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The morphological and physiological effects of a low-carbohydrate high-fat diet on coagulation parameters

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Abstract

The contribution of unhealthy diets to the emergence of chronic diseases which are a burden to both the society and health care costs, greatly increased over the last decades. Starch and sugar have been linked to the development of chronic diseases and therefore the World Health Organisation is endorsing efforts to cut the intake of digestible starch and sugar. A decrease in the intake of starch and sugar will potentially decrease the risk of chronic diseases like obesity, type 2 diabetes mellitus, hypercholesterolemia, hypertension, metabolic syndrome, coronary heart disease and cancer. A low-carbohydrate high-fat diet has emerged as a treatment strategy to reduce sugar and starch-linked chronic diseases. However, it has been hypothesized that the low-carbohydrate high-fat diet stimulates inflammatory pathways. In this research study, scanning electron microscopy, flow cytometry, and thromboelastography were used to compare blood parameters in participants either on a low-carbohydrate high-fat diet or on a control diet. Healthy volunteers, eating foods without specific restrictions on intake of dietary categories were recruited to form the control group (n=32) and individuals with food intake restrictions limiting carbohydrate intake while increasing fat intake were recruited to form the low-carbohydrate high-fat group (n=32). Platelet-rich plasma, with or without thrombin, was used to study platelet-fibrin network and red blood cell morphology. Distinct changes were noted in the morphology of red blood cells, platelets and fibrin networks of individuals on a low-carbohydrate high-fat diet. Fibrin network fibers for participants in the low-carbohydrate high-fat group were mostly discontinuous, with

some regions with matted deposits. Activated and aggregated platelets were identified morphologically using scanning electron microscopy and platelet activity was further quantified with flow cytometry. Flow cytometric results showed lower mean platelet aggregation in low-carbohydrate high-fat diet participants compared to controls. Thromboelastography was used to compare coagulation parameters of whole blood in low-carbohydrate high-fat diet participants with those of controls. Thromboelastography results show that in low-carbohydrate high-fat diet participants, on average, clot initiation time was shorter, the angle of the clot was greater but there was a decrease in clot strength. The time to maximum clot strength and total clot strength was lower in low-carbohydrate high-fat diet relative to controls. The results show that a low-carbohydrate high-fat diet may induce inflammation leading to a predisposition to hypercoagulability increasing the likelihood of the formation of an abnormal clot.

Key words: Low-carbohydrate high-fat diet, red blood cells, platelets, fibrin network, inflammation, coagulation

DECLARATION

I, Babalwa Cynthia Jobela hereby declare that this research dissertation is my own work and has not been presented for another degree at another university.

Signed: 

Date: 03/01/2019

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LIST OF ABBREVIATIONS

˚C	Degrees celsius
%	Percentage
µL	microlitre
µm	Micrometre
AD	Alzheimer's disease
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
APC	Allophycocyanin
CAD	Coronary artery disease
CRP	C-reactive protein
CVD	Cardiovascular disease
CD	Cluster of Differentiation
$CaCl_2$	Calcium chloride
Ca^{2+}	Calcium
DMDs	Dense matted deposits
DTS	Dense tubular system
ETD	Everhart-Thornley detector
FITC	Fluorescein isothiocyanate
Fl	Femtolitre
FVII, FIX, FX, FXI, FVIII, FV, FXII	Coagulation factors
FXIIIa	Activated form of FXIII
G	Grams
GP	Glycoprotein
GPCR	G-coupled receptors
HFD	High fat diet
HMDS	1,1,1,3,3,3- Hexamethyldisilazane
IL-6	Interleukin-6
IR	Insulin resistance
JNK	Jun-N-terminal kinase
Kcal	Kilocalories
Kg	Kilogram
LCHF	Low carbohydrate high fat diet
Mg	Milligrams

mL	millilitre
MPV	Mean platelet volume
MFI	Mean fluorescence index
MoAbs	Monoclonal Antibodies
NFkB	Nuclear factor kappa-beta
OCS	Open canalicular system
OSO_4	Osmium tetraoxide
$P2Y_{12}$	G_i-coupled platelet receptor for adenosine
PE	Phycoerythrin
PS	Phosphatidylserine
PBS	Phosphate buffered saline
PRP	Platelet-rich plasma
PPP	Platelet poor plasma
ROS	Reactive oxygen species
Rpm	Revolutions per minute
SFA	Saturated fatty acid
SEM	Scanning electron microscopy
sP-selectin	Soluble platelet selectin
T2DM	Type 2 Diabetes Mellitus
TEG	Thromboelastography
TF	Tissue factor
TNF-α	Tissue necrosis factor alpha
TLR	Toll-like receptors
TxA2	Thromboxane A2
vWF	Von Willebrand factor
WB	Whole blood
WHO	World Health Organisation

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CHAPTER 1: INTRODUCTION

1.1 Background summary

Previous research indicates that certain diets can cause not only excessive glucose and lipid levels, but also increase other cardio-metabolic risk factors such as oxidative stress, inflammation, and endothelial dysfunction.[1]

Nutritional excesses, specifically unhealthy foods, have contributed so much to the rise of chronic diseases, which are a burden to societal and health care costs.[2] There has been an urgency to explore dietary measures to prevent chronic diseases. One of these measures includes limiting the intake of carbohydrates.[3] The World Health Organisation (WHO) endorses this strategy, and suggests that it is essential in decreasing the risks of non-communicable diseases such as obesity, type 2 diabetes mellitus (T2DM), hypercholesterolemia, hypertension, metabolic syndrome, coronary heart disease, and cancer.[2,4] Several diets have been proposed in order to reduce the incidence of chronic diseases and specifically in the treatment of T2DM, obesity and metabolic syndrome.[5-6] The most prominent in recent years is the low-carbohydrate high-fat (LCHF) diet. The effectiveness and safety of the LCHF diet has not been determined, with differing opinions between health care professionals despite positive outcomes such as weight loss.[7] Also, controversy exists where some studies suggest that a high fat diet (HFD) can increase the risks of obesity, cardiovascular disease (CVD), and T2DM while other studies have documented that well controlled LCHF diets rather decrease the risk for these diseases.[8-9] However some uncertainty remains on the long-term effects of LCHF diets. These uncertainties regarding long-term effects are significant, given the fact that some chronic diseases like T2DM take a period of ten to twenty years or more to develop.[3] Some health care professionals are also concerned that vital nutrients may be lacking in the LCHF diet, increasing the risk of nutrient deficiencies, and also the possibility that a high intake of saturated fats may increase the risk of CVD.[7]

