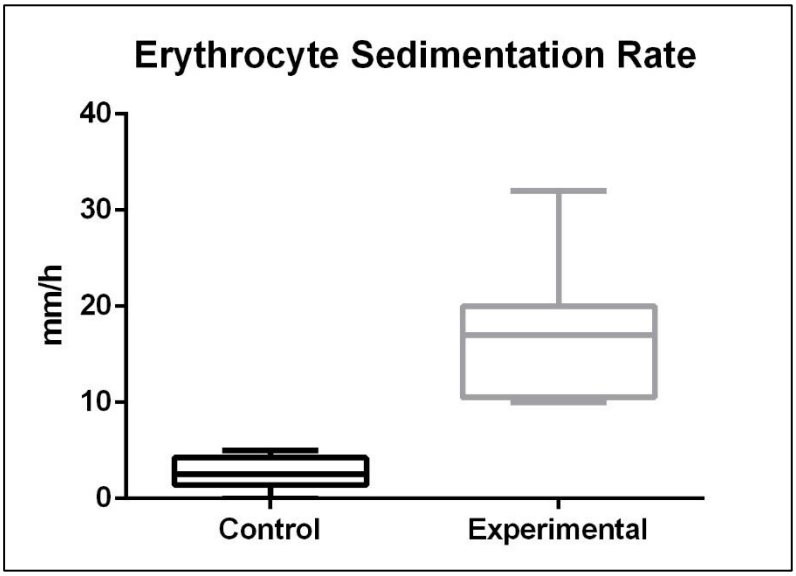


Figure 5.4.1 Box and whisker plot of the ESR results.

Table 5.4.1. Statistical analysis of results from the ESR test.

	Mean	Median	Standard Deviation	P-value	Statistically significant? (P < 0.05)
Control	2.61	2.50	1.71	<0.0001	Yes
T2DM	15.21	13.50	6.08		

The ESR measurements obtained in this study clearly shows a significant difference between the control and diabetic groups. All results from the control group are closely grouped

together around the range considered to be normal as can be seen in table 5.3.1. From the box plot and the low standard deviation for the control group, it is evident that ESR values have a very low spread, indicating consistency among this group. In contrast to the control group, the diabetic group had much higher values which are, according to table 5.3.1, above the normal range. Looking at the statistical analysis for the diabetic group, it is evident, by the high standard deviation, that there is a much higher spread and consequently, less consistency in this group. Statistical analysis also shows a very low p-value, indicating a very significant difference between the two groups.

5.5 Discussion

Analysis of the ESR results shows a statistically significant difference between the control and diabetic groups. This can be attributed to activated fibrinogen and pro-sedimentation factor which will cause blood to clot faster. The increased ESR values in the diabetic group, which all exceed the normal range, is indicative of the presence of inflammation in this group. This correlates with findings from past studies where ESR was also significantly increased among diabetics (113) (114) (115). The increase in ESR in the diabetic group can be ascribed to the increased ability of RBC agglutination as well as the decrease in negative membrane surface electric charge of RBC's (12) (13).

5.6 Conclusion

The statistically significant higher ESR measurement of participants from the T2DM group is an indication of the presence of inflammation in this group.

CHAPTER 6: SCANNING ELECTRON MICROSCOPY

6.1 Chapter objectives

In this chapter the biophysical ultrastructural changes of erythrocytes of poorly controlled T2DM individuals will be investigated and compared to those of healthy individuals using scanning electron microscopy.

6.2 Introduction

Scanning electron microscopy (SEM) makes use of a scanning electron microscope that produces images of a sample by scanning its surface with a concentrated beam of electrons. The sample is coated with a thin layer of conductive material (in this case we are using carbon) that is able to interact with the electrons in order for it to produce various signals that can be picked up by a detector that, in turn, will create an image based on the signals. SEM is very useful in investigating a sample's surface morphology. In this case we will be looking at the surface morphology of red blood cells and the changes associated with T2DM. SEM is used to view individual cells at very high magnifications which allows us to not only see the overall morphology of the cell but also a detailed image of the membrane surface and cell shape. Results from the SEM will give a better idea of how red blood cells are affected in individuals with poorly controlled T2DM and how their function is influenced.

6.3 Materials and Methods

Samples for SEM were collected from the two groups as discussed in chapter 3. For SEM, it is necessary to obtain whole blood samples in citrated tubes. Preparation for the microscope slides were all done *ex-vivo* and no coagulation agents were added to the samples.

The following reagents were used for SEM preparation:

- Hexamethyldisilazane (HMDS) from Sigma (440191-1L)
- Formaldehyde from Sigma (F8775-500ml)
- 1% Osmium tetroxide (OsO_4) from Sigma (75632-10ml)
- Ethanol from Sigma (24102-4x2.5L)

- Phosphate buffered saline from Sigma (P5493-1L) (When diluted to a 1X concentration, this product will yield a phosphate buffered saline solution with a phosphate buffer concentration of 0.01M and a sodium chloride concentration of 0.154M. The solution pH will be 7.4.)

All citrate tubes had to stand for at least 30 minutes after sample collection to allow for complete binding with calcium in tubes. Tubes were kept at room temperature and all preparation were done within 4 hours after sample collection.

10 µL of the sample was pipetted onto a 10 mm round cover slip and spread out with a bent pipette tip in order for the blood to cover the whole slide. It was left to dry at room temperature for 2-3 minutes until an outer ring of a quarter of the radius was dry. See figure 5.3.1 below:

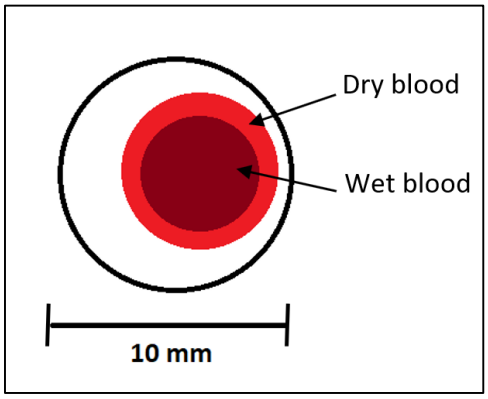


Figure 5.3.1. Drying of blood on microscope slide.
The amount of blood that needs to be dry before the microscope plate is submerged in 0.075 M PBS.

Cover slips were then transferred with a pair of tweezers to a labelled 24-well plate and submerged in 0.075 M PBS for 15 minutes after which the solution was discarded. Four percent formaldehyde was then added to each of the samples, just enough that the fixative covered them completely. Samples were then fixed for a minimum of 30 minutes after which the fixative was discarded. Samples were then washed three times with the PBS solution for three minutes for each wash. The PBS solution from the last wash was then discarded in a fume cupboard after which 1 drop of osmium tetra-oxide (OsO_4) was added to each sample. Sufficient distilled water was then added to each of the wells in order for the samples to be submerged in the solution. Samples were left in this solution and kept in the fume cupboard for 15 minutes after which the solution was discarded in an OsO_4 waste container. Samples

were then washed 3 times with PBS solution for 3 minutes for each wash. Waste from the first to washes were discarded in the OsO_4 container. Samples were then taken out of the fume cupboard. Waste from the last wash was discarded into a waste beaker. After the third wash, samples went through an ethanol series dehydration. Samples were washed in 30%, 50%, 70%, 90%, 100%, 100% and 100% for three minutes for each wash. Ethanol from the third wash was discarded in the fume cupboard after which samples were submerged in Hexamethyldisilazane (HMDS) for 30 minutes. After 30 minutes, HMDS was discarded in an HMDS waste container. One drop of HMDS was added on top of each of the slides. Cover slips were then lifted up from the 24-well plate with a pair of tweezers for approximately 15 seconds each to allow the bottom of the cover slip to dry, preventing it from sticking to the 24-well plate. The 24-well plate containing all of the samples was left overnight in the fume cupboard to allow for complete evaporation of HMDS.

In order for samples to be mounted, a strip of double-sided carbon tape was stuck to a labelled steel plate after which samples were lifted out of the 24-well plate with a pair of tweezers and stuck gently to the carbon tape, each sample according to its label on the steel plate. Once all cover slips were mounted, they were carbon coated 3 times in a carbon coater. For the first coat, samples were not tilted so that evaporated carbon could coat the samples at a 90° angle. For the second and third coats, samples were tilted to the left and to the right in such a fashion that evaporated carbon would coat the samples at an angle of approximately 120°. Coated samples were stored in labelled containers in the microscopy laboratory of the University of Pretoria on the Hatfield campus until an appointment was made so that they could be viewed.

A Zeiss Crossbeam 540 FEG-SEM was used to view the samples. Samples were viewed at an accelerating voltage of 1 kV using the in-lens detector. Micrographs of a population of red blood cells were taken to give an idea of the overall shape of the cells in the particular sample. Single cells were then zoomed in on, to look at the finer ultrastructural properties. All samples were inserted into the microscope by a laboratory technician from the microscopy laboratory.

6.4 Results

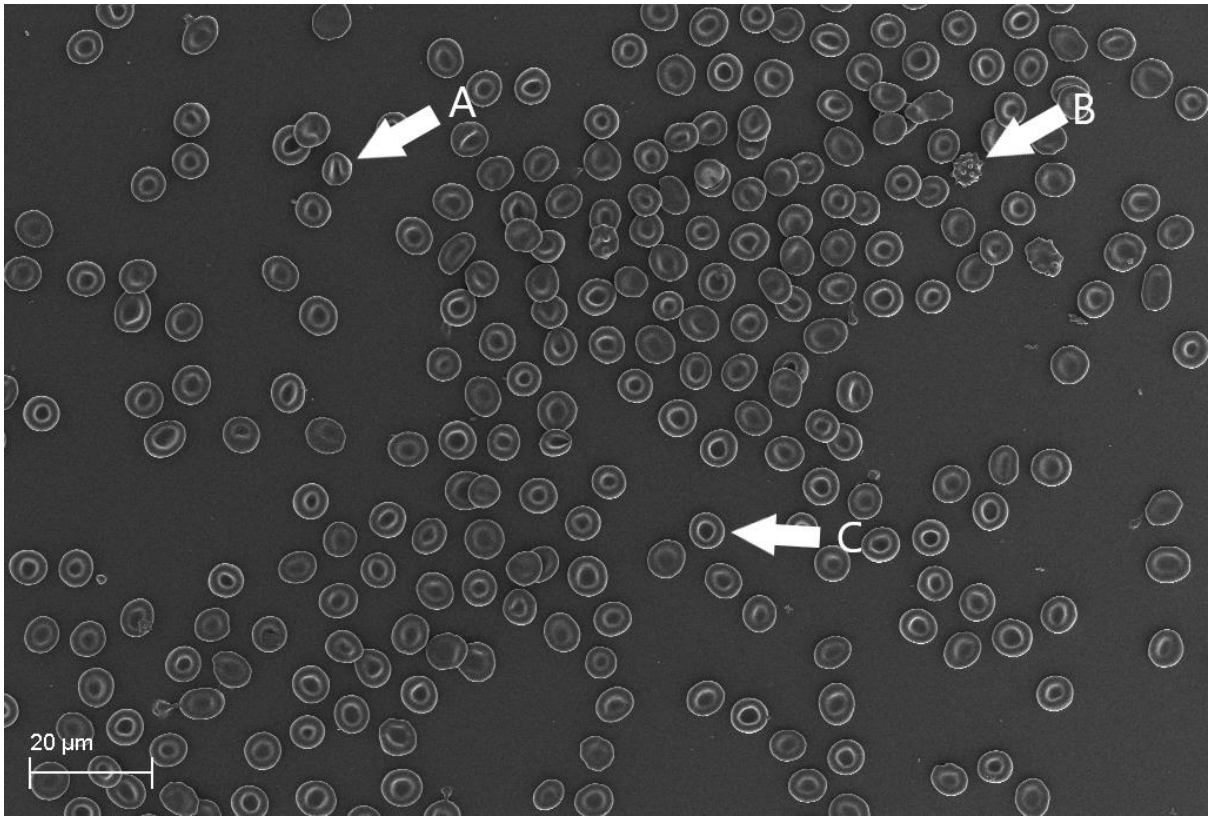


Figure 6.4.1. RBC population from the healthy control group. This micrograph depicts a healthy RBC population. A: Very few RBC's have an irregular shape, B: some are undergoing eryptosis, C: whereas most of the RBC's are healthy with a biconcave shape.

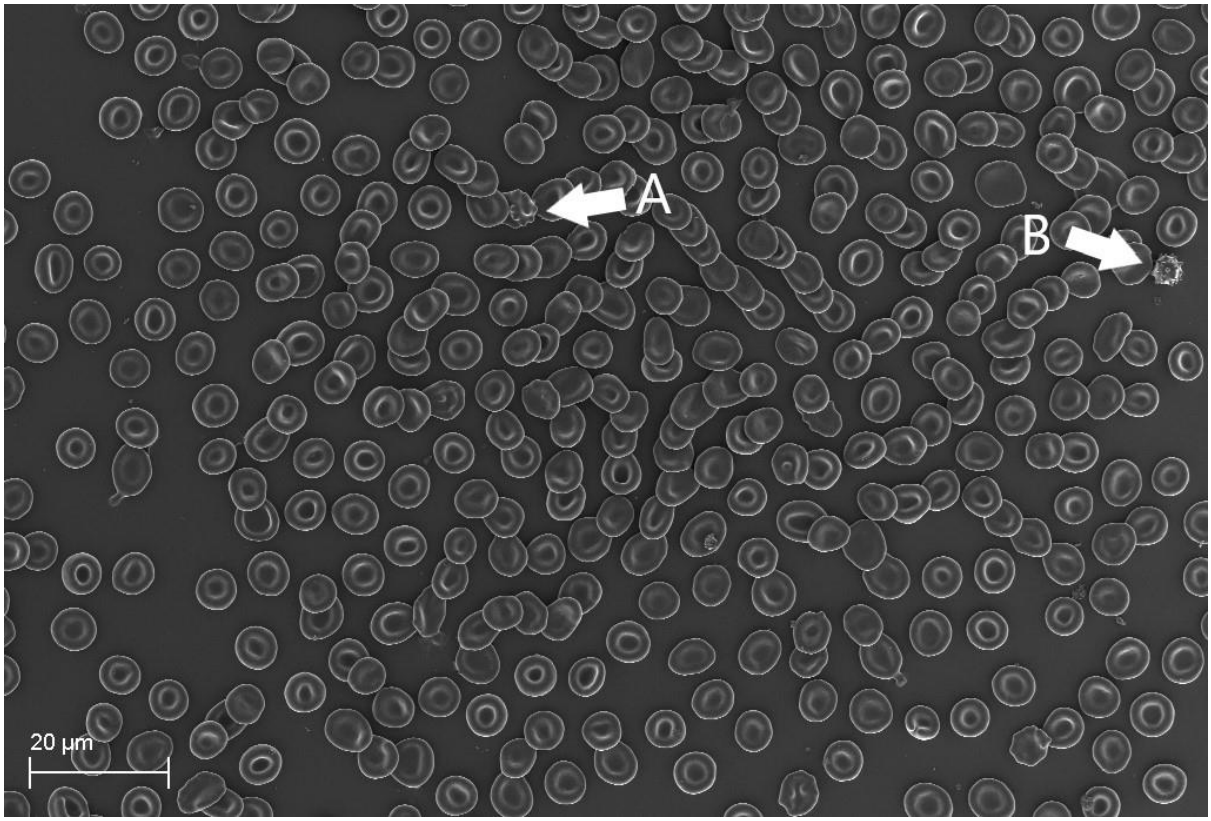


Figure 6.4.2. RBC population from the healthy control group (2). Most of the cells are healthy with a biconcave shape and very few eryptotic cells can be observed (arrows A and B)**.**

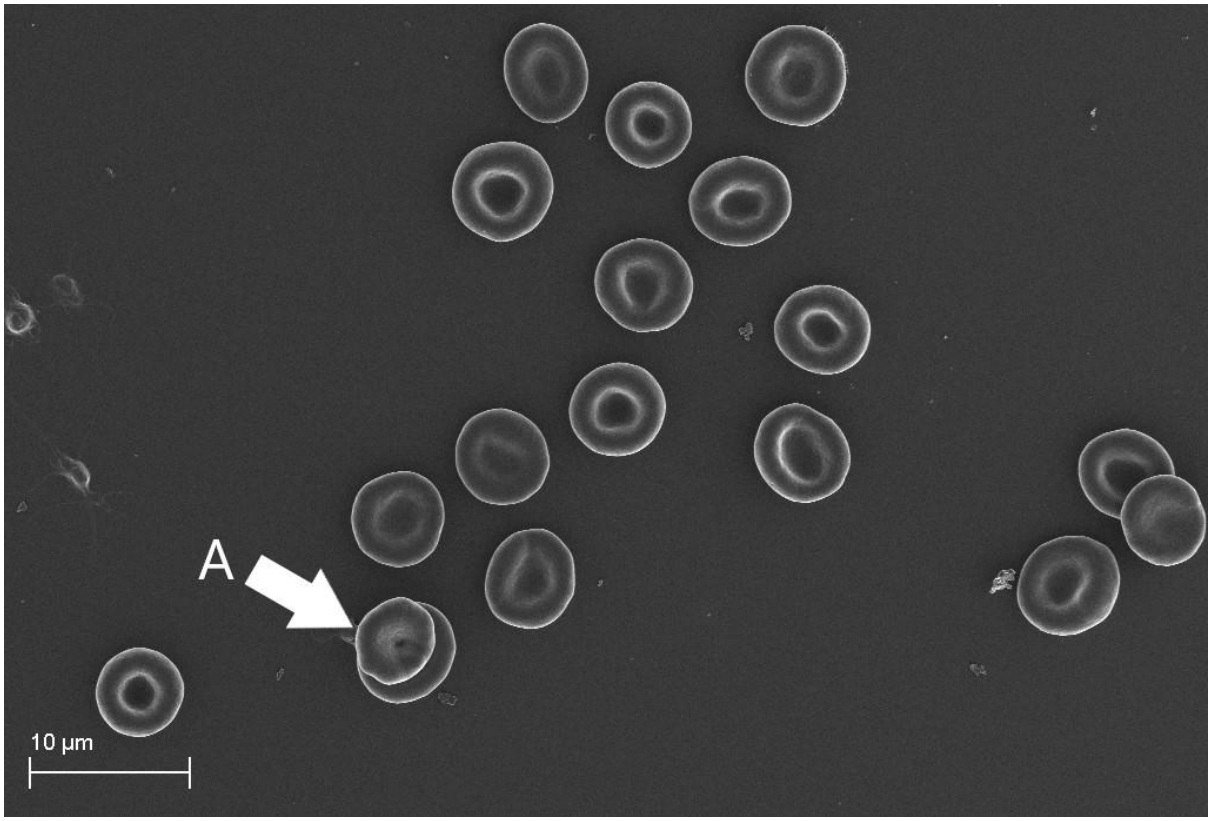


Figure 6.4.3. RBC population from the healthy control group at higher magnification (3). Another RBC population at a higher magnification, also from the control group. There are very few irregular cells and majority of the cells have a healthy biconcave shape.