Food intake during an LCHF diet depends on individual preference and may include moderate proportions of carbohydrates with a high proportion of fat, or a very restricted low-carbohydrate diet known as the ketogenic diet. The LCHF diet is defined as restricting carbohydrate intake to less than one hundred and thirty grams per day.[10] A ketogenic diet restricts carbohydrate intake to twenty to fifty grams, an

amount which constitutes less than 10% of the daily required calories.[10] A ketogenic diet results in a state called ketosis. A physiological response to limited carbohydrates and decreased insulin levels may result in the body burning fat for energy leading to ketosis.[10] With restriction of carbohydrates, glucose supply to the liver is reduced and less glycogenesis (glycogen formation) will take place. The depletion of glucose will consequently activate gluconeogenesis (the formation of glucose from non-carbohydrate sources like amino acids in the liver).[3] The other process is ketogenesis, a metabolic process whereby the liver converts non-esterified fatty acids to ketone bodies.[11] When the amount of glucose being produced is not sufficient to cover the cells energy needs, ketone bodies are produced so as to substitute energy sources.[3] Low-carbohydrate consumption may lead to a decrease in circulating insulin levels, a stimulus for the metabolism of fatty acids.[12] The digestion of these fatty acids lead to the formation of acetyl-coenzyme A and subsequent production of ketone bodies (acetoacetate and D-β-hydroxybutyrate).[13] The brain, muscles and other organs can process the ketone bodies, via their mitochondria, as their source of energy (adenosine triphosphate) when carbohydrates are restricted.[11,13]

A review of the literature has suggested that maintaining the LCHF diet over months or years is not necessarily associated with metabolic changes in the body, provided that the diet supplies sufficient energy for the body with adequate proteins.[8,14] While it is suggested that LCHF diets reduce metabolic risk factors, there is a lack of evidence supporting the long-term efficacy, safety and health benefits of LCHF diets.[8-9] Despite the popularity of LCHF diets, the physiological and morphological effects, in particular, on blood cells and the coagulation profile is not known. Inflammation is a key indicator of poor physiological health and platelets, red blood cells (RBCs) and coagulation profiles are very sensitive to systemic inflammatory changes and cytokine levels.[15-16] Circulating pro-inflammatory cytokines have been shown to be elevated during systemic inflammation in most diseases. A study done by Bester and Pretorius (2016) showed that inflammatory cytokines such as interleukins (IL-1β, IL-6 and IL-8) have visible impact on platelets and RBCs, where the *ex vivo* addition of the cytokines caused platelet hyper-reactivity, and RBCs structures resembled cells undergoing death (eryptosis).[16] There was also evidence of hypercoagulability triggered in the

presence of these cytokines leading to the formation of abnormal clots, verified via thromboelastography (TEG).[16-17]

1.2 Aims and objectives

As these key inflammatory cytokines have been proven to effect visible changes to blood, the aim of our study, therefore, was to determine the effect of an LCHF diet on the morphology of RBCs, platelets and fibrin networks, and the overall coagulation profile.

In order to achieve the stated aims, our objectives are to:

- Obtain blood samples from individuals screened into two groups - either on an LCHF diet or on a control diet inclusive of carbohydrates
- Apply techniques such as scanning electron microscopy, flow cytometry, and thromboelastography to the examination of the acquired blood samples.
- Use applicable software to convert all findings to quantifiable and analysable data associated with coagulation
- Use statistical software to compare data outcomes from both groups
- Determine any differences existing between the groups so as to draw inferences on how diet affects coagulation parameters and potential associations with inflammation.

CHAPTER 2: LITERATURE REVIEW

2.1 Diet and Inflammation

Evidence suggests that one's diet can influence inflammation within the body.[18-19] A diet containing a lot of processed starch, sugar, saturated fat and fiber from fruits, may promote inflammation.[20] Inflammatory indicators include: increased levels of factors such as IL-6, tissue necrosis factor alpha (TNF-α) and low levels of adiponectin.[21] The imbalance of inflammatory and anti-inflammatory cytokines, creates a pro-inflammatory milieu in for example adipose tissue, which can lead to insulin resistance (IR) and endothelial dysfunction resulting in the formation of blood clots. This eventually predisposes affected individuals to the metabolic syndrome, T2DM and CVD.[20]

Inflammation can also be induced by oxidative stress involving the presence of free radicals and reactive oxygen species (ROS). ROS is essential to normal physiological processes but may become pathological when their production exceeds their removal, by antioxidants. Both oxidative stress and inflammation are linked by nuclear factor-κB (NFκB).[22] NF*κ*B is a primary regulator of inflammation and can be activated by ROS leading to an increase in the release of inflammatory cytokines such as IL-6.[23] Evidence has indicated that a diet rich in saturated fatty acids (SFAs) activates pro-inflammatory responses via a mechanism involving toll-like receptors (TLRs) specifically, TLR-4.[24-26] SFAs are said to bind to TLRs, which then activate the NFκB pathway.[27-28] Activation of NFκB results in impaired insulin signalling with decreased production of nitric oxide (a potent vasodilator).[29] Factors such as IL-6, TNF-α and SFA stimulate kinases such as Jun N-terminal kinases (Jnk) and IkB kinase of kappa kinase (IKK), which then phosphorylate insulin receptor substrate at tyrosine serine residues thereby inactivating it, leading to IR. Thus, SFAs are implicated in the mechanisms underlying obesity, which is supposedly linked to inflammation and IR in endothelial cells. However, the mechanism linking the two is not yet known.[30]