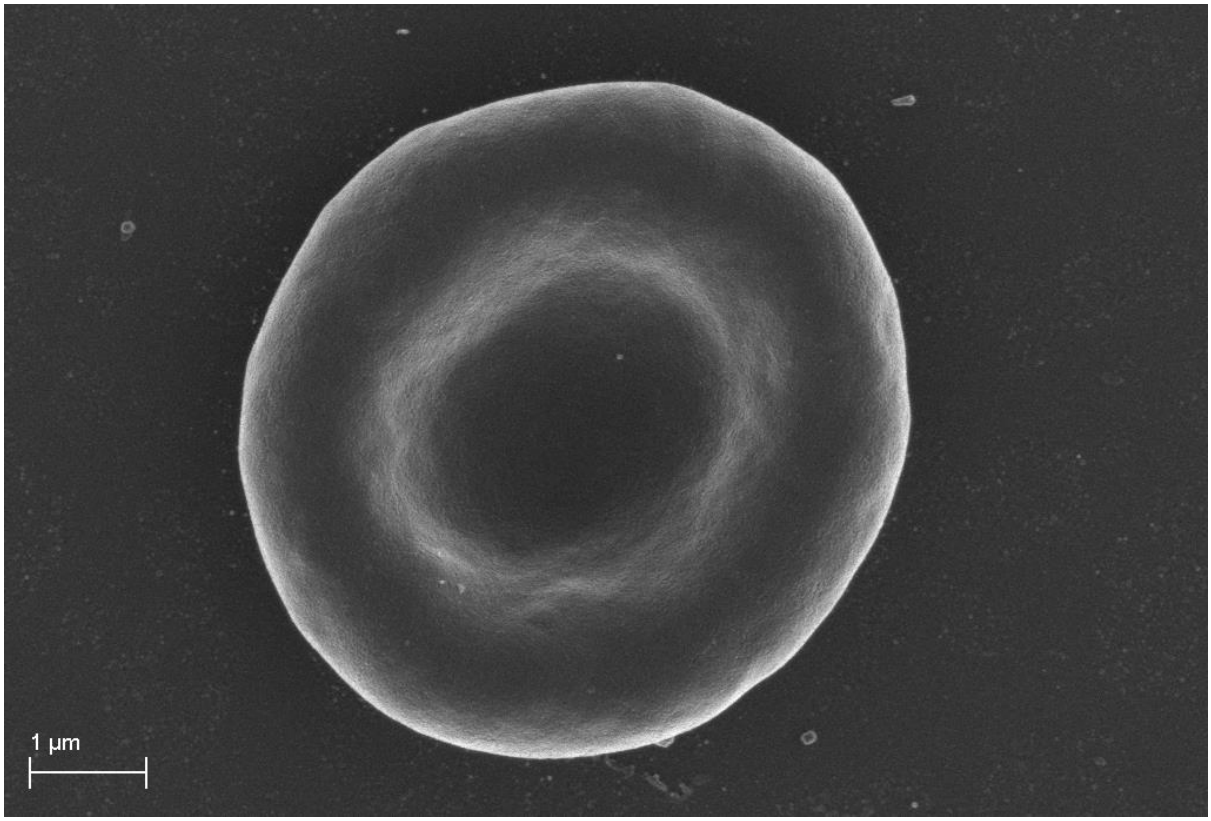


Figure 6.4.4. A single RBC from the healthy control group. A 30 000x machine magnification of a single RBC of a control. The smooth outer membrane and biconcave, round shape indicates the overall health status of the cell.

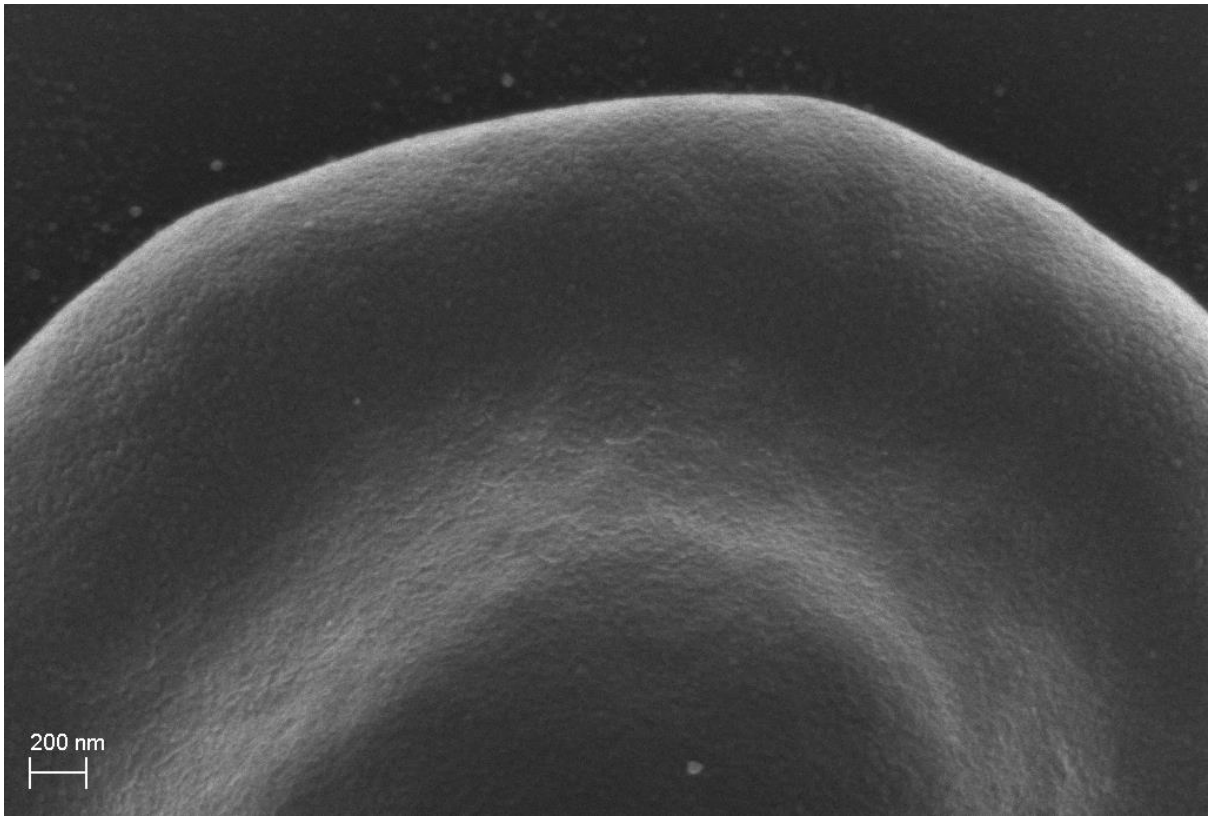


Figure 6.4.5. Higher magnification of single RBC from the healthy control group. An 80 000x machine magnification of the same RBC as above shows no signs of irregularities on the cell membrane of a control

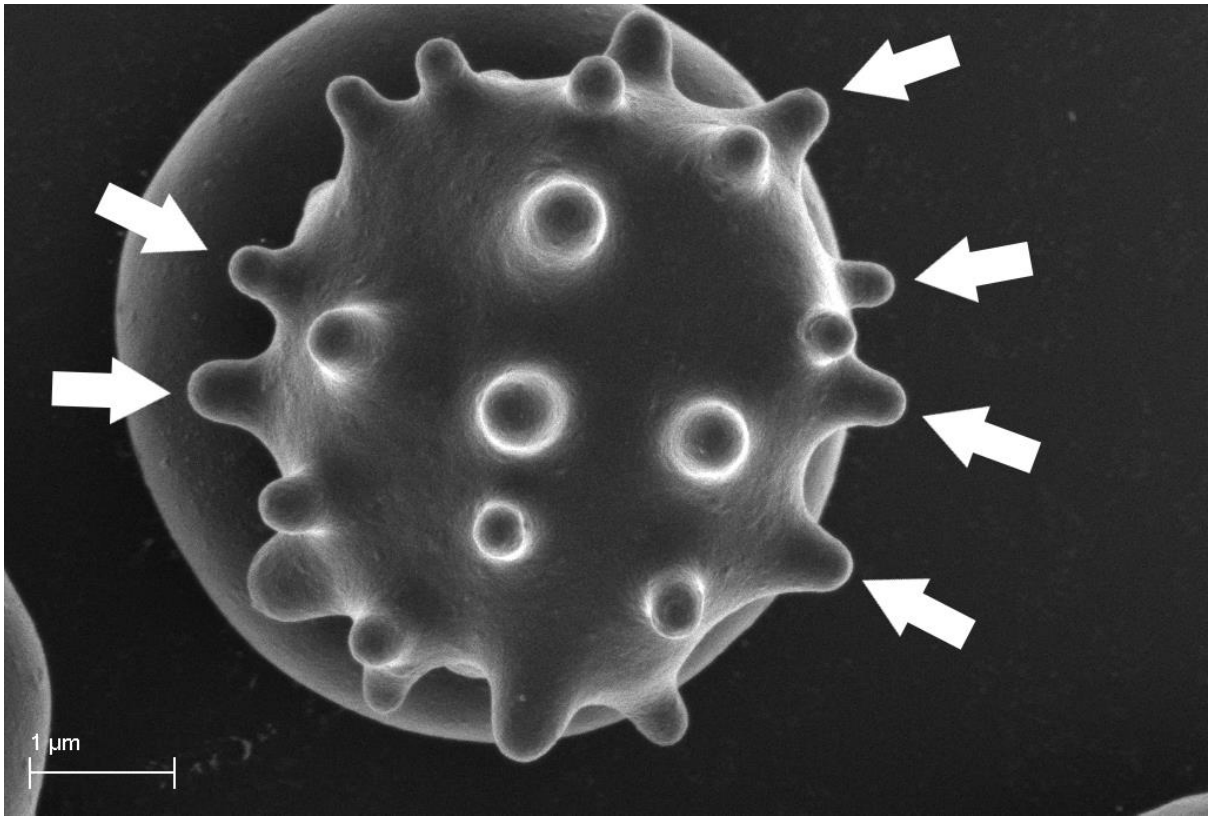


Figure 6.4.6. An eryptotic RBC from the healthy control group. Since the lifespan of an RBC is limited to approximately 120 days, they all have to undergo eryptosis, the process of programmed RBC death. Eryptotic cells are easily identifiable by the starting stages of RBC defragmentation or cell 'blebbing' (indicated by the white arrows). In healthy eryptosis, the cell membrane shows little to no sign of irregularity as can be seen below.

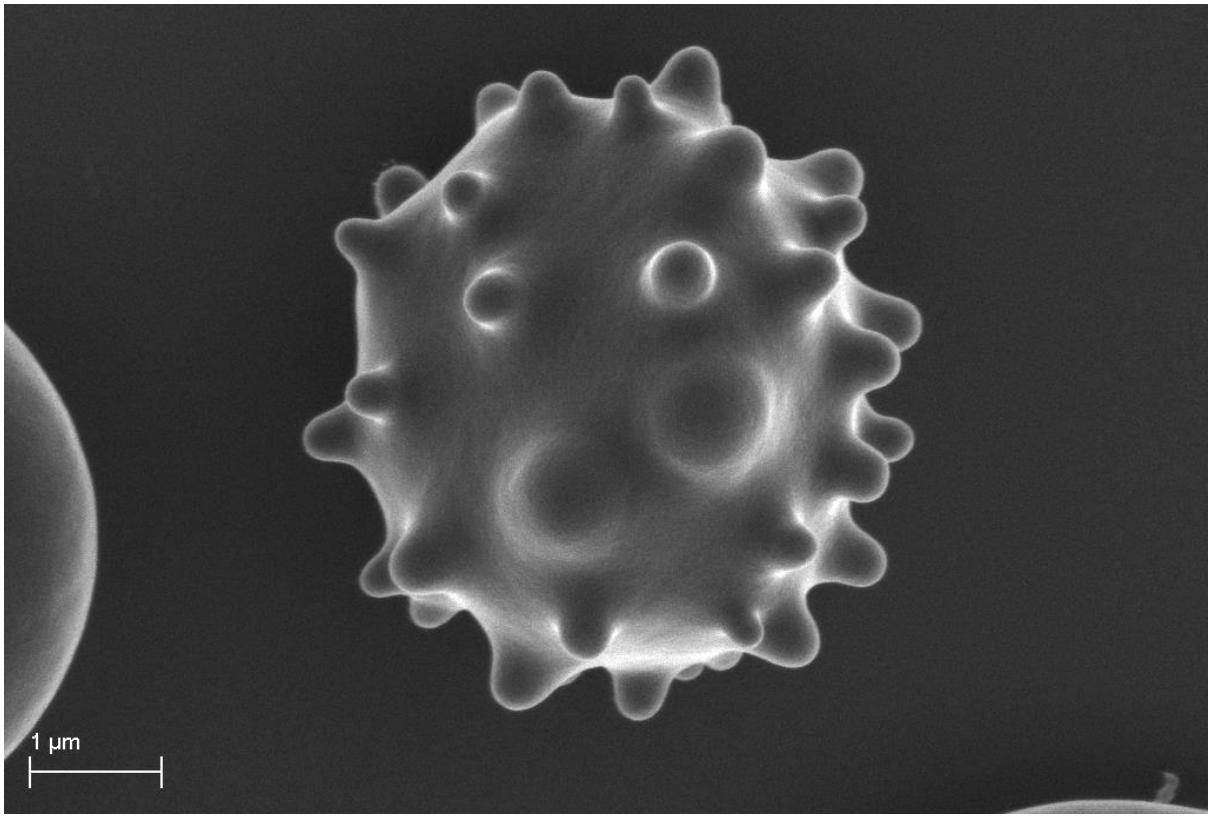


Figure 6.4.7. Eryptotic RBC from the healthy control group (2). Another healthy eryptotic RBC. The same characteristics can be observed here as in figure 6.4.6 above.

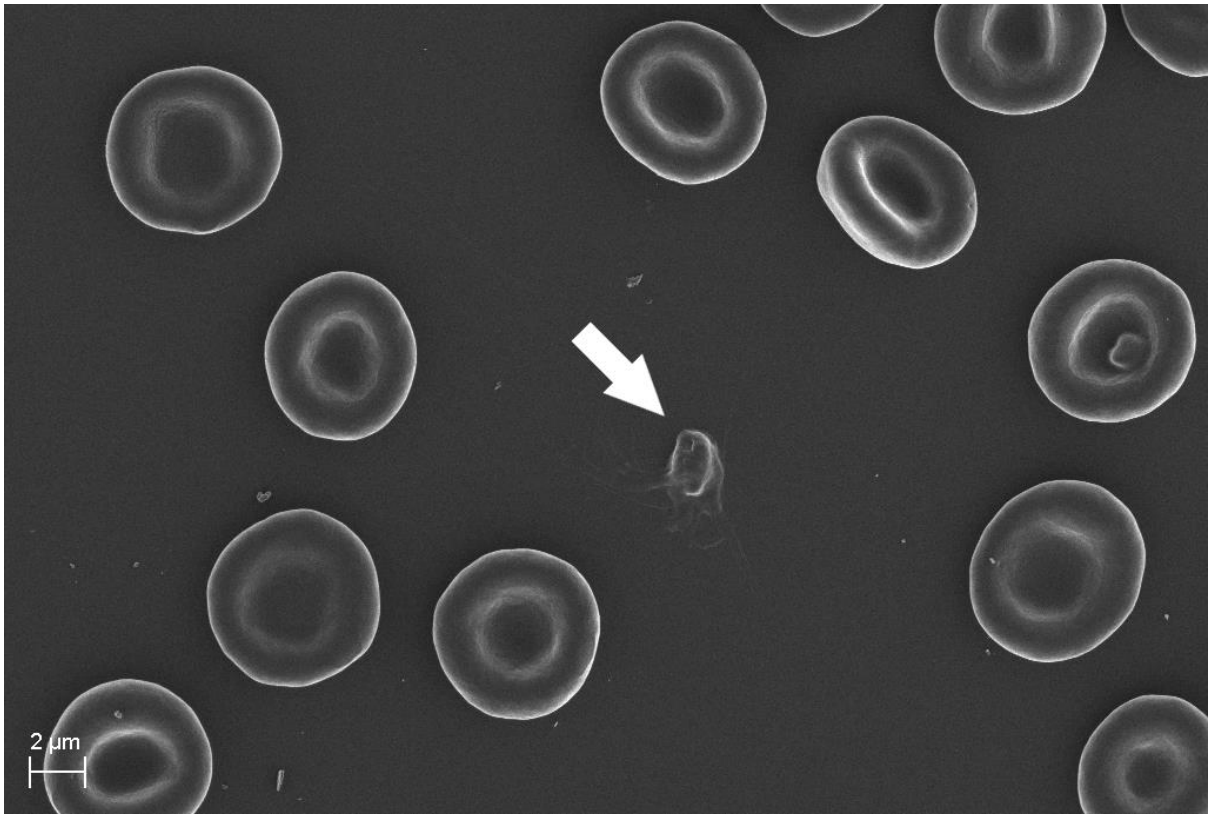


Figure 6.4.8. A platelet from the healthy control group. A healthy platelet (indicated by the white arrow) from the control group surrounded by healthy RBC's.