In addition, diets with increased proportion of saturated fat are associated with endothelial dysfunction, a precursor to atherosclerosis.[31] Endothelial dysfunction can be induced with as little as a single high fat meal.[32] A study done by Unruh and colleagues (2015) examined the effects of HFD on RBCs function in mice.[33] RBCs from HFD-fed mice showed increased levels of ROS and increased membrane phosphatidylserine (PS) externalization, a marker of RBCs aging as well as a marker

for apoptosis.[34] An increase of PS externalization may result in enhanced phagocytosis of RBCs by macrophages *in vitro.* These changes can lead to disruptions on RBC function. RBC dysfunction was found to contribute to endothelial dysfunction, a manifestation of diet-induced obesity, which may serve as a marker of atherosclerotic pathogenesis.[33]

In obese participants, the LCHF diet was shown to influence markers of inflammation but there was no evidence to support if this was linked to oxidative stress.[35] Adherence to the LCHF diet (1 month) by these participants led to an increase in C-reactive protein (CRP) and IL-6 during weight loss.[35] A contradictory finding was made in another study of obese participants taking a much lower caloric LCHF diet and in this case, there was an improvement of inflammatory markers, CRP, total adiponectin, high density lipoprotein levels but no difference in TNF-α.[12,36]. Previous studies also showed the presence of high levels of inflammatory cytokines in obesity.[37-39] Adipose tissue responds to a high intake of calories by increasing the volume and the size of adipocytes. With the increase in the size and volume of adipose tissue the blood supply becomes limited which then increases a demand for oxygen in the adipose tissue. This results in hypoxia and eventually infiltration of macrophages into the adipose tissue. The consequence of this is the increased stimulation and production of pro-inflammatory factors. The subsequent inflammation in the adipose tissue culminates into systemic inflammation. The systemic inflammation is linked to the development of obesity-related comorbidities such as metabolic syndrome and T2DM.[40] Multiple factors produced by adipose tissue can modulate inflammation, atherosclerosis, and blood coagulation. These factors are known as adipokines and include TNF-α, IL-6, CRP, plasminogen activator inhibitor, adiponectin, and leptin.

In obesity, TNF-α levels are high and decrease with weight loss. It influences metabolism of lipids and insulin signalling in adipose tissue. It also stimulates the release factors like IL-6 while suppressing the release of anti-inflammatory factors. The increased levels of TNF-α result in oxidative stress.[41]

IL-6, a central cytokine that functions in the immune response, activates coagulation by increasing tissue factor expression by monocytes. IL-6 also promotes the production of CRP by the liver, a protein that indicates an increased systemic

inflammatory response.[42] Elevated levels of CRP is a predictor of coronary heart disease and diabetes.[41]

Plasminogen activator inhibitor is a factor that plays a key role in coagulation by inhibiting fibrinolysis of clots.[43]

Leptin is a hormone that is increased with obesity. It stimulates oxidative stress systemically. [44]

Adiponectin is an anti-inflammatory cytokine which is decreased in obesity, suppresses inflammation and counteracts insulin sensitivity.[44]

Evidence from human and animal studies established an association between inflammation and IR.[45] However, mechanisms linking the two pathways are yet unknown. IR is where the body is unable to respond to the insulin it produces. People with IR are prone to develop T2DM. IR is known to be an integral aspect of metabolic syndrome, which also includes features like obesity and hypertension.[45] Insulin serves important functions in glucose homeostasis, lipid and protein metabolism. In the endothelium, insulin increases the availability of nitric oxide thereby stimulating vasodilation. [46]

2.2 Inflammation and Coagulation

Inflammation and coagulation are intricately linked processes that affect each other as shown in Figure 2.1.[47] Inflammation is known to influence coagulation by increasing production of coagulation proteins, decreasing activity of anticoagulant pathways and impairing fibrinolysis.[48-49] Tissue factor (TF) is a principal initiator of coagulation and seems to play an essential role in the relationship between the two. Under inflammatory conditions monocytes express TF on their surface.[50] Inflammatory cytokines like TNF-α, IL-6 act as mediators of inflammation, activating the coagulation cascade. They do this by increasing expression of TF and this results in formation of fibrin facilitated by thrombin release.[51-52] Also TNF-α decreases the expression of the anticoagulant pathway and fibrinolysis is impaired. Therefore, inflammatory cytokines play a role in increased activation of coagulation. The increased activation of coagulation could lead to hypercoagulability and increased formation of clots.

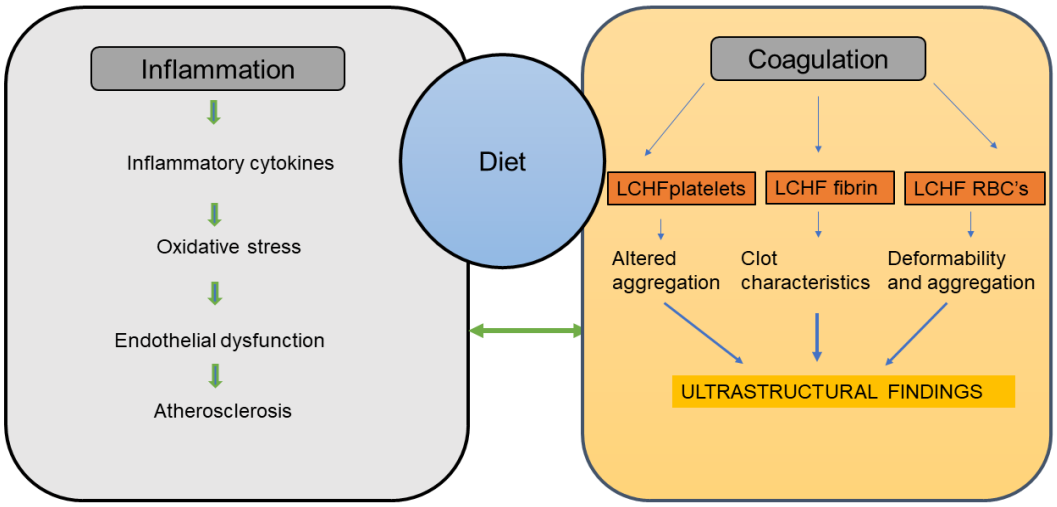

Figure 2.1: An outline showing potential effects of diet on inflammation and coagulation parameters. Redrawn from (Esposito and Giugliano,2006).[21]