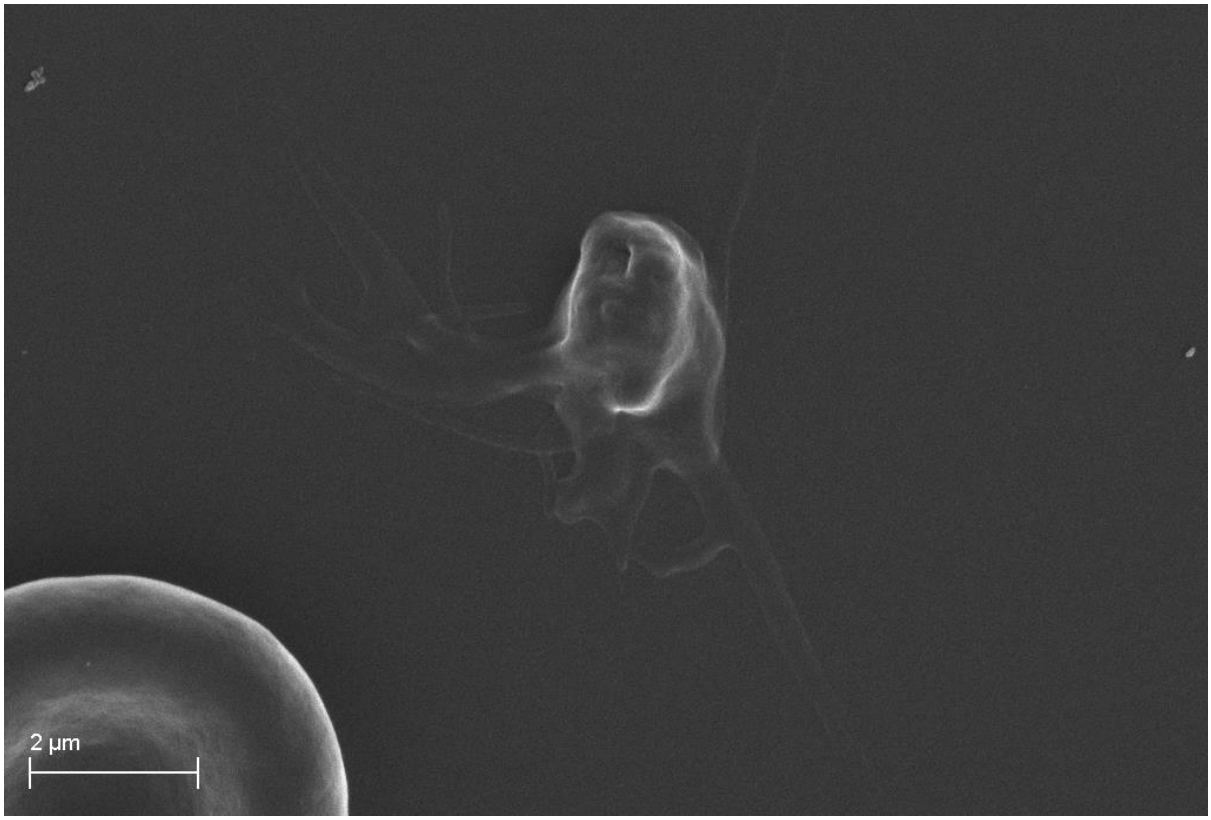


Figure 6.4.9 Platelet from the control group (2). A higher magnification of the platelet in 6.4.8 shows an overall smooth surface with only a few pseudopodia attached to the microscope slide.

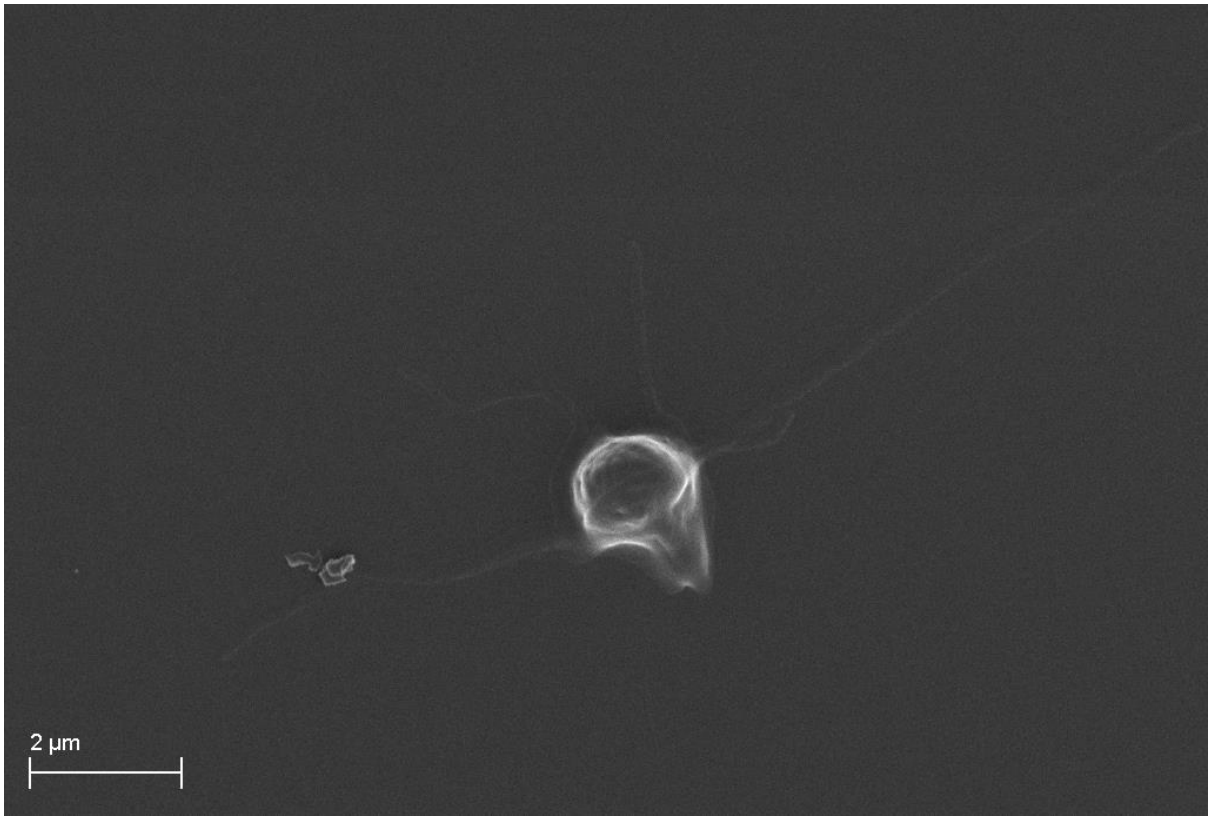


Figure 6.4.10. A platelet from the control group (3). A single healthy platelet from the control group. Pseudopodia extend from the centre of the platelet but are barely visible.

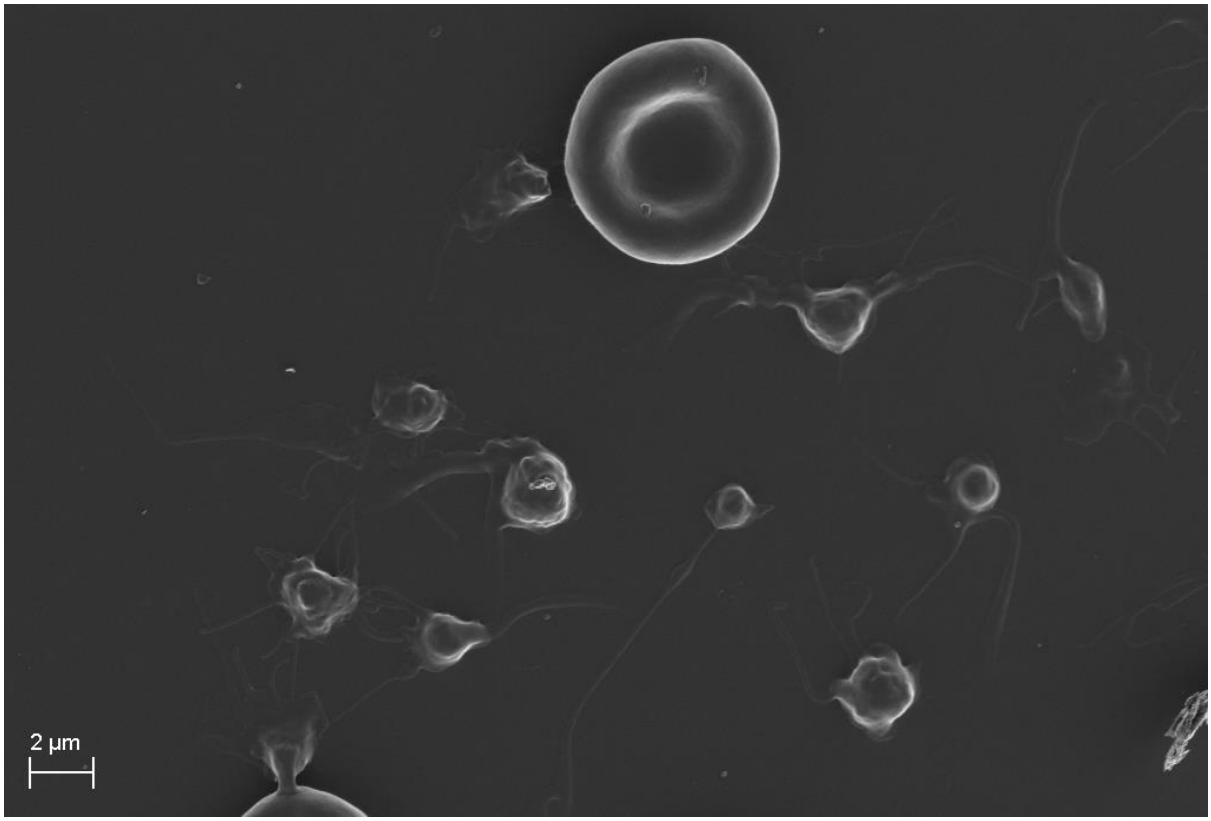


Figure 6.4.11. A group of platelets from the control group. Similar to figures 6.4.8-10, the centre of the platelet is clearly visible but pseudopodia that extend from it are not as clear.

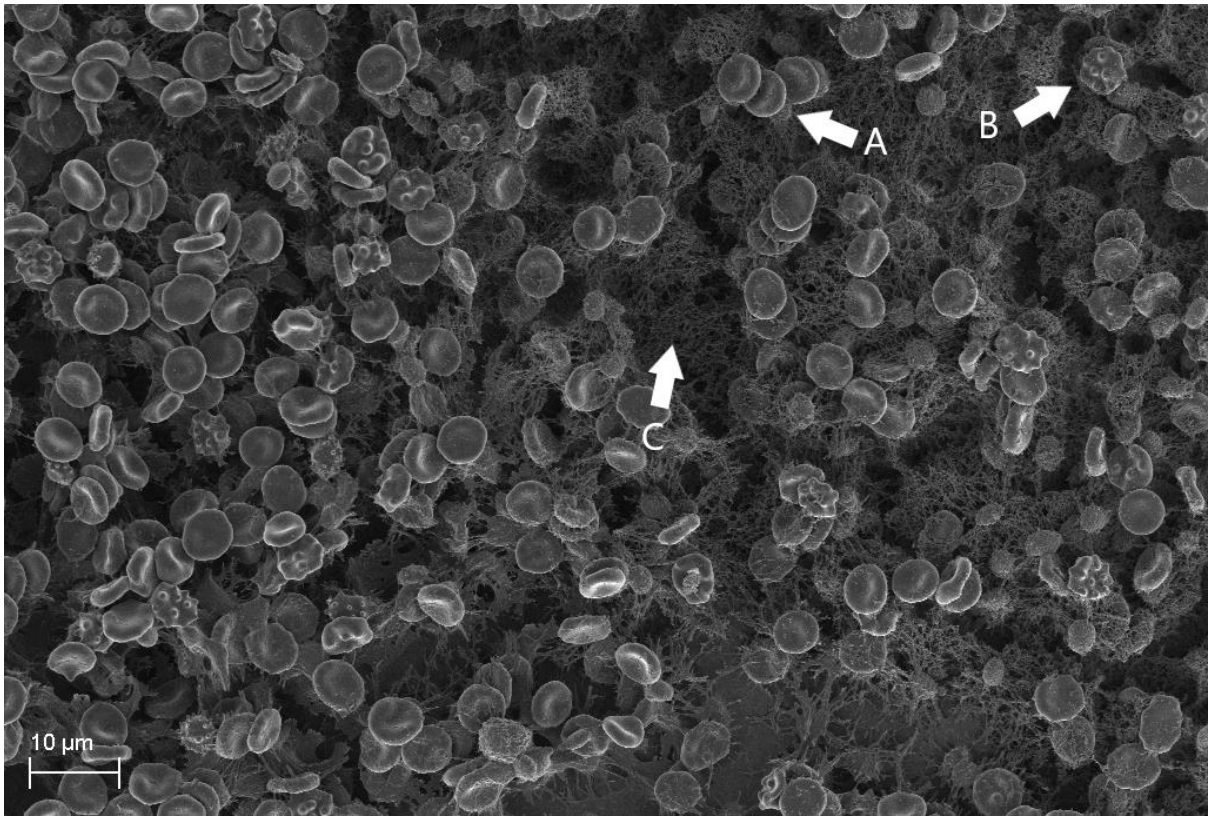


Figure 6.4.12. Tightly packed blood clot from the poorly controlled T2DM group.

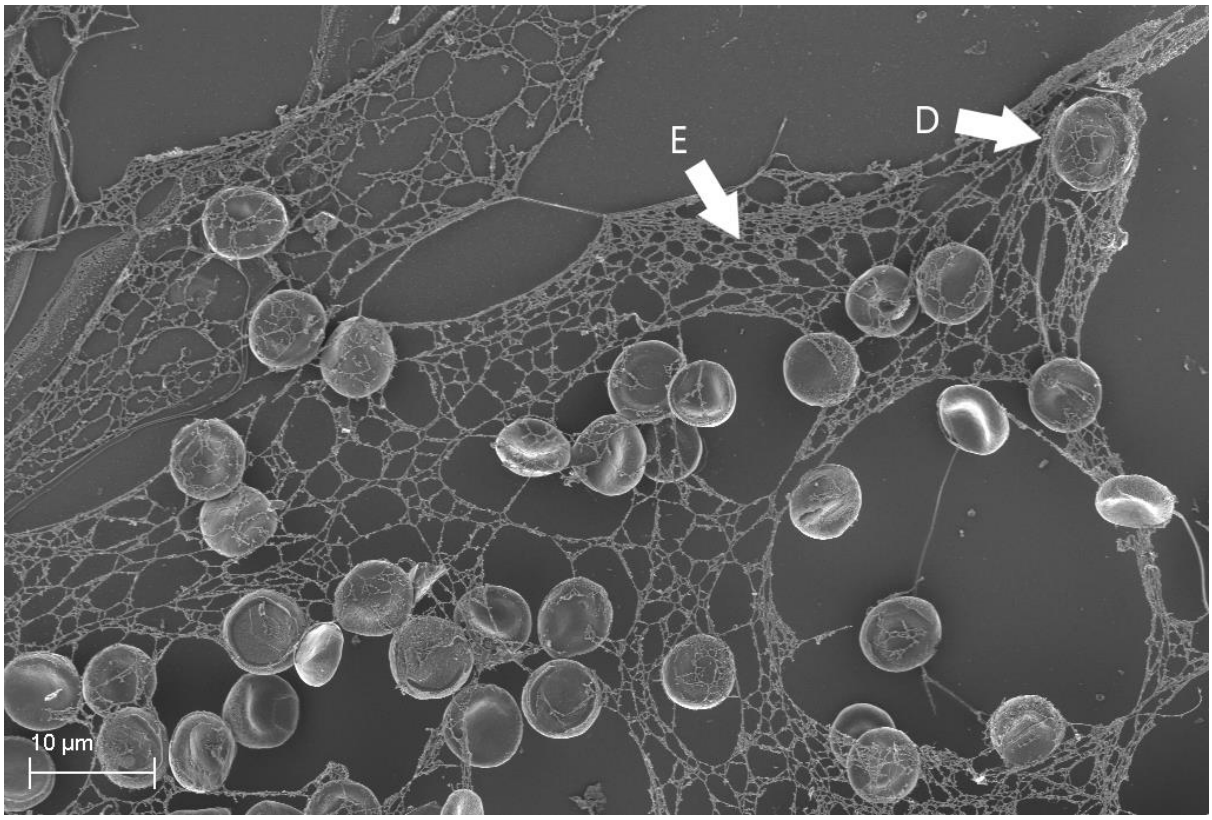


Figure 6.4.13. Loosely packed blood clot from the poorly controlled T2DM group.

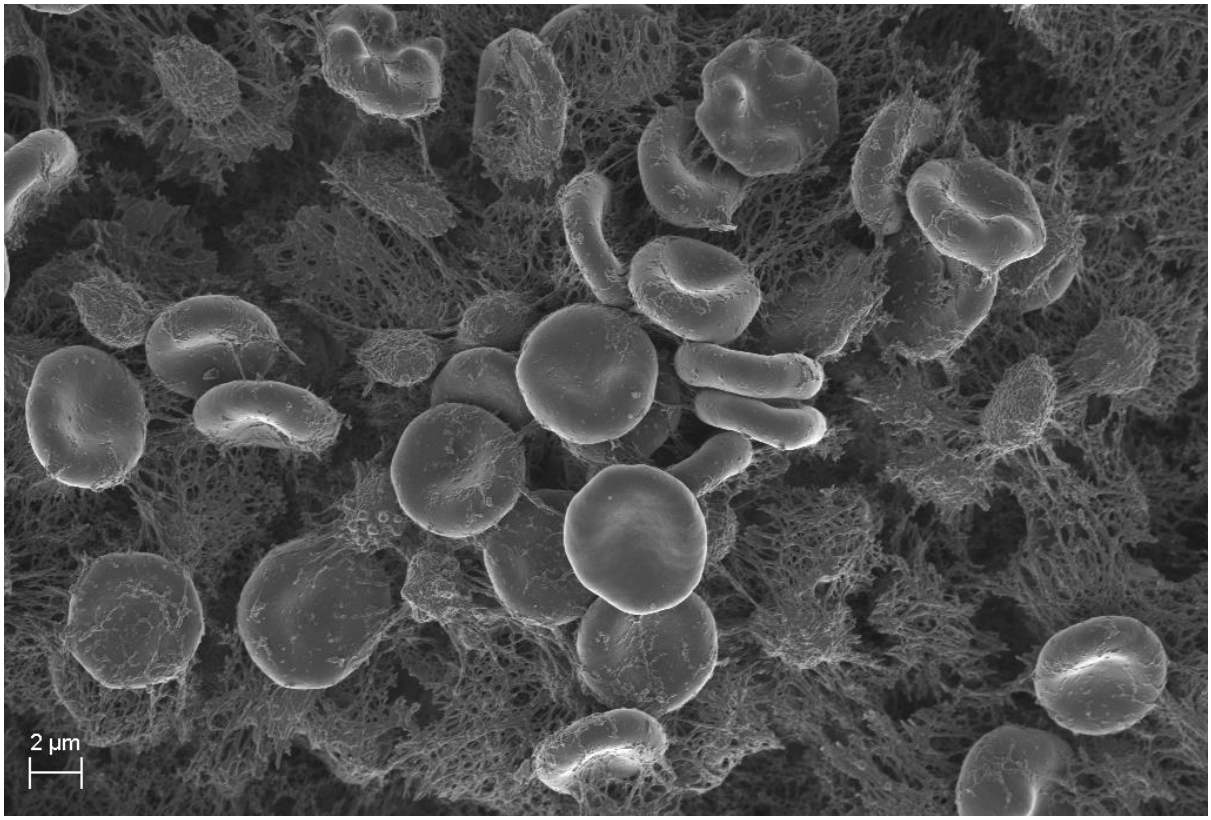


Figure 6.4.14. Tightly packed clot from the poorly controlled T2DM group (2).

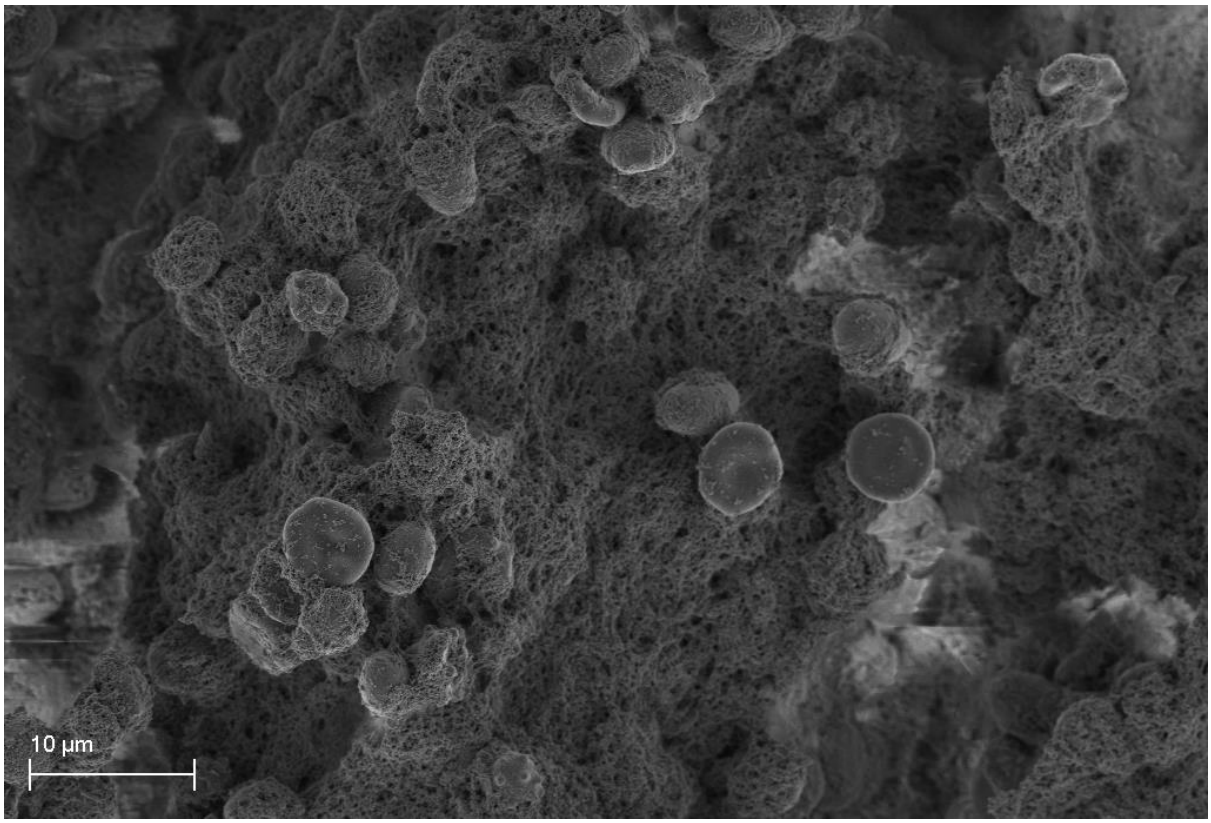


Figure 6.4.15. Tightly packed clot from the poorly controlled T2DM group (3).

Figures 6.4.12, 6.4.13, 6.4.14 and 6.4.15. Micrographs from the T2DM group showed 2 types of clotting. The first type, tightly packed clots in figures 6.4.12, 6.4.14 and 6.4.15, were observed in all but two of the diabetic individuals. Tight clots contained A: normally shaped RBC's, B: eryptotic cells and were held together by C: a tight fibrin network. The second type of clotting, 'loosely' packed clotting in figure 6.4.13, were seen in only a two of the participants. Here the D: RBC's were entangled in E: looser fibrin network that looks distinctively different to the tightly packed clot.

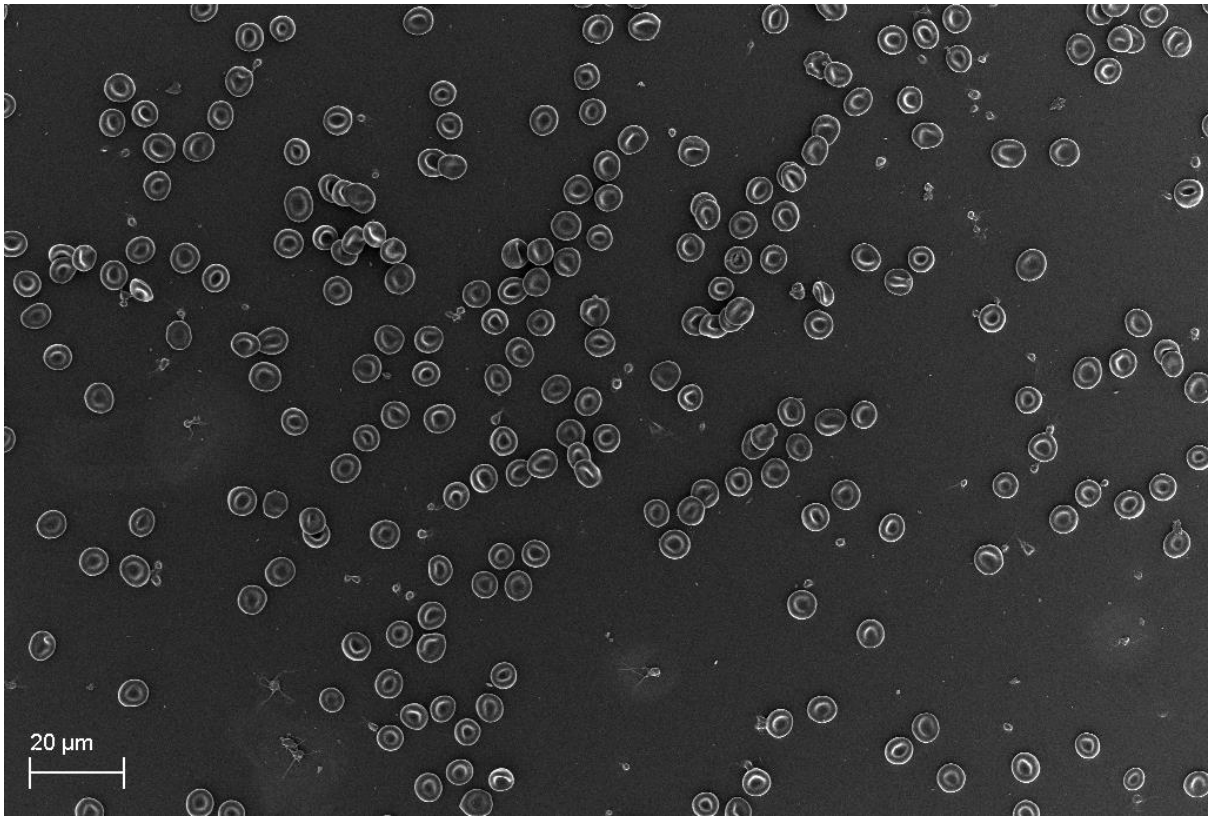


Figure 6.4.16. RBC population from the from the poorly controlled T2DM group.

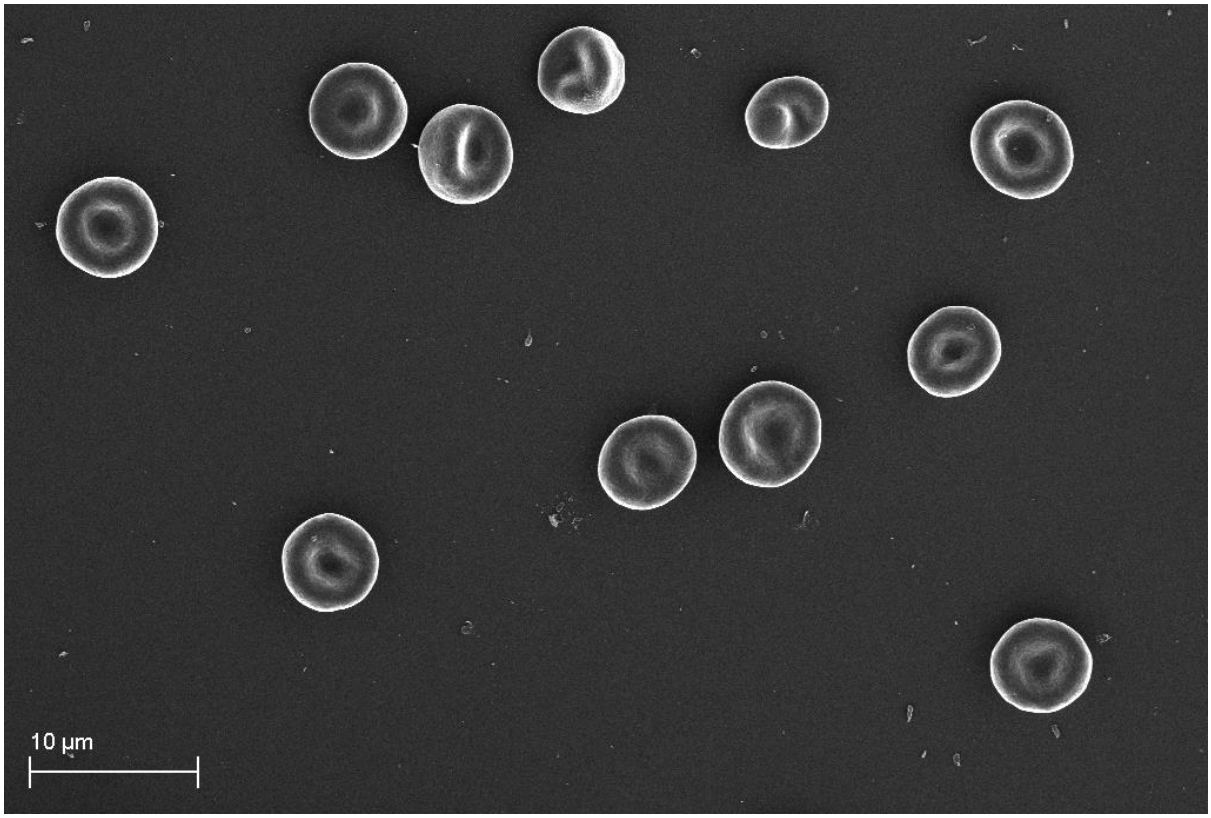


Figure 6.4.17. RBC population from the from the poorly controlled T2DM group (2).

Figures 6.4.16 and 6.4.17. Even though samples from the diabetic group contained clots such as the ones in figures 6.4.12-15, there were also RBC populations that presented with a normal appearance, similar to that of the control group.

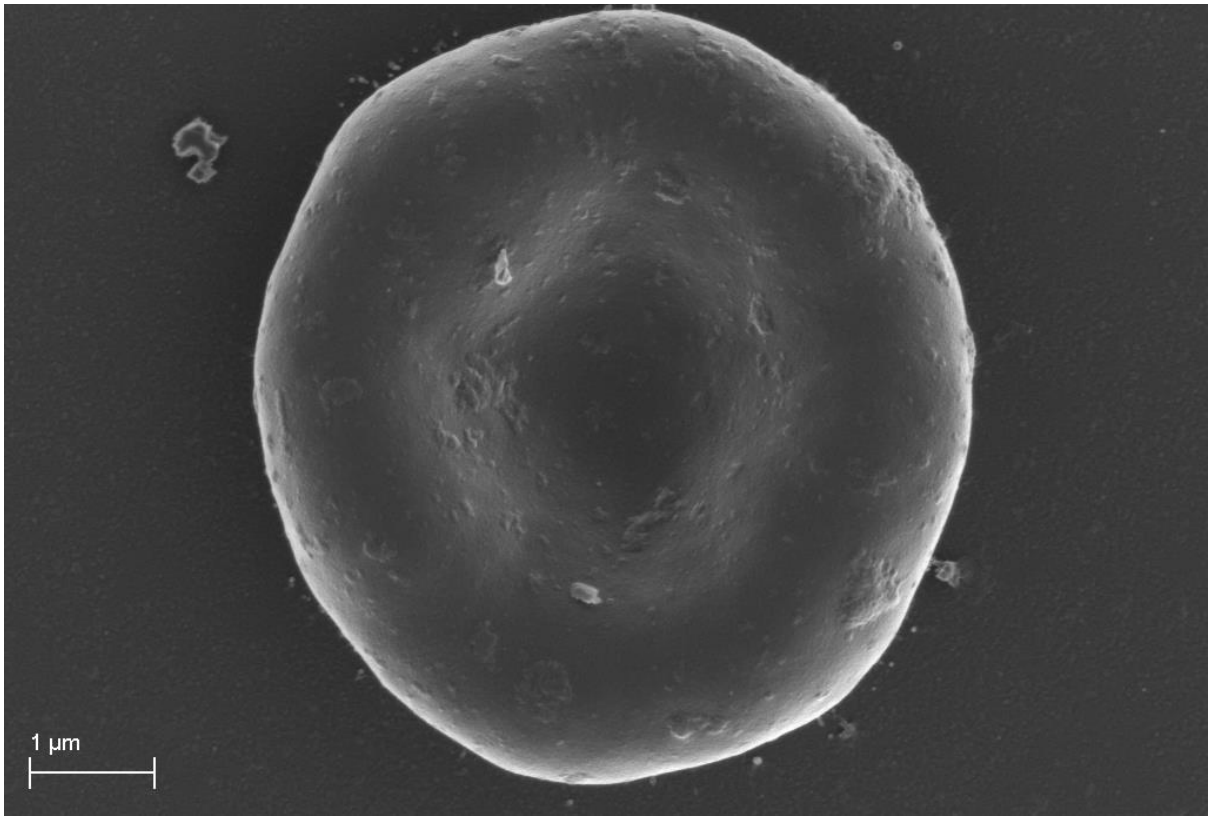


Figure 6.4.18. A single RBC from the from the poorly controlled T2DM group. Even though the cell has the distinct biconcave shape, overall the membrane surface is very irregular.