Coagulation forms an important part of haemostasis and the process is well known. Haemostasis is a process that stops bleeding when there is an injury of tissue.[53] It has two main components, namely, primary and secondary haemostasis. Primary haemostasis refers to platelet aggregation and formation of a platelet plug.[54] The formation of the fibrin clot involves two complementary processes: coagulation, where thrombin is activated and activation of platelets at the site of injury. Activation of platelets at the injury site involves tethering, rolling, adhesion, secretion and thrombus formation.[55] At the site of injury, platelets adhere with subendothelial matrix mediated by interaction of receptors such as glycoprotein (GP) Ibα and Von Willebrand factor (vWF) followed by activation of platelets by collagen through binding to its receptor GPVI.[56] This binding triggers the conversion of integrin receptors to their high affinity modes, thereby enabling binding of fibrinogen to GPIIb/IIa which is important for platelet bridging and platelet aggregation.[57] Platelets become degranulated by secreting their granule contents like adenosine diphosphate (ADP), thromboxane A2 (TxA2) and thrombin. The secretory products such as ADP binds to G protein-coupled receptors (GPCR) and activates them which then strengthens the growing thrombus.[58] More platelet shape changes and secretion of products occurs as to recruit more platelets to a growing platelet plug.

After formation of the platelet plug, fibrin networks are formed to create a stable platelet-fibrin plug. Secondary haemostasis is initiated to heal the wound. Two

pathways can initiate the formation of fibrin clot, namely, the extrinsic and intrinsic pathway which merge to a common pathway as summarized in Figure 2.2.

In the extrinsic pathway, TF triggers thrombin generation by binding to activated form of Proconvertin (FVII) present in the blood.[59] The two form a complex (FVIIa/TF) with calcium called the tenase complex, which then activates FIX and FX.[60] Activated FX (FXa) then converts prothrombin to thrombin in the common pathway described afterwards.

In the intrinsic pathway, activated platelets trigger the conversion of FXII to its activated form, FXIIa. FXIIa, along with calcium activate FXI to FXIa. The FXIa and calcium subsequently activate FIX to FIXa (A small amount of thrombin is generated which can also convert FVII to FVIIa and FV to FVa). FIXa, in the presence of FVIII activated by platelet phospholipids and calcium, converts FX to FXa.

In the common pathway, platelet phospholipids, calcium, activated FV (FVa) as well as FXa generated from both pathways activate prothrombin,[61] converting it to thrombin. Thrombin, in the presence of calcium, will then cleave FXIII (to form FXIIIa) and fibrinogen to fibrin.[62] FXIIIa then promotes cross-link of fibrin polymers forming a stable fibrin network.[63]

To prevent unnecessary clot formation, blood coagulation has to be regulated.[64] Activation of coagulation is regulated by three major anticoagulant pathways, including: Antithrombin (a serine protease inhibitor of thrombin, FXa and FIXa),[65] the protein C pathway (which inhibits FVa, FVIIIa) and tissue factor pathway inhibitor (a natural inhibitor of TF/FVIIa complex at the initiation phase).[66] During inflammation-induced activation of coagulation, the function of all three pathways can be impaired.[47] These alterations lead to imbalanced haemostasis and an increased risk of thrombosis.

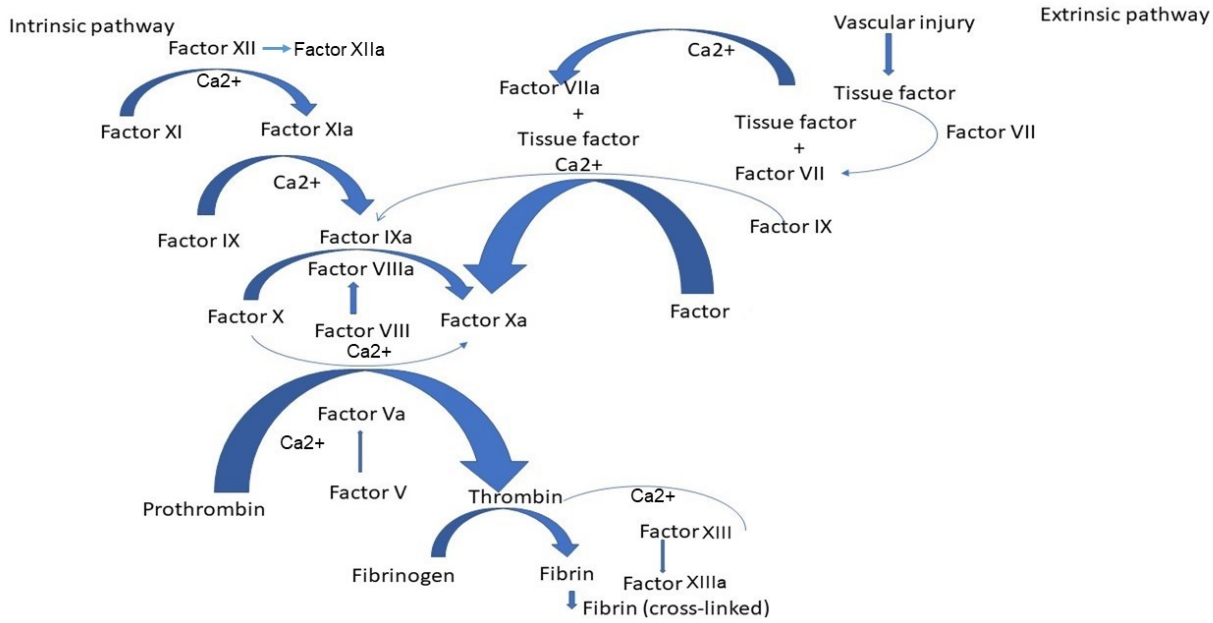

Figure 2.2: A diagram showing the coagulation pathway, where thrombin initiates the conversion of fibrinogen to fibrin clot formation. Redrawn from (Swanepoel et al 2015).[63]

2.3 Roles of Fibrinogen and Fibrin

Fibrinogen plays a major role in thrombosis, haemostasis and inflammation.[67] Fibrinogen serves as a precursor to fibrin. For the clot to expand and to gain strength, fibrinogen must be converted to fibrin.[68] Fibrinogen circulates in plasma at a concentration of two to five mg/mL over a period of approximately for four days. However, during severe inflammation fibrinogen levels increase and exceed approximately to seven mg/mL.[69] The strength of the clot depends on thrombin cleaving fibrinogen into fibrin monomers, which then form fibrin polymers. Fibrin plays a vital role in haemostasis as both the main product of the coagulation cascade and a substrate for fibrinolysis. [17]

In coagulation, fibrinogen increases blood viscosity.[68] It also acts as a bridge between platelets by binding to GPIIb/IIIa receptor on platelet surface, resulting in platelet aggregation and eventually leading to thrombus formation.[70] Fibrinogen is also a substrate for thrombin which converts it to fibrin, the final step in the coagulation cascade. After tissue damage, fibrin strands cover the damage site, by protecting the tissue while it heals. Together, the platelet aggregates and cross-linked fibrin form a stable clot, which covers the site of injury, and prevent excessive blood loss. The fibrin

network also serves as a framework for the binding of endothelial cells, leukocytes, platelets, and plasma proteins to the clot.[71]

During inflammatory conditions, circulating fibrinogen, FVII, FVIII as well as TF are elevated which might increase predisposition to clot formation.[72] The increased fibrinogen, FVII, FVIII as well as TF are associated with both inflammation and IR.[73] The concurrent increase in circulating TF and FVII might also increase risk of initiation of the coagulation cascade. FXII has been linked with clot compactness suggesting that the elevated level of FXII increases clot formation by forming a denser clot.[74]

With inflammation and cardiovascular events clot resolution is impaired. During thrombotic events elevated levels of fibrinogen and oxidative stress causes formation of abnormal fibrin fibers. These abnormal fibrin fibers have been detected as dense matted deposits (DMDs) and RBCs may be trapped in the mesh of these fibers.[75] Formation of tighter clots with impaired fibrinolysis have been detected in conditions such inflammatory diseases.[76-77] Evidence has shown that one of the features of inflammatory conditions is hypercoagulability, which is defined as abnormal coagulation and is a risk factor for thrombosis.[78-80] Elevated levels of fibrinogen is linked with hypercoagulability and this can result in a changed clotting profile.[79] Researchers have shown how fibrin is changed in inflammatory conditions.[80-81] Instead of a typical net appearance seen in healthy individuals, the fibrin fibers form DMDs with fiber diameters being smaller when compared to normal healthy fibers.[82] These DMDs may be the cause of higher prevalence of thrombotic events.[83] Usually with healthy fibrin clot fibers there are both thick major and thin minor fibers with the thick fibers being prominent.[84]

2.4 Role of RBCs in coagulation

RBCs are responsible for oxygen transport throughout the body, however, they are also active in clotting processes.[85] RBCs contribute to coagulation in the following ways: (a) by increasing blood viscosity in shear stress and (b) aligning of platelets close to the site of injury.[86] Evidence suggests that the RBCs increase coagulation factors and platelet aggregation.[87] Mature RBCs contain a plasma membrane with proteins, lipids and carbohydrates.[88] The proteins connect the bilayer with integral membrane proteins.[88] The outer bilayer is made up of membrane lipids including phospholipids, glycolipids and cholesterol. Four major classes of phospholipids are

arranged asymmetrically, PS and phosphatidylethanolamine on the inside and phosphatidylcholine and sphingomyelin on the outside.[88] Cholesterol is distributed equally between the phospholipid bilayer. The phospholipids and cholesterol play a key role in the structural integrity and stability of RBC membrane.[15] Disruption of the lipid asymmetry leads to externalization of PS, which is thought to contribute to the premature destruction of RBCs.[88] Effective blood coagulation involves an adequate prothrombotic surface for the assembly of FXa and FVa. These factors catalyse the conversion of prothrombin to thrombin. The prothrombotic surface can be provided by cells that express PS, a negatively charged phospholipid. It was thought that only activated platelets can express PS. However, in conditions like apoptosis, inflammation or oxidative stress RBCs lose their membrane asymmetry resulting in the exposure of PS.[89] Thus, a percentage of RBCs express PS on their outer surface and this helps in the assembly of coagulation factors FXa and FVa, which catalyzes formation of thrombin from prothrombin ultimately resulting in clot formation. *In vitro* studies have also shown that RBCs can support thrombin generation and promote fibrin deposition during venous thrombosis.[90] RBCs play an additional role in fibrinolysis by suppressing plasmin generation and inhibiting clot dissolution.[91] They also increase the sizes of pores in the fibrin network structure but decrease fibrin network permeability.[92-93]

2.4.1 RBC deformability

Deformability refers to the ability of the RBC to change shape.[94] Under shear stress, healthy RBCs are flexible and can deform from biconcave-discoid shapes to elongated structures enabling them to pass through tiny capillaries. Impaired RBC deformability has been associated with inflammatory diseases like T2DM.[95] Inflammatory conditions such as T2DM, rheumatoid arthritis (RA), hereditary hemochromatosis, Parkinson's disease and Alzheimer's disease (AD), and stroke impair RBC deformability and alter RBC membrane properties such as rigidity, stiffness and elasticity.[95-96] Oxidative stress and chronic inflammation are known to decrease RBCs deformability that alters RBC morphology.[97]

2.4.2 RBC aggregation

RBC aggregation is associated with inflammation.[98] Increased RBC aggregation is related to increased plasma fibrinogen.[98] An increase in plasma concentration of fibrinogen can result in RBC aggregation associated with thrombosis.[86] Inflammatory

conditions are reported as examples of clinical situations with increased RBC aggregation.[98-99] The fibrinogen molecules attach to the membrane surface of RBC and this encourages RBC aggregation.[100] The attachment of fibrinogen to the RBC membrane not only increases RBCs aggregation but also blood viscosity is influenced. An increase in blood viscosity makes it difficult for RBCs to move through microvessels, resulting in decreased blood flow in numerous clinical states.[101-102] An increase in blood viscosity and fibrin concentration correlate with and are predictors of cardiovascular diseases and are key factors in the development of atherosclerosis.[103-104] Atherosclerosis is an inflammatory disease caused by the formation of plaque inside the artery walls.[43] The plaque is likely to contain RBCs, fibrinogen, and platelets. The stages of atherosclerosis includes immune cells with reduced nitric oxide and increased levels of ROS which causes endothelial dysfunction.[105]