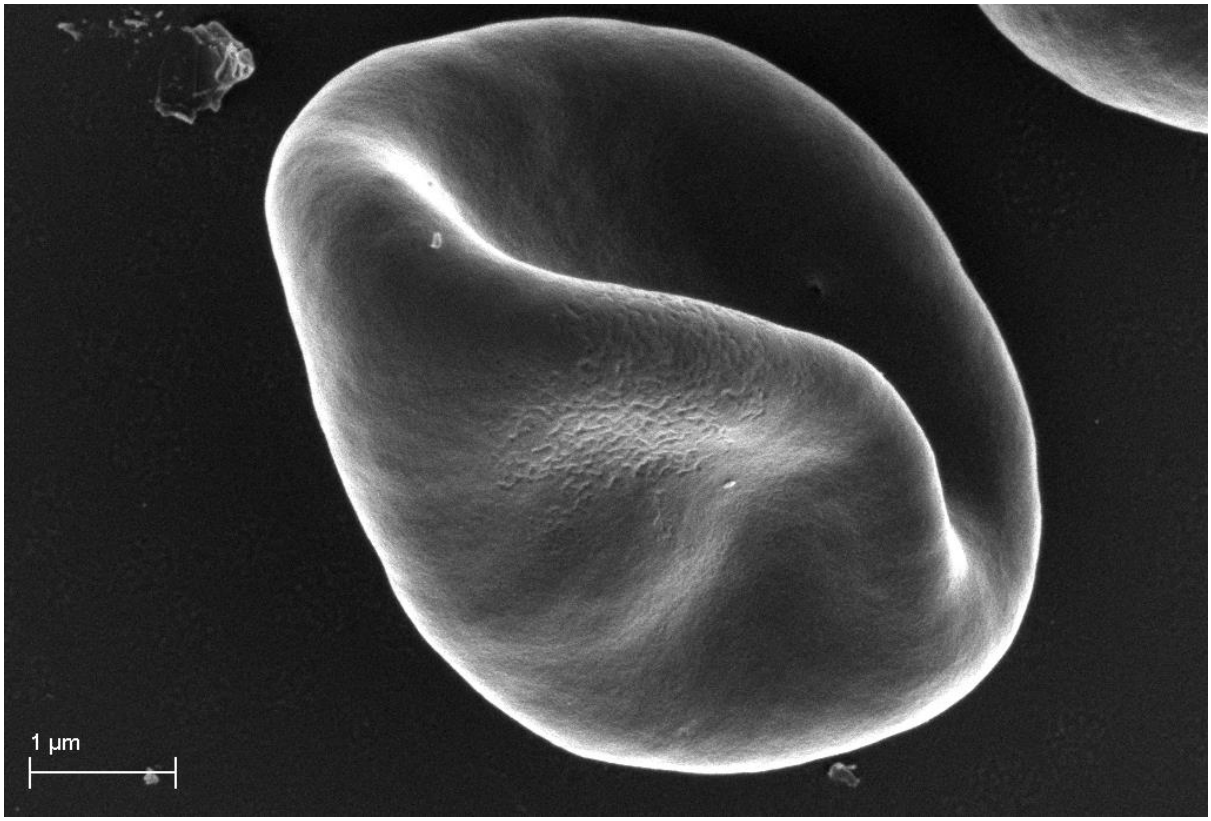


Figure 6.4.19. A single RBC from the from the poorly controlled T2DM group (2). Individuals from the T2DM group also presented with irregular shaped RBC's as opposed to the distinctive biconcave disc.

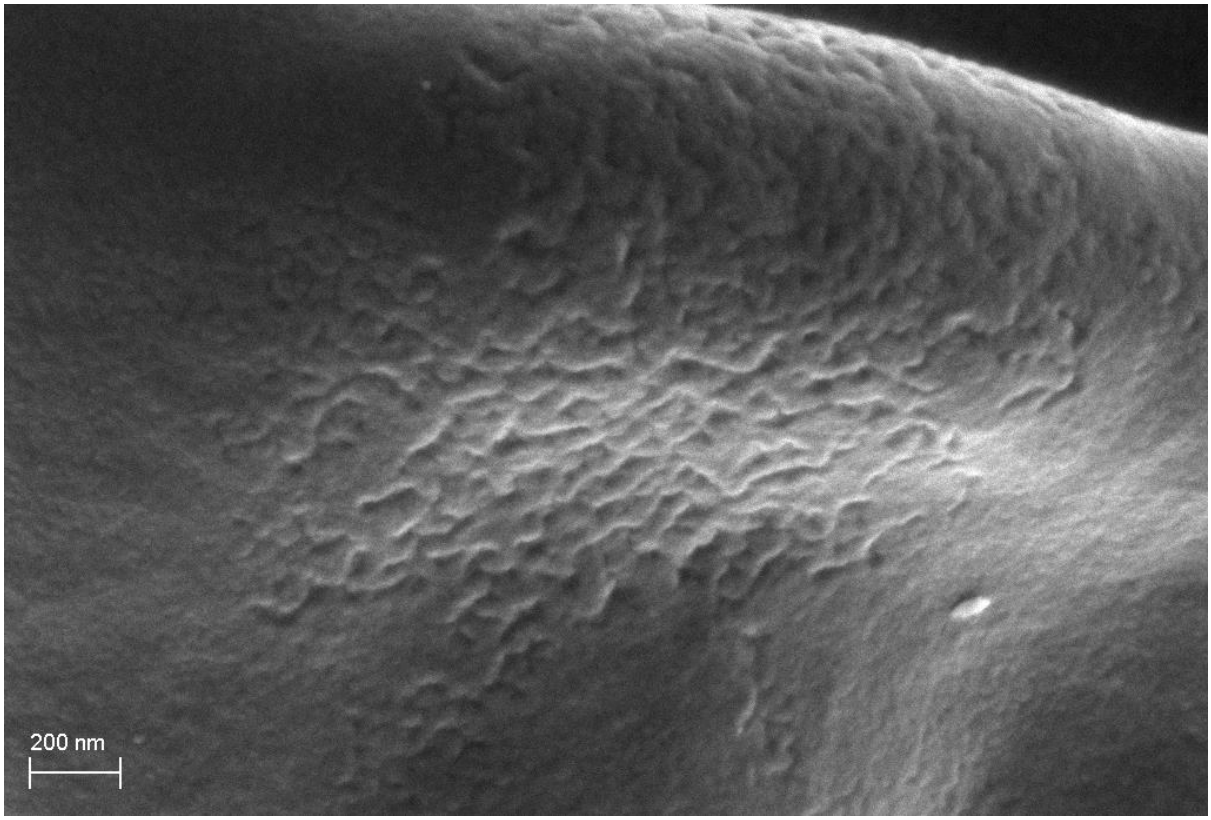


Figure 6.4.20. The surface membrane of a RBC from the poorly controlled T2DM group. A higher magnification of figure 6.4.19 above, showing the irregularities on the cell membrane, a feature not observed in the control group.

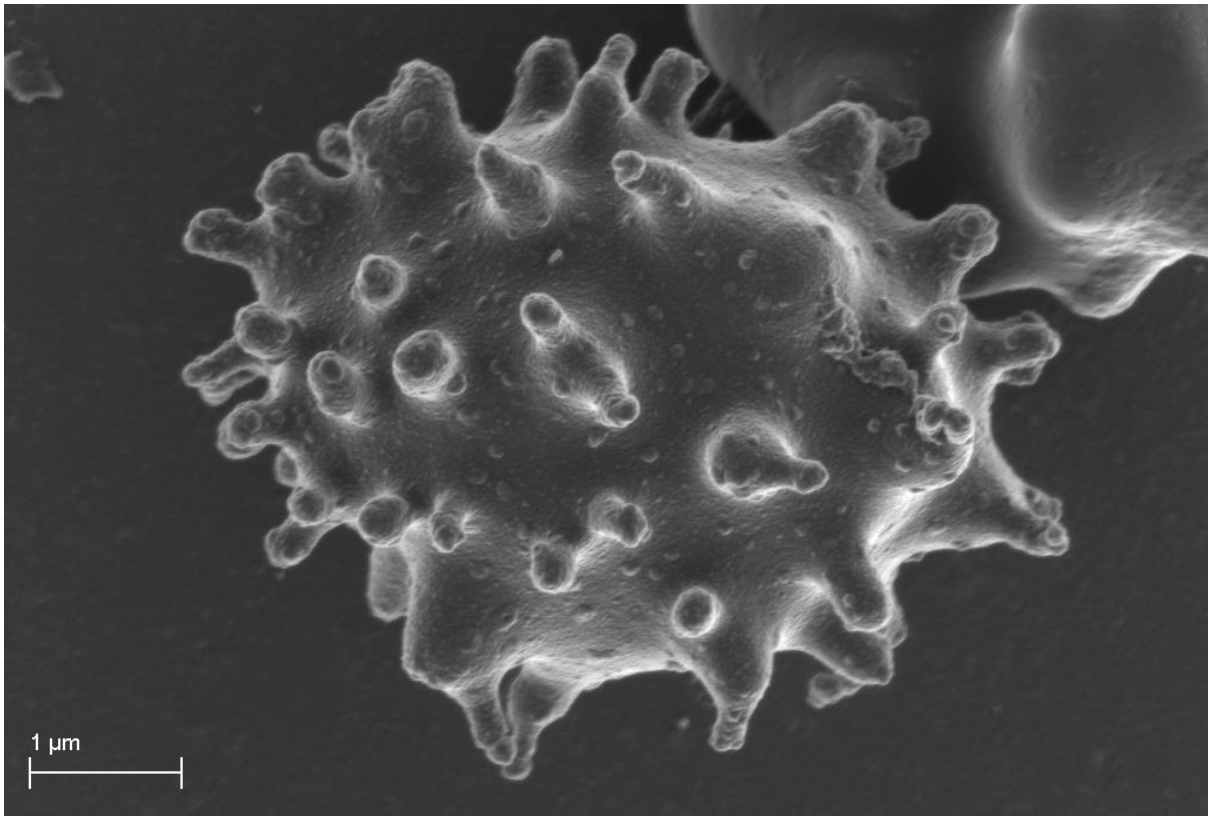


Figure 6.4.21. One of the eryptotic RBC's from the from the poorly controlled T2DM group.

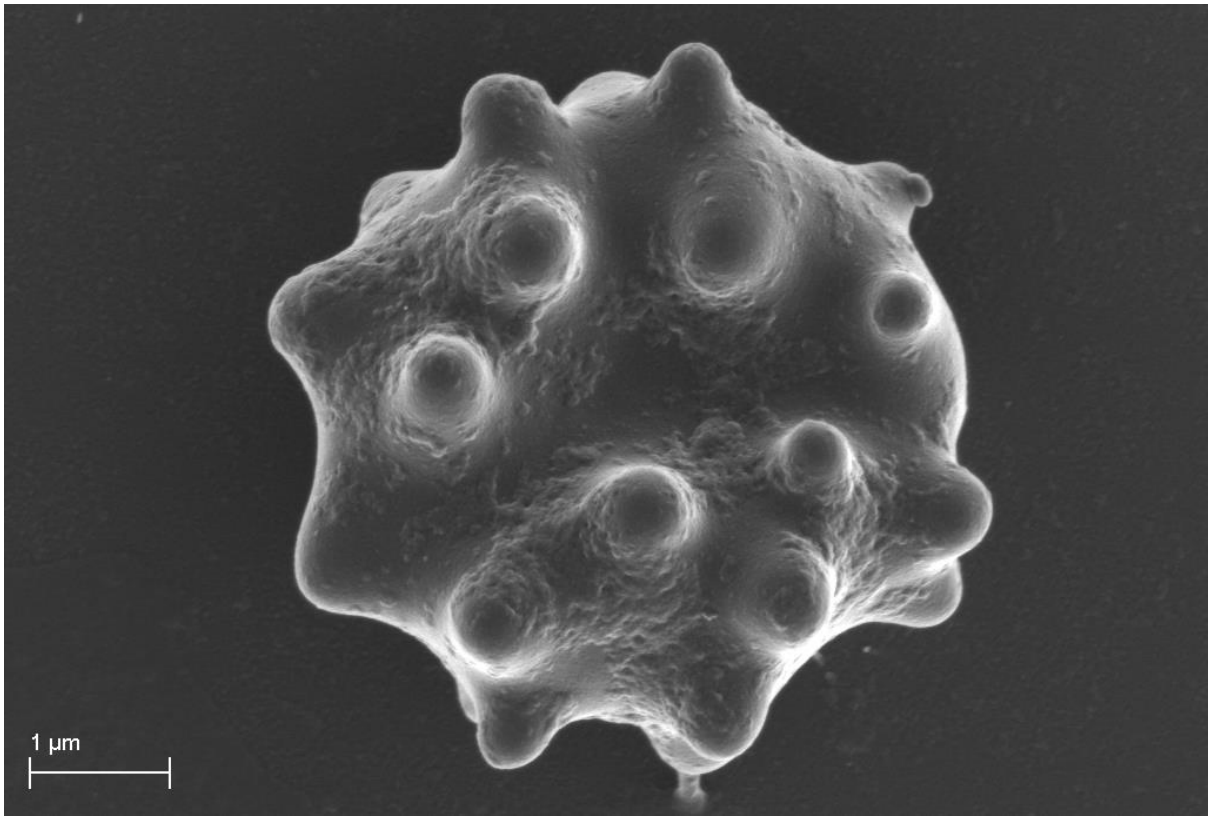


Figure 6.4.22. One of the eryptotic RBC's from the from the poorly controlled T2DM group (2).

Figures 6.4.21-22. Eryptotic RBC's from the T2DM group. These cells presented with irregularities on the cell membrane.

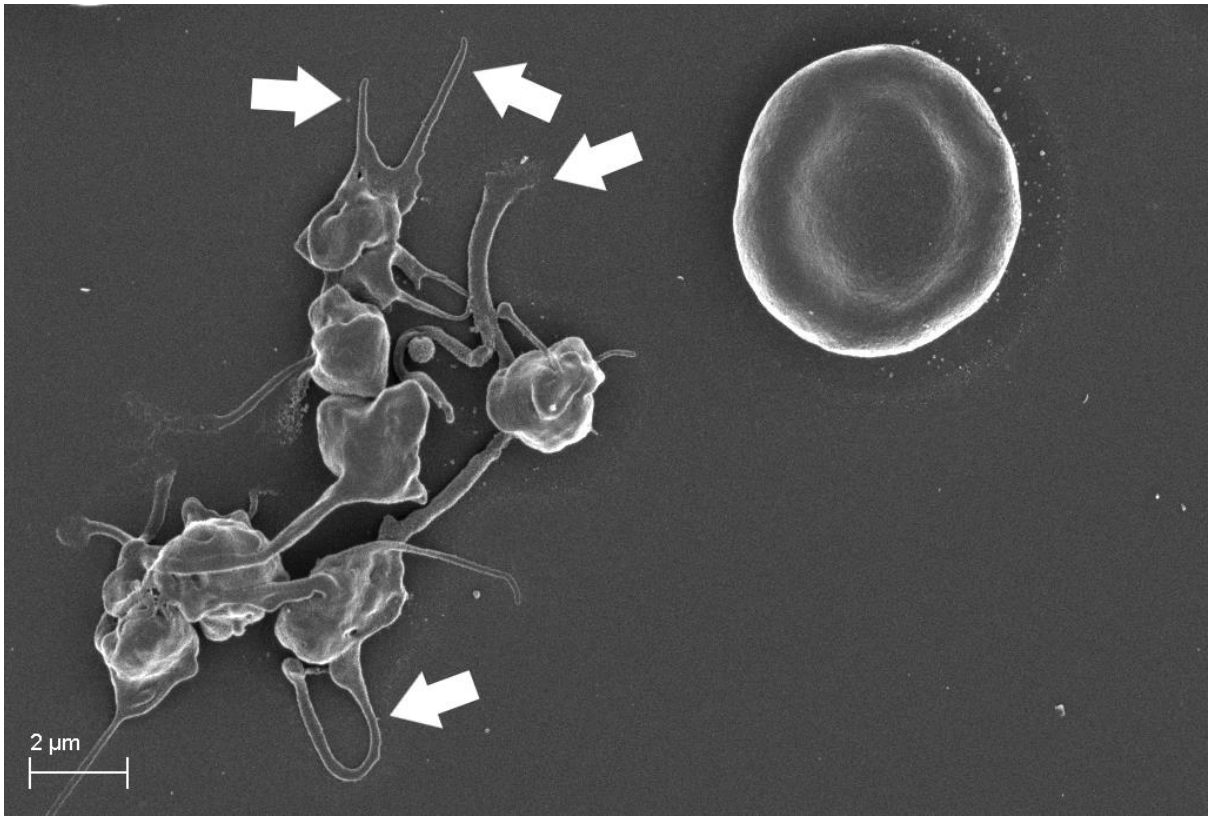


Figure 6.4.23. Activated platelets from the diabetic group.

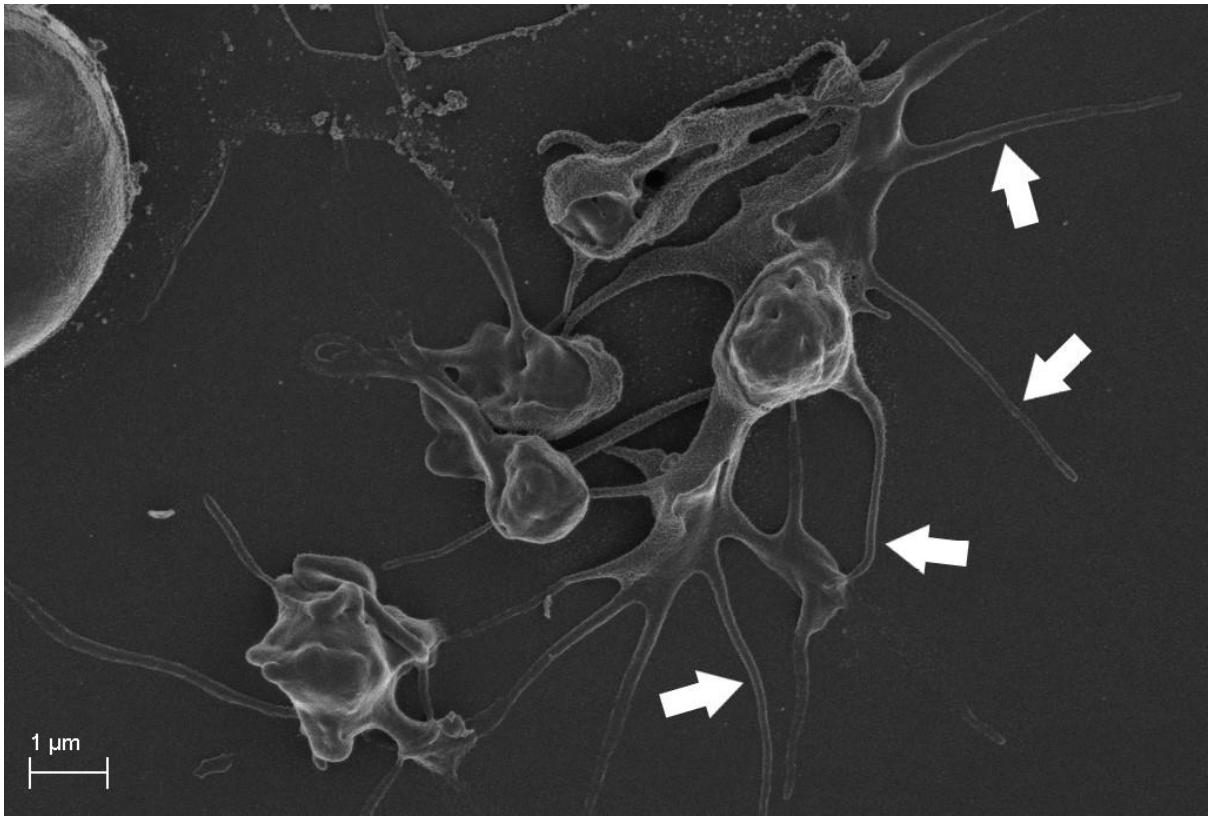


Figure 6.4.24. Activated platelets from the diabetic group (2).

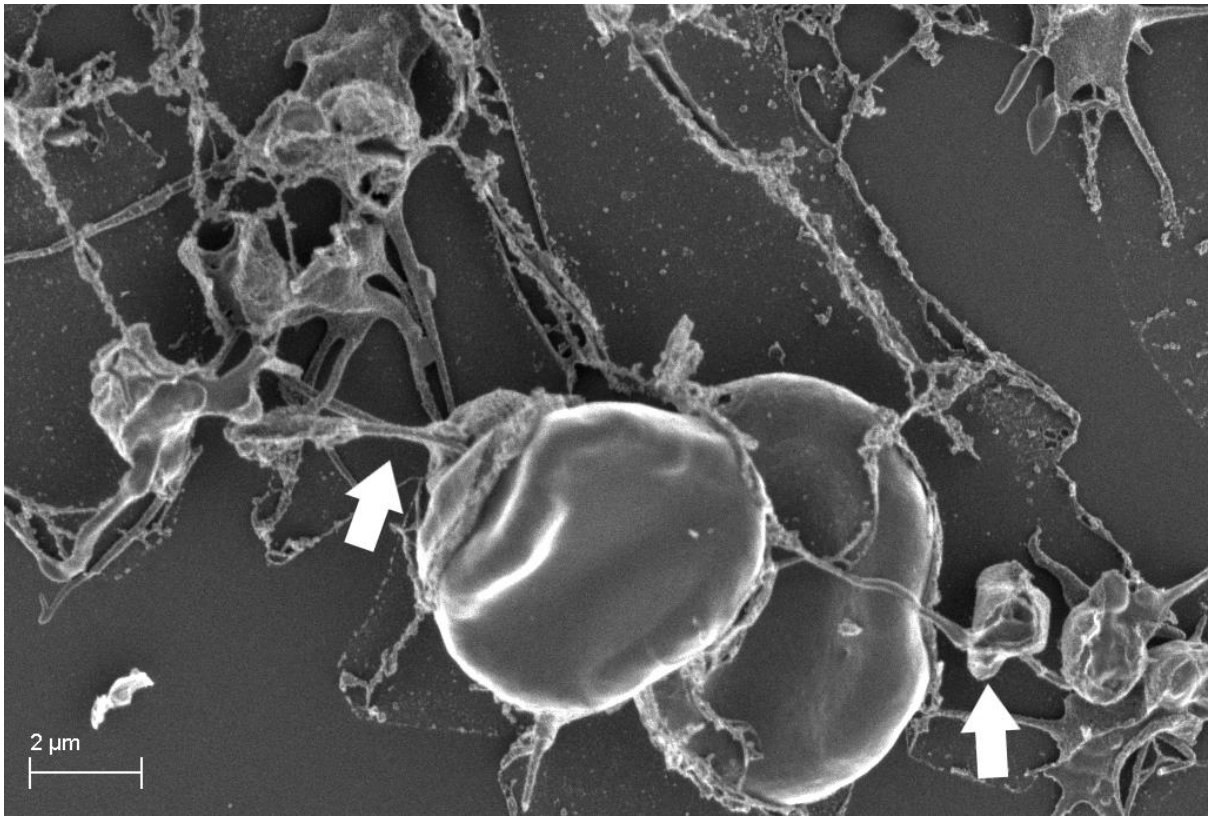


Figure 6.4.25. Activated platelets from the diabetic group (3).

Figures 6.4.23 - 6.4.25. Observed platelets from the T2DM group appeared to be agitated with distinctive pseudopodia (indicted by the white arrows) adhering onto each other (6.4.23, 6.4.24) and onto RBC's (6.4.25). This indicates activated platelets due to inflammation.

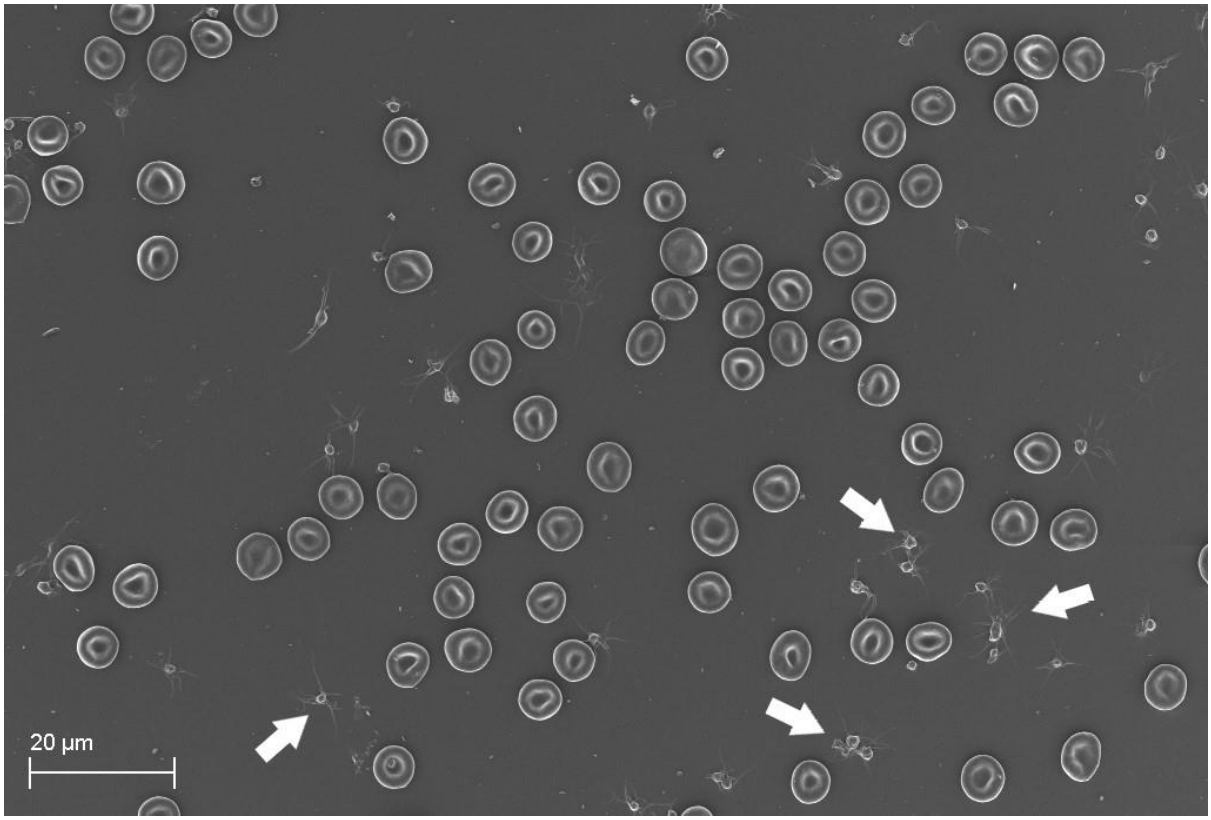


Figure 6.4.26. A RBC population from the diabetic group. Even though the RBC's appear to be healthy, there are a lot of activated platelets on the slide (indicated by the white arrows).

Table 6.4.1. Descriptive summary of SEM results.

	Control	**T2DM**
RBC membrane	Smooth overall	Rough, irregular
Overall RBC shape	Biconcave disk	Folded and irregular
Eryptosis	Yes, with smooth membrane surfaces.	Yes, with irregular membrane surfaces and more prevalent
Clot formation	No	Yes, tight clot
Platelet shape	Spherical	Irregular
Pseudopodia	Very few	Yes

6.5 Discussion

Before discussing the biophysical differences between the two groups, it is important to know that morphological data gathered from SEM is qualitative and has to be backed up by quantitative data to carry any significant meaning.

All clotting occurred spontaneously and as mentioned earlier, no clotting agents were added to the samples.

6.5.1 Morphology of cell in the control group

Looking at the control group and comparing the results to previous studies (116) (117) (118), it is clear that this group is representative of a healthy control group. A typical healthy red blood cell will have the distinct shape of a biconcave disc which is what can be seen in figure 6.4.4. Looking at figures 6.4.1 and 6.4.2, some RBC's do appear abnormal but their numbers are minimal. The larger proportion of the population has the typical biconcave disc shape of a RBC. Taking a closer look at the membrane surface of one of these cells in figure 6.4.5, there is no evidence of irregularities on the surface of the membrane. No clots were observed in any of the samples from the control group.

Some cells in the population are undergoing eryptosis. This is to be expected and not necessarily an indication of any health risks since all RBC's will undergo this process when they reach the end of their approximately 120-day lifespan. The eryptotic cells from individuals in the healthy group all presented with similar characteristics that are depicted in figures 6.4.6 and 6.4.7, a crenated spherical shape with a smooth membrane surface.

Platelets from the control group were rarely observed and when they were, they were mostly isolated from other cells, attached to the surface of the microscope slide. The number of platelets seen were very low, however, this is to be expected since the preparation method used is for viewing of whole blood samples and not for platelets only. Since the platelets in the control group are not activated, they will not form pseudopodia and attach to their surroundings, making it easy for them to be washed off of the microscope slide during preparation. Platelets that were seen such as the one in figures 6.4.8 – 6.4.11 presented with a spherical shape with very little pseudopodia.

6.5.2 Morphology of cells in the diabetic group

Figures 6.4.12 – 6.4.15 shows the blood clots that were observed in samples from the diabetic group. Though figure 6.4.13 shows clot formation, it is distinctly different from the other clots. Even though all four of these figures are displaying clot formation, it is important to know that not all of the RBC populations are forming clots and some (figures 6.4.16 and 6.4.17) look similar to the population in figures 6.4.1 and 6.4.2. The control group however, did not have any clot formation.

Figures 6.4.12, 6.4.14 and 6.4.15 shows the first type of clot that were present in most of the diabetic samples. This clot has a tightly packed formation so much so that the surface of the

microscope slide cannot always be seen. RBC's are held together by numerous fibres, adhering to the RBC surface. There is also a much higher ratio of eryptotic cells in this population, as well as excessive fibrin formation, indicating possible health risk.

The second type of clot observed in the T2DM group, is the loosely packed clot shown in figure 6.4.13. The RBC's in this clot are caught in a mesh-like structure created by fibrin fibres. Only 2 out of the 20 participants presented with this type of clot, indicating that there may be some underlying cause for the formation of this type of clot.

In addition to clot formation, the overall health status of erythrocytes from the T2DM group are poor when comparing them to the control group. Figure 6.4.18 shows an erythrocyte with irregularities on the membrane surface, in contrast to the smooth surface observed in the control group (figure 6.4.4-6.4.5). Red blood cells from the diabetic group also tend to have irregular shapes and are more prone to bending and deformability, as can be seen in figure 6.4.19.

Furthermore, eryptotic cells (figures 6.4.21-6.4.22) from the diabetic group also display the same irregularities on the surface membrane as well as an irregular crenated shape contrary to the almost symmetrical crenated shape that a healthy eryptotic cell has.

The platelets of diabetic individuals are more clumped together (figures 6.4.23 and 6.4.24) when comparing them to the control group. They also tend to attach to nearby RBC's in order to form clots (figure 6.4.25). The general platelet shape are also less spherical and more prominent pseudopodia can be seen. When these pseudopodia attach to nearby RBC's, they tend to disfigure and change the shape of RBC's.

6.6 Conclusion

Analysis of SEM micrographs indicates a clear difference between the biophysical properties of whole blood in the healthy control group and the group of diabetic individuals. The fact that the blood of individuals with poorly controlled T2DM spontaneously form tightly packed clots, albeit *ex-vivo*, confirms the hypercoagulable state they are constantly in. This again confirms that diabetics are prone to accelerated atherosclerosis.

Swanepoel *et al,* did similar studies on the ultrastructural and viscoelastic changes on whole blood because of progesterone, synthetic progestins and endogenous and synthetic

estrogens (116) (117). Gyawali *et al,* did a study of RBC morphology with oxidative stress and inflammation in metabolic syndrome (119). These studies reported results similar to what was seen in the diabetic group of this study with changes in RBC morphology, damage to the cell membrane as well as activated platelets. To further investigate what was observed in the SEM results, thromboelastography will be used to determine the viscoelastic properties of the blood.

CHAPTER 7: THROMBOELASTOGRAPHY

7.1 Chapter objectives

In this chapter, viscoelastic characteristics of the whole blood in poorly controlled T2DM individuals will be compared to those of healthy individuals using the thromboelastograph.

7.2 Introduction

Thromboelastography (TEG) is a technique based on the assumption that the end result of the haemostatic process is a clot and its physical properties determine the patients' haemostatic status. TEG measures the physical properties of a clot in whole blood using a pin that is suspended in a cup from a torsion wire connected to a mechanical-electric transducer (120) (121).

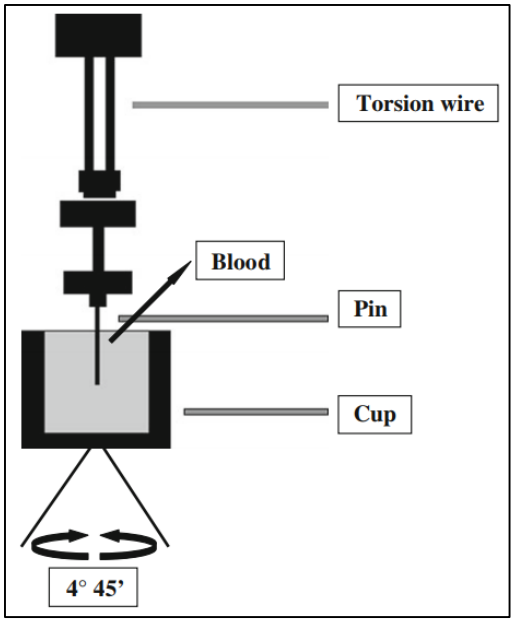


Figure 7.2.1. Illustration of the mechanism of thromboelastography.

A pin, suspended by a torsion wire, is immersed in a cup filed with whole blood, maintained at 37 °C. The wire is connected to a mechanical-electrical transducer. The cup is oscillated through and angle of 4°45' in order to simulate sluggish venous flow and to activate coagulation. The speed and strength of the clot formation is measured and processed by computer and represented graphically by a thromboelastograph tracing (120) (121).

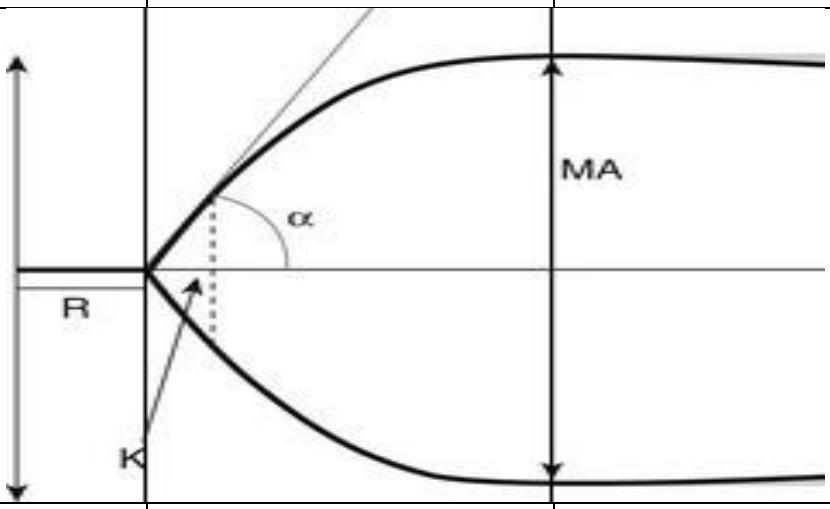
Parameter	Clot time	Clot rate	Maximum clot strength
			
Hypercoagulable	↓ R (min)	↓ K(min) α (degrees)	↑ MA (mm)
Hypocoagulable	↑ R (min)	↑ K(min) ↓ α (degrees)	↓ MA (mm)

Figure 7.2.2. Thromboelastograph tracing.

An example of a typical thromboelastograph tracing with parameter changes according to hyper- or hypocoagulibilty.

For the purpose of this study, the following parameters in table 7.2.1 were studied to determine clot formation and clot strength:

Table 7.2.1. Parameters and their descriptions that were measured with the thromboelastograph (122) (123).

Reaction time (R)	The period of time of latency from calcium chloride addition to initial clot formation.
Kinetics (K)	The time taken to achieve a certain clot strength (amplitude of 20 mm on thromboelastograph).
Angle in degrees (α)	A measurement of the rate of clot formation.
Maximum amplitude (MA)	a direct function of the maximum dynamic properties of fibrin and platelet bonding via glycoprotein IIb/IIIa and is a measurement of the maximum strength/stiffness of the clot.
Time to maximum rate of thrombus generation (TMRTG)	the time interval(s) observed before maximum velocity of clot growth.
Maximum rate of thrombus generation (MRTG)	is the maximum velocity of clot growth observed (dynes.cm^{-2}.min^{-1}).

Total thrombus generation (TTG)	the total area under the velocity curve during clot growth (dynes/cm^2), representing the amount of clot strength generated during clot growth.

Version 4.2 TEG software were used to analyse all data.