2.5 Role of Platelets in Coagulation

Platelets are not only central to primary haemostasis but are also involved in secondary haemostasis where they assist in coagulation, thrombin generation and fibrin formation. Platelets activate coagulation by interacting with glycoprotein receptors or through exposed phospholipids on the surface of the platelets following activation. During injury, vWF binds to platelet receptor GPIb/IX/V that causes activation of platelets. The organization of the phospholipids between the inner and outer leaflets of the platelet membrane is asymmetrical and this regulates coagulation.[106] When platelets are in resting state PS along with anionic phospholipids are located on the inner leaflet of bilayer.[107] Upon platelet activation with thrombin and collagen or shear stress, PS moves from inside to outer leaflet of the platelet membrane. The movement enables thrombin generation by an increase in the activation of prothrombin to thrombin and FXI to FXIa and provides a negatively charge surface binding sites for FVa and FVIIIa.[108] These are both integral steps in the coagulation cascade. Also, platelets take over the initiating role of TF and FVIIa in the coagulation cascade.

When platelets adhere to high concentrations of collagen via GPVI, they expose PS at their outer surface and when the PS is exposed, there is a distinctive change in platelet shape and aggregation.[109] During this shape change, platelets lose most of their cytoskeletal structure, round off to balloon-like structures forming membrane

blebs. The membrane bleb is a bulging or protrusion in the plasma membrane of the cell that is caused by decoupling of the cytoskeleton from the plasma membrane.[110] The blebbling of plasma membrane is a morphological feature of cells that are undergoing late stages of apoptosis.[110] Platelets that undergo severe blebbing in this manner generate microparticles which also contribute to thrombin generation which increase coagulation in blood.[111]

Platelets also function as storage sites for proteins involved in blood coagulation and its regulation.[59] The signalling events that are involved in platelet activation also induce a conformational change in the GPIIb/IIIa complex, enabling it to bind to vWF and fibrinogen, which strengthens the clot and induces platelet aggregation.[112] Platelets also provide a framework for the formation of fibrin fibers. Once the fibrin clots are formed, they regulate the process of clot dissolution.[113]

2.5.1 Platelet morphology and function

Platelets are the smallest blood cells with a diameter of two to three micrometers (μm), discoid in shape, anucleate with a mean cell volume of seven to ten femtolitre and live for eight to ten days. Platelets are formed from precursor cells called megakaryocytes in the bone marrow. Their normal count is (150–400) × 10^3 per microliter in whole blood (WB).[114]

Platelets perform several functions in blood coagulation. When there is injury to blood vessels, platelets respond by changing shape, secreting their granule contents and progressively adhere to each other by forming aggregates.[115] Platelets function to minimize blood loss when endothelial injury occurs and also to maintain vascular tone, participate in inflammation, host defence and tumor biology.[116]

A visible component of the plasma membrane of platelets, are indented invaginations called open canalicular system (OCS), a tunnel that is present throughout the cell and is connected with plasma membrane. OCS performs three vital functions: i) it allows external elements to enter into platelets and facilitates the release of its granule contents to the outside; ii) it is a main storage site for plasma membrane glycoproteins; iii) during platelet activation, it enables formation of filopodia.[117] Present in platelets are a closed network channel called the dense tubular system (DTS) of residual endoplasmic reticulum which function to sequester calcium ions.[118] The discoid shape of a resting platelet is characterized by a well-defined and highly specialized

cytoskeleton and this is maintained by three major components including the spectrin-based membrane skeleton, the actin cytoskeleton, and the marginal microtubule coil. Besides mitochondria, peroxisomes, and lysosomes, granules are also present. Platelets are characterised by a variety of biologically active molecules in their granules. These molecules are essential for coagulation and inflammation.[119] There are two main granules namely alpha and dense granules. The most abundant granules are alpha (α) granules, and they store most proteins that are important for platelet adhesion such as fibrinogen. Dense granules function primarily to recruit more platelets to a site of injury. Also stored in the dense granules are some molecules secreted to induce further platelet activation. The molecules in dense granules include catecholamine, serotonin, calcium, adenosine 5′-diphosphate (ADP) and adenosine 5′-triphosphate (ATP).[120]

Platelets contain membrane receptors that are integrated within their outermost layer of the platelet membrane. The receptors include GPIIb, GPIV, GPV and are an important component of platelet function. These receptors bind adhesive agents, aggregating agents, inhibitors and procoagulant factors essential to normal platelet function.[121-122] GPIIb/IIIa is a protein that binds fibrin and fibrinogen thus facilitating platelet-platelet interaction, while GPIa/IIa facilitates adhesion to collagen.[123-124] Platelets contain a number of active molecules stored in their granule contents. In case of tissue injury the molecules are delivered to an injury site and their role is to recruit other cells.[120] Before platelet activation, these granules are in close proximity with the OCS. With platelet activation, the granules fuse with the platelet plasma membrane and are secreted into the OCS.[125] Alpha granules contains proteins like GPIIbIIIa, P-selectin, and CD36.[125] Upon platelet activation an adhesive protein (P-selectin) redistributes from its storage site in alpha granules and is exposed at the platelet membrane surface. Once P-selectin is on platelet membrane surface, it recruits neutrophils via neutrophil receptor P-selectin glycoprotein ligand (PGSL).[126-127] The increased expression of P-selectin on the membrane and in plasma occurs during thrombin-induced platelet activation.[128] CD36 reacts with oxidized lipids in hyperlipidemic mice, enhancing prothrombosis and increasing risk for atherosclerosis.[129]

The organization of phospholipids between the inner and outer layer is asymmetrical and this enables platelets to regulate coagulation.[120] Present on the inner layer are an

abundance of negatively charged phospholipids, which maintain the platelet surface in a non-procoagulant state. The phospholipids also play a role in coagulation by activating coagulation FX to Xa and prothrombin to thrombin eventually leading to formation of the clot.[130] Also expressed on the membrane surface of platelet following activation are different surface receptors such as CD63 and CD9.[114] CD63 is a lysosomal integral membrane protein. When the platelet is in an inactive state, CD63 is present on the plasma membrane of dense granules and lysosomes. Upon platelet activation, CD63 relocates to the plasma membrane where it binds with platelet integrin $\alpha IIb\beta_3$-CD9 complex.[131] CD63 enables activated platelets to spread over fibrinogen. CD63 also allows the activated platelet to form a complex with the platelet main fibrinogen receptor (GPIIb/IIIa/$\alpha IIb\beta_3$) and CD9. Figure 2.3 is a scanning electron micrograph of a healthy platelet. The membrane of platelets is filled with surface receptors that play a role in signal-dependent platelet activation.