7.3 Materials and methods

The following reagents were used in thromboelastography:

- 0,2 M calcium chloride ($CaCl_2$) (7003) from Barker Medical
- TEG® (TEG ® 5000 computer-controlled device, Haemoscope Crop. Niles, IL, USA)

340 µL of whole blood (WB) sample mixtures from citrate tubes were placed in a disposable cup in a computer-controlled TEG hemostasis system (Model 5000, Hemoscope, Niles, IL), with addition of 20 µL $CaCl_2$ as the last step to initiate clotting. Thromboelastographic data were collected until maximum elastic modulus were reached or until one hour had elapsed.

7.4 Results

The readings from the 7 parameters that were measured are displayed below. The two groups are denoted as “Control” for the control group, and “Experimental” for the T2DM group.

All parameter means from TEG will be described using the Mann-Whitney *U* test in GraphPad Prism 7® for Windows. All test results were evaluated at a 5% level of significance.

7.4.1 Reaction time (R) (min)

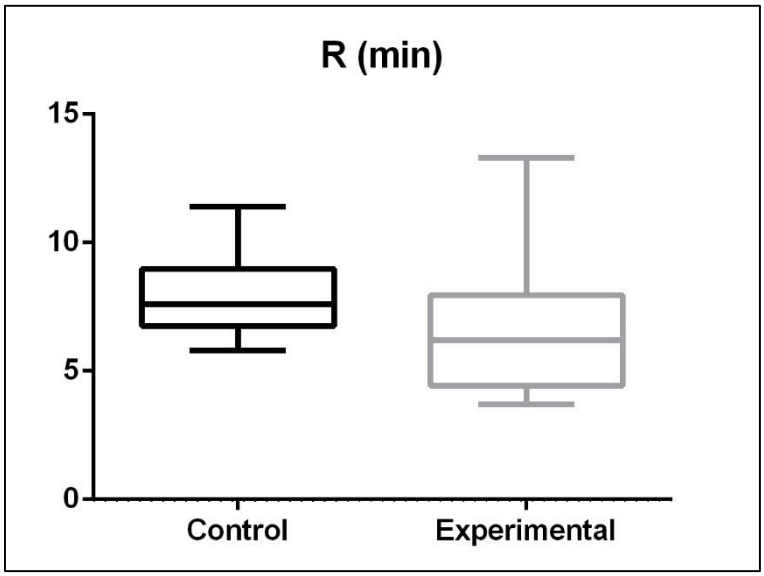


Figure 7.4.1. Box-and-whisker diagram of the reaction time.

Comparison between the control and T2DM groups.

Table 7.4.1. Statistical analysis of the reaction time.

	Mean	Median	Standard Deviation	P-value	Statistically significant? (P < 0.05)
Control	7.92	7.6	1.40	0.0153	Yes
T2DM	6.60	6.2	2.46		

A statistically significant difference between the reaction times of the two groups, where the reaction time for the T2DM group was significantly lower than that of the control group, reinforces the state of hypercoagulability of diabetics. Reaction time is directly dependant on clotting factors, which correlates with the inflammatory state and hypercoagulability of participants from the T2DM group. According to figure 7.2.2, a decreased reaction time corresponds to a hypercoagulable state.

7.4.2 Kinetics (K) (min)

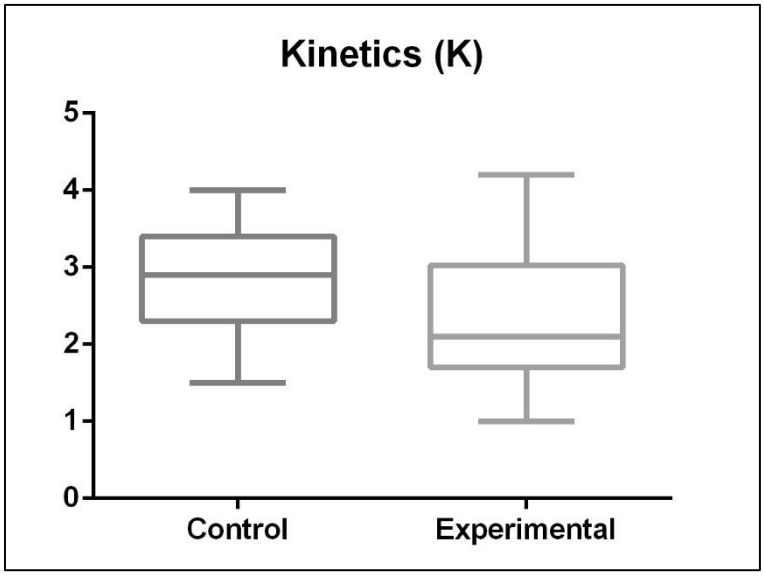


Figure 7.4.2. Box-and-whisker plot of the kinetics.

Comparison between the two groups.

Table 7.4.2. Statistical analysis of the kinetics.

	Mean	Median	Standard Deviation	P-value	Statistically significant? (P < 0.05)
Control	3.13	2.9	1.32	0.0148	Yes
T2DM	2.73	2.1	1.75		

Kinetics represent the time it takes for the clot to achieve its maximum strength. A statistically significant difference between the two groups, where the T2DM group has a shorter time than the control group, indicates that clots will achieve their maximum strength at a faster rate in individuals with type 2 diabetes as opposed to healthy individuals. A decreased time to maximum strength (K-value) corresponds with a hypercoagulable state (figure 7.2.2).

7.4.3 Angle (degrees)

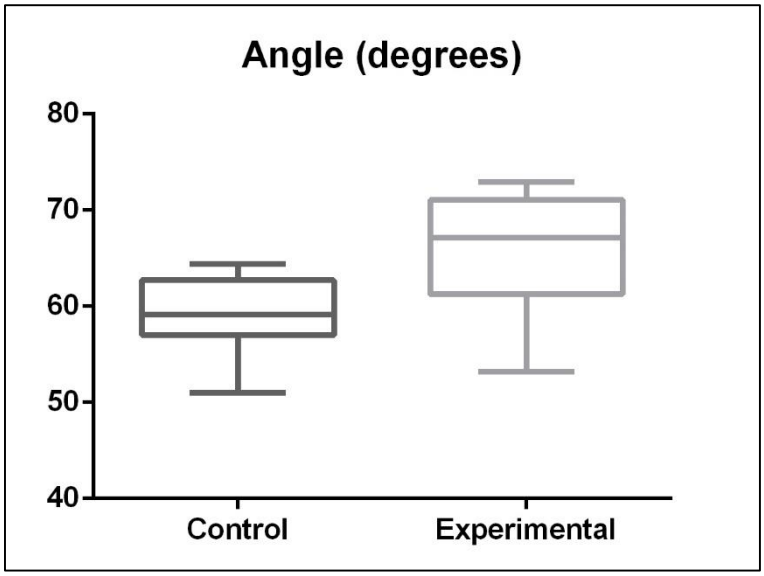


Figure 7.4.3. Box-and-whisker plot of the difference in angle.

Comparison between the two groups.

Table 7.4.3. Statistical analysis of the angle.

	Mean	Median	Standard Deviation	P-value	Statistically significant? (P < 0.05)
Control	58.22	59.15	6.55	0.0008	Yes
T2DM	65.01	67.15	8.97		

The angle, which is measured in degrees, is a measurement of the rate of clot formation. The greater the angle, the faster the rate of clot formation. A significant difference is seen between the two groups where the angle for the T2DM group is greater than that of the control group, representing a faster rate of clot formation. This also correlates with what was seen in 7.4.2, where diabetic individuals have a shorter time for clots to achieve maximum strength, meaning increased rate of clot formation. An increase in the angle corresponds with a hypercoagulable state (See figure 7.2.2).

7.4.4 Maximum Amplitude (MA) (mm)

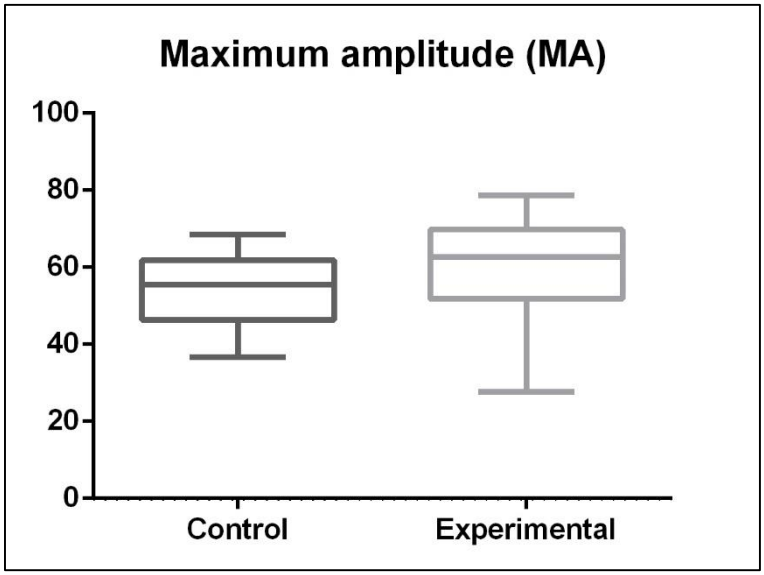


Figure 7.4.4. Box-and-whisker plot of the maximum amplitude.

Comparison between the two groups.

Table 7.4.4. Statistical analysis of the maximum amplitude.

	Mean	Median	Standard Deviation	P-value	Statistically significant? (P < 0.05)
Control	58.22	55.50	9.52	0.0371	Yes
T2DM	59.93	62.70	12.23		

Maximum amplitude is a measurement of the maximum strength/stiffness of the clot. There is a statistically significant difference between the control and T2DM groups where the T2DM group has a higher maximum clot strength. This correlates with the increased platelet count seen in the haematology analysis as well as micrographs from the scanning electron microscope.

7.4.5 Maximum rate of thrombus generation (MRTG) (dynes.cm^{-2}.S^{-1})

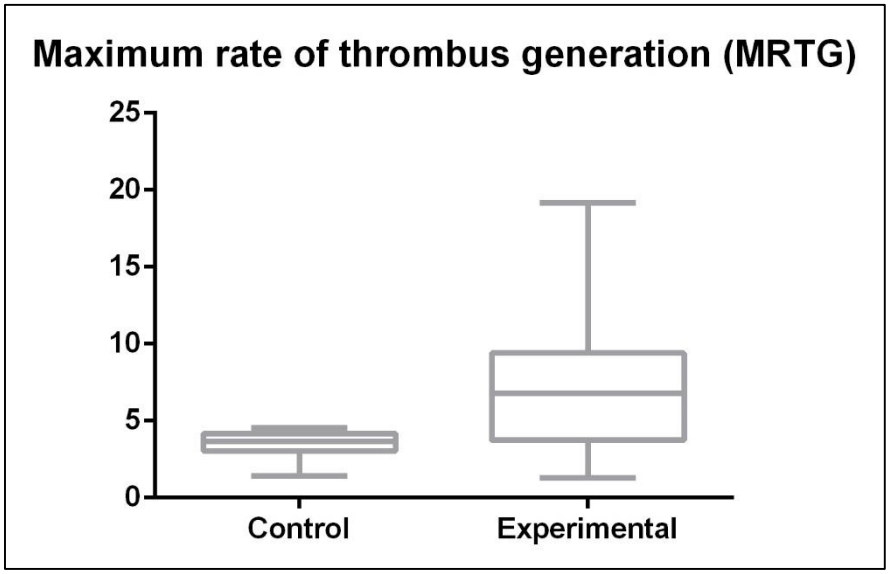


Figure 7.4.5. Box-and-whisker plot of the maximum rate of thrombus generation.

Comparison between the two groups.

Table 7.4.5. Statistical analysis of the maximum rate of thrombus generation.

	Mean	Median	Standard Deviation	P-value	Statistically significant? (P < 0.05)
Control	3.54	3.665	0.74	0.0007	Yes
T2DM	7.71	6.780	5.37		

A parameter measured in dyne-second per square centimetre (dynes.cm^{-2}.S^{-1}) which is a unit of dynamic viscosity, MRTG measures the maximum velocity of the clot growth. Analysed MRTG results showed a statistically significant difference between the control and T2DM groups where the T2DM group had much higher values as well as a higher standard deviation than the control group. The low standard deviation of the control group shows consistency among healthy individuals, as opposed to the T2DM group that had a much higher standard deviation, indicating inconsistency in this group. The higher MRTG values in the T2DM group is indicative of faster growing clots in diabetic individuals.

7.4.6 Time to maximum rate of thrombus generation (TMRTG) (min)

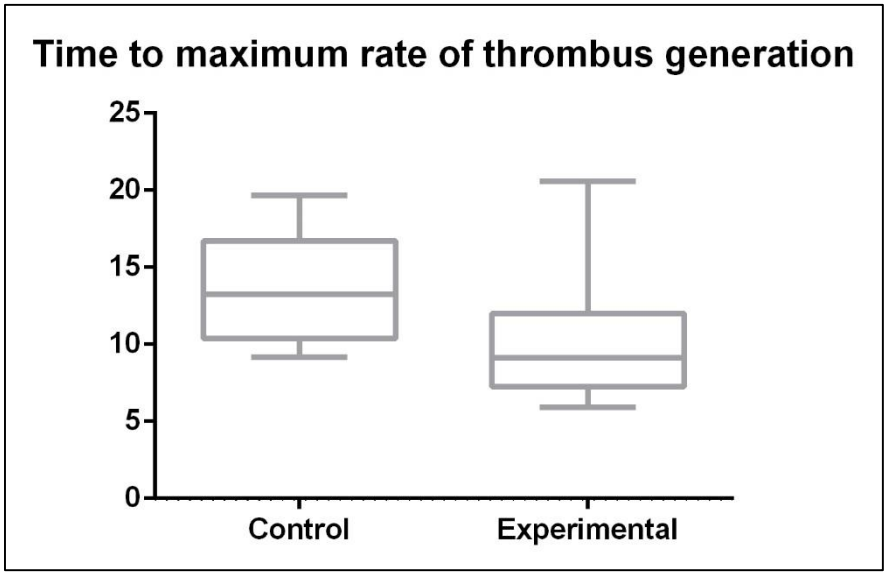


Figure 7.4.6. Box-and-whisker plot of the time to maximum rate of thrombus generation.

Comparison between the two groups.

Table 7.4.6. Statistical analysis of the time to maximum thrombus generation.

	Mean	Median	Standard Deviation	P-value	Statistically significant? (P < 0.05)
Control	13.67	13.27	3.28	0.0001	Yes
T2DM	10.37	9.125	4.31		

TMRTG represents the time it took to reach MRTG, the parameter discussed in 7.4.5. Results from the TEG showed a statistically significant difference in the TMRTG values of the two groups where the T2DM group had a much shorter time to achieve maximum velocity of clot growth with a mean of 10.37 minutes as opposed to the 13.67 minutes of the control group. This means that clots in diabetics grow at a faster velocity (7.4.5) but also achieve that velocity in a shorter time span.

7.4.7 Total thrombus generation (TTG) (dynes/cm^2)

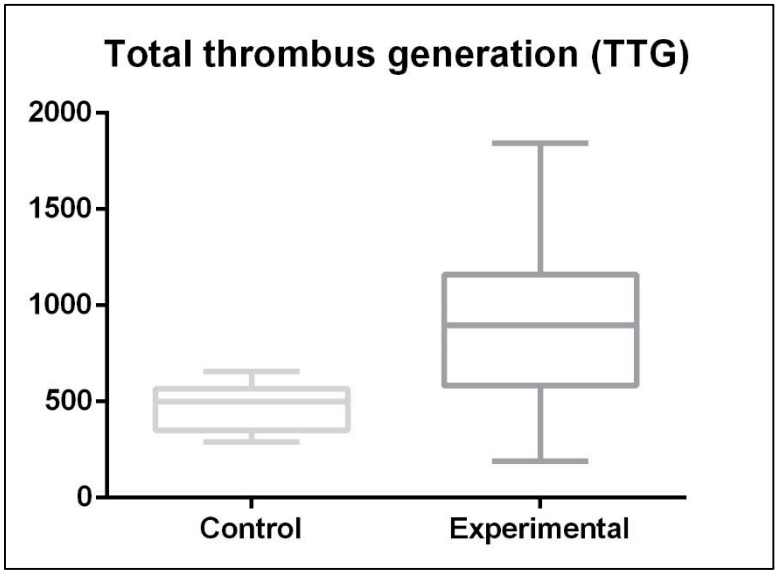


Figure 7.4.7. Box-and-whisker plot of total thrombus generation.

Comparison between the control and T2DM groups.

Table 7.4.7. Statistical analysis of total thrombus generation.

	Mean	Median	Standard Deviation	P-value	Statistically significant? (P < 0.05)
Control	476.23	499.8	113.78	<0.0001	Yes
T2DM	898.15	896.9	394.75		

TTG is a measurement of the area under the velocity curve (see figure 7.2.2) during clot growth, a unit of pressure used to measure the clot strength generated during clot growth. Analysed TTG results showed a statistically significant difference between the control and T2DM groups where the T2DM group had a much greater clot strength during clot growth. This means that the clot is a lot harder to break down in diabetics than in healthy individuals.

7.5 Discussion

All results obtained from the TEG for the T2DM group showed values that are favourable towards a hypercoagulable state. Using figure 7.2.2 as reference, it is evident that the T2DM group had a significant decrease in reaction time and kinetics and a significant increase in angle and maximum amplitude. All of these parameter measurements correspond with a hypercoagulable state and reinforces the results from the haematology analysis as well as the micrographs from the scanning electron microscope.

A hypercoagulable state can be dangerous, especially to diabetics, if not managed properly. It increases the chance of clot formation and as discussed in the introduction of this script, this will have a greater effect on smaller vascular structures where aggregated RBC's will block the flow of blood (11) (12). This can ultimately lead to complete loss of blood flow to certain parts of the body which means permanent damage due to lack of oxygen. An individual in a hypercoagulable state will also have a greater risk of developing a thromboembolic event which can cause loss of limb or stroke (124).

7.6 Conclusion

Based on the results from the TEG, it can be concluded that individuals with poorly controlled T2DM are in a hypercoagulable state and run a greater risk of developing a thromboembolic event.

CHAPTER 8: CONCLUSIONS AND FUTURE WORK

8.1 Chapter objectives

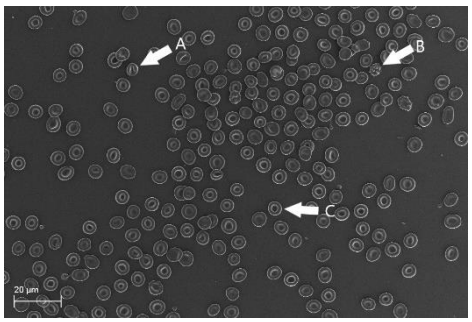
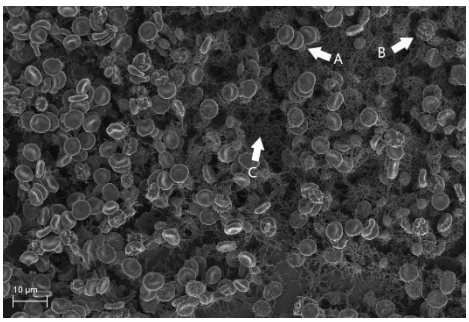
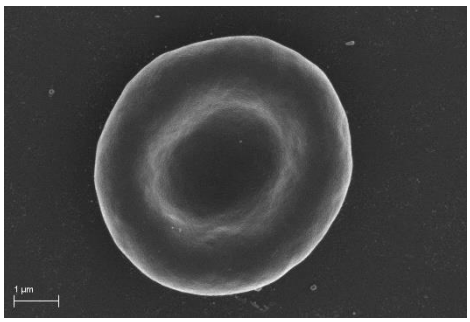
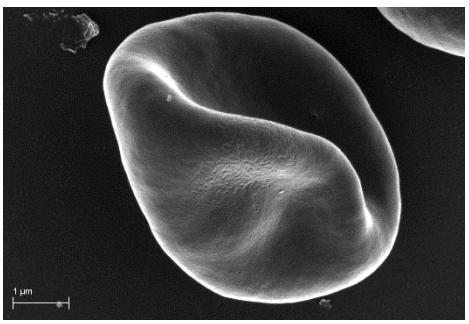
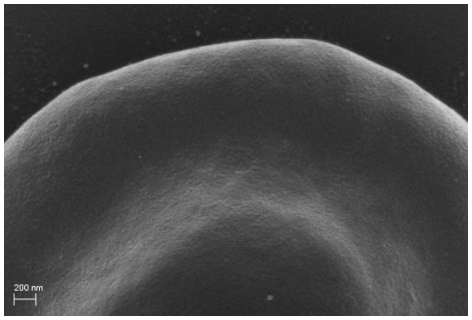
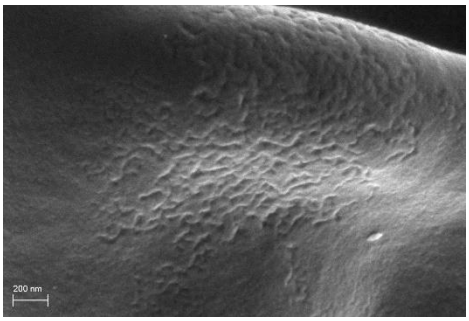
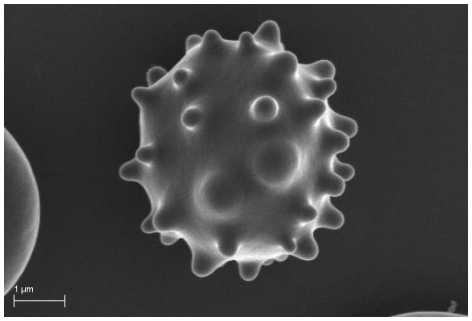
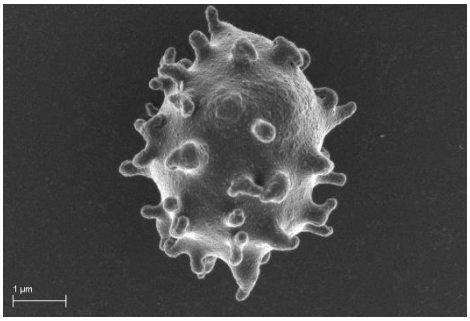
This chapter will aim to summarize all the findings obtained in the study, i.e. the ESR values, parameters measured using the haematology analyser, TEG measurements and SEM findings.

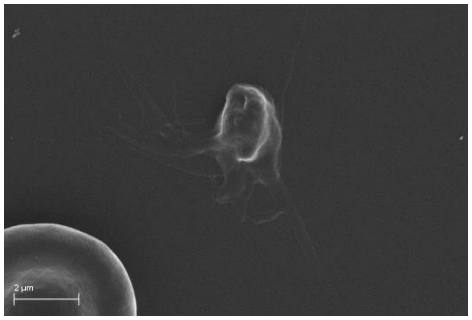
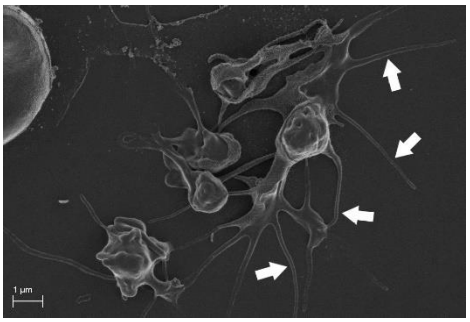
8.2 Results

Table 8.2.1. Summary of all script results comparing controls to poorly controlled T2DM.

	Control group	Diabetic group
Haematology analysis		
WBC count	WBC count fell in normal ranges for healthy individuals.	Contrary to what was expected, since an increased WBC count is a marker for inflammation, the WBC count for the T2DM group was not significantly higher than that of the control group.
RBC count	RBC count fell in the normal ranges for healthy individuals.	No statistically significant difference was observed between the two groups. The RBC count for the diabetic group was slightly lower than that of the control group which can be ascribed to numerous factors.
MCV	MCV values fell in the normal ranges for healthy individuals.	A statistically significant difference between the two groups were observed where values for the diabetic group were lower than that of the control group. This may be due to the chronic use of aspirin by diabetics but needs to be investigated before assumptions can be made.
Platelet count	A normal platelet count was observed.	A significantly higher platelet count was observed in the T2DM group, an indication of the presence of inflammation. This supports the results from ESR.

ESR		
Inflammation	No signs of inflammation. ESR values were all in the normal range for healthy individuals	ESR values were significantly higher, indicating the presence of inflammation.
TEG		
Summary	Results from the control group fell in normal ranges (122)	Results from all seven parameters in the T2DM showed a statistically significant difference, compared to the control group, that favours a hypercoagulable state.

	Control	T2DM
SEM		
RBC population	 Population of healthy RBC's.	 Blood clot consisting of mainly RBC's and fibrin.
RBC	 RBC with classic biconcave shape.	 Deformed RBC.
RBC (higher magnification)	 Smooth surface of RBC membrane.	 Irregular surface of RBC membrane.
Eryptotic RBC	 Eryptotic cell with regular crenated shape and smooth membrane surface.	 Eryptotic cell with irregular shape and rough membrane surface.

Platelets	 Lone platelet with smooth membrane surface and little pseudopodia .	 Platelets adhere to each other and microscope slide with multiple distinct pseudopodia.

8.3 Discussion and conclusion

Table 8.2.1 gives a detailed summary of all findings obtained in this study. ESR confirmed the presence of inflammation in poorly controlled diabetic individuals which was supported by the increased platelet count seen in the haematology analysis. As discussed in the literature review, chronic inflammation is associated with a hypercoagulable state which is confirmed by the TEG results where all seven parameters in the T2DM group differed significantly from the control group in such a way that it favours a hypercoagulable state. This was corroborated by morphological features seen in micrographs using SEM, which confirmed the presence of spontaneous clot formation in the T2DM group as well as increased platelet activation and damaged RBC's health in both mature and eryptotic RBC's.

Regarding the haematology analysis where some results were inconsistent to that of an inflammatory state; as described in 4.5, these inconsistencies can be ascribed to numerous factors and should be investigated in a controlled setting. The similarity of WBC count between the two groups was one of these "inconsistencies". Aspirin is often prescribed to individuals with T2DM in order to treat the hypercoagulable state of these individuals. Some studies have shown that the chronic use of aspirin may affect the bone marrow, the site of production of most blood cells (98). Another study found that chronic alcohol abuse correlates with a decrease in white blood cell production (97).

For SEM, the micrographs indicated evidence of a clear difference between the biophysical properties of the two groups; from the populations of red blood cells down to the membrane texture. Samples from the T2DM group formed "spontaneous" blood clots as opposed to no clot formation in samples from the control group. This spontaneous clotting did occur *ex vivo* and does not necessarily mean that individuals with type 2 diabetes will spontaneously form

clots inside of their vascular system however, it does reinforce the hypercoagulability associated with diabetes. Individual RBC's from the T2DM group also had a rough membrane surface as well as an irregular shape compared to RBC's from the control group which had a biconcave shape with a smooth membrane surface. This difference in membrane texture can also be seen in the eryptotic cells of the two groups. Platelets from the control group were rarely observed and had very few and unclear pseudopodia which adhered on to the surface of the microscope slide. On the other hand, many platelets could be seen in samples from the T2DM group and had very prominent pseudopodia which adhered onto other platelets and RBC's.

All 7 parameters tested with TEG showed a significant difference between the two groups where all of these parameters for the T2DM group are consistent to that of a hypercoagulable state. These are: A decrease in the reaction time, a decrease in kinetics (the time taken to achieve a certain clot strength), an increase in the angle (a measurement of the rate of clot formation), an increased maximum amplitude (a measurement of the maximum clot strength), an increase in maximum rate of thrombus generation, a decrease in the time to maximum rate of thrombus generation and an increase in total thrombus generation.

In conclusion, all techniques used in this study showed the pronounced effect that type 2 diabetes has on the cells involved in coagulation. Some degree of damage was consistently seen in RBC's from the T2DM group. This will most certainly have an effect on the function of these red blood cells and can be investigated in future studies. This could open up possibilities to better prevent thromboembolic events and improve wound healing in individuals with T2DM.

8.4 Limitations

The main limitations of this study are the small sample size and the use of aspirin in the T2DM group. Participants were limited due to the fact that a lot of individuals with poorly controlled T2DM were observed to be smokers, an exclusion criterion for this study. Chronic aspirin use in, order to treat some of the comorbidities of the disease, is fairly common among type 2 diabetics. Aspirin is known to have a positive effect on the circulatory system but it was deemed unreasonable to ask of participants to stop taking aspirin for 2 weeks prior to sample collection.

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10. ADDENDA

10.1 Ethics approval

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.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

12/10/2017

Approval Certificate
New Application

Ethics Reference No: 363/2017

Title: Investigating the ultrastructural and viscoelastic properties of whole blood, with specific focus on erythrocytes, in poorly controlled type 2 diabetes

Dear Mr Peter Meijer

The **New Application** as supported by documents specified in your cover letter dated 5/10/2017 for your research received on the 5/10/2017, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 11/10/2017.

Please note the following about your ethics approval:
- Ethics Approval is valid for 1 year
- Please remember to use your protocol number (**363/2017**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

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

We wish you the best with your research.

Yours sincerely

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

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

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10.2 Confirmation of statistician

MRC

BIOSTATISTICS UNIT

28 March 2017

To whom it may concern,

This letter confirms that **W. Meijer** from the **Department of Physiology, Faculty of Health Sciences of the University of Pretoria** discussed his project: **"Health complications in poorly controlled Type 2 Diabetes associated with erythrocyte health status"** with me. I confirm that I will assist with the statistical analysis of the study data.

Data analysis

The parameters from the TEG and other tests will be described using mean, median, standard deviation and range. The parametor means will be compared for the healthy and diabetic groups using the t-test, or a non-parametric alternative if non-normal data. Tests will be evaluated at 5% level of significance. All analysis will be done using STATA 14.

Sample size

The sample size of 40 per group will be sufficient for this study.

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10.3 Informed consent form

INFORMATION LEAFLET AND INFORMED CONSENT FORM

TITLE OF STUDY: Investigating the ultrastructural and viscoelastic properties of whole blood, with specific focus on erythrocytes, in poorly controlled type 2 diabetes

Principal investigator: Wikus Meijer

Ethical clearance number: 363/2017

Department of Physiology

University of Pretoria

INTRODUCTION

You are invited to participate in a laboratory-based research study conducted by the Department of Physiology (School of Health Sciences) from the University of Pretoria. You are free to choose if you would like a family member to be present in the information session and with the decision making. This information leaflet is to help you to decide if you would like to participate. Before you agree to take part in this study you should fully understand what is involved. If you have any questions, which are not fully explained in this leaflet, do not hesitate to ask the investigator.

PURPOSE OF STUDY

The researcher is investigating the health complications in poorly controlled type 2 diabetes associated with erythrocyte normality, specifically focusing on how your blood clot, found in your blood (consisting of platelets and fibrin), as well as how red blood cells look like under a specialized microscope that can magnify up to 100 000x (Scanning Electron Microscope).

PROCEDURES

We will draw 3 tubes of 4.2 mL blood. Two tubes will be used for measuring haematological parameters. This includes test that determines blood count (the amount of white- and red blood cells as well as platelets) in a blood sample, as well as how fast the blood sample clots and settles down. Values outside of the normal ranges will affect the shape of red blood cells. The 3rd tube will be used for microscopic analysis in order to investigate any changes in the blood clotting mechanism. The 4th tube will be used for flow cytometry where we will determine the percentage of red blood cells that are dying.

The total amount of blood that will be drawn will be approximately 15 mL

HAS THE TRIAL RECEIVED ETHICAL APPROVAL?

This protocol was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria and written approval has been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki which deals with the recommendations guiding doctors in biomedical research involving human/subjects. A copy of the Declaration may be obtained from the investigator should you wish to review it.

WHAT ARE MY RIGHTS AS A PARTICIPANT IN THIS TRIAL?

Your participation in this study is entirely voluntary and you can refuse to participate or stop at any time without stating any reason. Your withdrawal will not affect your access to other medical care. The investigator retains the right to withdraw you from the study if it is considered to be in your best interest.

RESEARCH KNOWLEDGE OBTAINED IN THIS STUDY

The current study will have no benefit for the participant but will add to our knowledge of understanding the involvement of red blood cells in type 2 diabetes and may add to future research on this disease.

MAY ANY OF THESE TRIAL PROCEDURES RESULT IN DISCOMFORT OR INCONVENIENCE?

Venipunctures (i.e. drawing blood) are normally done as part of routine medical care and present a slight risk of discomfort. Drawing blood may result in a bruise at the puncture site, or less commonly fainting or swelling of the vein, infection and bleeding from the site. Your protection is that the procedures are performed under sterile conditions by experienced personnel. A total of three times 4.2 ml of blood will be collected during your single donation.

WHAT ARE THE RISKS INVOLVED IN THIS STUDY?

In previous studies, some participants have reported experiencing side effects, which included bruising, or swelling of the vein.

ARE THERE ANY WARNINGS OR RESTRICTIONS CONCERNING MY PARTICIPATION IN THIS STUDY?

None

INSURANCE AND FINANCIAL ARRANGEMENTS

Neither you nor your medical scheme will be expected to pay for the study. During a study-related injury (thrombophlebitis) the Department of Physiology assumes no obligation to pay

for the medical treatment of other injuries or illnesses, the medical practitioner performing the procedure will have insurance to cover any injury related to the procedure.

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. In connection with this study, it might be important for domestic and foreign regulatory health authorities and the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, as well as your personal doctor, to be able to review your medical records pertaining to this study.

Any information uncovered regarding your test results or state of health as a result of your participation in this study will be held in strict confidence. Your medical practitioner will be informed of any finding of importance to your health. Information will not be disclosed to any third party in addition to the ones mentioned above without your written permission.

INFORMED CONSENT

I hereby confirm that I have been informed by the investigator, Wikus Meijer about the nature, conduct, benefits and risks of the study: Investigating the ultrastructural and viscoelastic properties of whole blood, with specific focus on erythrocytes, in poorly controlled type 2 diabetes.

I have also received, read and understood the above written information (Participant Information Leaflet and Informed Consent) regarding the research study.

I am aware that the results of the study, including personal details 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.

Participant's name (Please print)

……

Participant's signature ……Date……..

_____

Witness's name (Please print)

……

Witnesses's signature …… Date……

_____

I, Dr ……. herewith confirm that the above participant has been informed fully about the nature, conduct and risks of the above trial.

_____

Investigator's name

Wikus Meijer

Investigator's signature …… Date……

Contact details for Investigator: 0711312416

Contact details for ethics committee: 0123541677

INFORMATION FILLED BY MEDICAL PRACTITIONER/PRINCIPAL INVESTIGATOR

Age:	Gender:
Height:	Weight: BMI:
Diabetic status	Type 1 / Type 2 / Gestational / No history of diabetes
Any other chronic condition (including anaemia)?	Y / N
Do the participant smoke?	Y / N
If female, is the participant on an oral contraceptive?	Y / N
If female, is the participant pregnant?	Y/ N
Medication that the participant uses? If yes, please specify.	Y / N

10.4 Proof of Ethical Clearance

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.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

12/10/2017

Approval Certificate
New Application

Ethics Reference No: 363/2017

Title: Investigating the ultrastructural and viscoelastic properties of whole blood, with specific focus on erythrocytes, in poorly controlled type 2 diabetes

Dear Mr Peter Meijer

The **New Application** as supported by documents specified in your cover letter dated 5/10/2017 for your research received on the 5/10/2017, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 11/10/2017.

Please note the following about your ethics approval:
- Ethics Approval is valid for 1 year
- Please remember to use your protocol number (**363/2017**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

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

We wish you the best with your research.

Yours sincerely

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

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

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