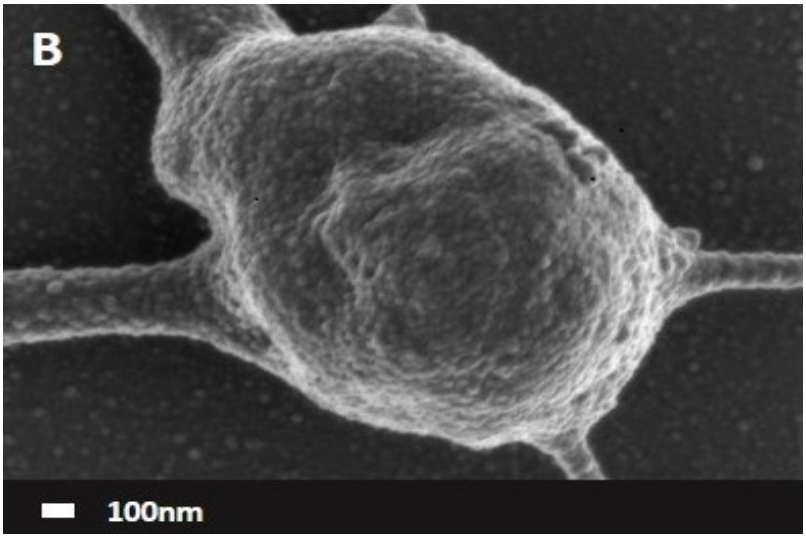

Figure 2.3: Scanning electron micrograph (SEM) of WB showing a platelet from a control participant on a relatively healthy diet inclusive of carbohydrates. 100X magnification of platelet with slightly projected pseudopodia. Scale= 1µm.

2.5.2 Platelets, Inflammation and atherosclerosis

Platelets play an important role in conditions such as inflammation, atherogenesis and atherothrombosis as they adhere to vasculature by releasing their bioactive mediators. Studies have shown that some markers of platelet activation are elevated in diabetes and obesity.[132-133] These markers include mean platelet volume (MPV), soluble CD40 ligand and P-selectin. The MPV is a marker of interest as it is linked with *in vivo* platelet activation, hence platelet activation is associated with diseases that involve thrombosis and inflammation.[133] Increased MPV has been observed in diabetic

patients, hypertension, hypercholesterolemia, smoking and obesity.[134-135] Furthermore, a number of studies have reported MPV as a marker for predicting CVD risk.[134-135] Of interest to our study is the inflammatory marker, sCD40 ligand which is released from activated platelets and is also increased in people with diabetes and CVD.[136] Also, important to our study is a protein called P-selectin, which is synthesized by platelets and stored in α-granules. During the inflammatory response, this protein is released onto the platelet surface.[136-137] P-selectin and CD40L are important to the platelet's capacity to form aggregates with each other, with leukocytes and facilitates their adhesion to the endothelium.[138-139] These proteins, thus facilitate platelets participation in atherothrombosis.

Research performed on rats fed a HFD, showed that the HFD alters platelet aggregation and signalling through its ADP receptors.[140]

2.6 The Banting Diet in South Africa

In South Africa, one of the popular versions of the HFD currently trending among individuals avoiding the pitfalls of high carbohydrate intake is the Banting diet. The promoter of this diet is Tim David Noakes, a medical doctor and scientist with an extensive background in Exercise Physiology who took an interest in dietary impact on this field. Dr Noakes named the diet after an eminent Englishman who popularized the diet following successful application of it in overcoming obesity. His curricular and extracurricular advocacy for limiting carbohydrate intake via scientific publications and participation in popular sports made him a respected icon in South Africa as well as internationally. Several of his publications provide insight into research outcomes showing the impact of limiting carbohydrates (and or high fat intake) on physiological status of individuals involved in sports [141-143],affected by obesity[10,144] or psychological health.[145-146] Due to the prominence of the Banting diet, being the most common LCHF diet in South Africa, we focused on selecting individuals on this diet as participants for the LCHF group in our study.

CHAPTER 3: (SCANNING ELECTRON MICROSCOPY)

3.1 Aims and Objectives

The aim in this chapter is to demonstrate the differences between ultrastructural findings on RBCs, fibrin, and platelets in participants on an LCHF diet compared to controls.

To achieve this aim, our objectives are to:

- Apply scanning electron microscopy (SEM) to examine cells within blood samples obtained from individuals in both groups
- Generate and present findings as qualitative and quantitative data using relevant software
- Use statistical software to compare quantitative data outcomes from both groups
- Discuss differences detected between the groups so as to draw inferences on how diet may affect the morphology of red blood cells, platelets and the overall structure of fibrin fibers.

3.2 Introduction

Electron microscopy started with the new venture of electron optics first used in 1926 when Busch studied the trajectories of charged particles in axially symmetric electric and magnetic fields. In 1963, Pease and Nixon collectively put all the improvements in one instrument: the SEM V with three magnetic lenses and an Everhart–Thornley detector.[147] From the original instrument in 1965 a first from first commercial SEM, was developed in 1965.[148] Therefore the principle behind the SEM as is used now, is not very different from the first instrument except in better resolution at higher levels of magnification. Currently SEM is a widely used instrument to study in detail the surface structure of samples of interest. SEM is an evolving and a very sensitive ultrastructural technique that can convey very specific changes to individual cells and fibrin packaging, involved in clotting.[85,149]

The SEM used in the current study can produce a very high magnification of up to 1,000,000 x magnification to and is able to reveal the details of cells up to less than ten nanometers. This allows the study of the surface properties of a cell producing an image of high quality.

RBCs are very specialized cells which are round, discoid in shape and whose main function is to transport oxygen throughout the blood. RBCs also play a vital role in coagulation and inflammation [150]. RBCs are different from most cells due to the fact that they are without organelles such as nuclei, mitochondria and ribosomes. Their plasma membrane serves as their most revealing feature when studying age related alterations changes in terms of structure and function. The fact that they are devoid of organelles makes them sensitive to oxidative stressors.[151] RBCs are also sensitive to increased inflammatory cytokines and in a number of diseases this is associated with inflammation.[15] The presence of inflammation alters RBC structure including shape, elasticity, structural deformability and changes in blood rheology/viscosity. In a study recently done on HFD rats, it was reported that chronic high fat feeding in mice induced structural, biochemical and functional alterations in RBCs.[152] During inflammatory conditions more cells are different from the normally shaped RBCs (discocytes) due to change to their shapes and sizes. The term that is used for the diverse shapes is poikilocytosis and these include leptocytes, stomatocytes, knizocytes, echinocytes, acanthocytes and dacrocytes.

Table 3.1: RBC morphology with description.

RBC morphology	**Description**
Discocytes	Have a biconcave shape with round hollow depression in the center.[153]
Leptocytes	Are thin flat cells, peripheral with a large diameter, center area filled with hemoglobin.[154]
Stomatocyte	Are oval with cup-shaped with a c-shaped central cavity.[153]
Knizocytes.	Have a pinched appearance in the center and the area can be increased.[155]
Echinocytes	Have short spicules and the spicules are spaced symmetrically.[156]
Acanthocytes	Have irregularly shape projections which are asymmetrical.[157]
Dacrocytes	Have a tear-drop shape with one end round and the other one pointed.[157]
RBC Agglutination	RBCs are clumped together forming a stack.

Platelets perform several functions, one of their primary functions being to stop bleeding. When there is damage to blood vessels, platelet activation respond by changing shape, spreading, secreting their granule contents, progressively adhering to each other, forming aggregates and eventually forming a clot.[158] The formation of the clot minimizes the loss of blood at the site of injury. Activation of platelets result in platelet degranulation where different molecules are released. This is to recruit more platelets to support in the process of tissue repair.[63] Platelet signalling pathways involved during platelet aggregation includes G_i-coupled platelet receptor for adenosine ($P2Y_{12}$) which activate platelets by stimulating the release of ADP from dense bodies.[72,159] The release of ADP then stimulate platelet shape change forming pseudopodia.[160] The anti-aggregatory effect of insulin has been demonstrated in platelets. In healthy people insulin has been shown to inhibit platelet aggregation by suppressing the $P2Y_{12}$ receptor.[161] A recent study in rats also showed that HFD alters platelet aggregation and purinergic signalling.[140] Using SEM, we can detect shape changes that are associated with hyperactivity and hyper-aggregation.

Also involved in coagulation are fibrin networks that form during the secondary haemostasis where they form a network over the wound and protects it as it heals. During tissue injury, fibrin networks form a loose net-like structure that acts as a plug to seal the injured tissue.[63] Usually with healthy fibrin clot fibers, there are both thick major and thin minor fibers with the thick fibers being predominant.[72] For the fibrin network to be formed, thrombin is needed to convert fibrinogen to fibrin. The concentration of thrombin is so important in fibrin formation due to its effects on the thickness and density of the clot.[162] Thrombin plays a very important role in regulating blood clotting where increased levels in blood can lead to hypercoagulability, increased clot stiffness, and inhibit fibrinolysis.[17] Hypercoagulability is a feature of inflammation and its presence can result in dense fibrin clots composed of abnormally highly branched thin fibers.[163-164] Visible characteristics such as dense fibrin clots and highly branched thin fibers were shown to be related to coronary heart disease.[165]

3.3 Materials and Methods

3.3.1 Participants

Sample population

Ethical clearance for the study was obtained from the University of Pretoria, Faculty of Health Sciences Research Ethics Committee. Sixty-four individuals volunteered as participants in this study. Thirty-two individuals on a LCHF diet were recruited from a market, selling products that are low in carbohydrates and high in fat, specifically established for people on the Banting diet (hereon termed “Banting Market”) in Pretoria, South Africa. The inclusion criteria were: (a) participants older than eighteen years willing to provide full written informed consent, (b) participants on LCHF diet for at least three months or more. Exclusion criteria included: a) smoking, b) excessive alcohol use (more than two glasses of red wine per day), c) chronic diseases (and those on medication) such as T2DM, hypertension, d) chronic and acute use of any medication. Familial hypercholesterolemia was considered a potential confounder, and so participants with the disorder were excluded from the study. All participants signed informed consent before any procedures were performed. Once LCHF diet participants were identified, they were asked to fast for at least twelve hours prior to their visit. We provided a questionnaire to obtain information on each participant’s diet history, medical history, smoking habits, alcohol intake and use of chronic medication. Height and weight were measured with the participants being in a standing position barefoot. Body mass index was calculated as weight in kilograms divided by the square of height in meters. The body mass index was categorized according to WHO cut-offs.[166] The systolic and diastolic blood pressure as well as heart rate were measured.

Blood sample collection

All procedures were performed on two groups: (1) Participants on a normal diet (control group) (2) Participants on the LCHF diet (LCHF group). Blood samples were collected from all individuals meeting the inclusion criteria. Blood was drawn by a qualified doctor upon completion of an informed consent form by each participant. Blood samples were collected via venipuncture from all participants. Two milliliters (ml) of blood from each participant was drawn into a citrate tube. Within thirty minutes of the collection of blood, sample preparation was performed on the samples for microscopic analysis.

3.3.2 Sample Preparation for SEM of RBCs, platelets and fibrin fibers

To prepare the WB samples for SEM analysis, ten microlitre (µL) of WB was pipetted from the sodium citrate tube and smeared onto a glass coverslip (LASEC, South Africa) and prepared in triplicate. To make smears for platelets, WB was centrifuged at 6.70 xg for fifteen minutes and ten µL of supernatant platelet-rich plasma (PRP) was pipetted onto a glass coverslip. To obtain fibrin fibers, the PRP was transferred to a 1.5 ml eppendorf tube and further centrifuged for ten minutes to obtain platelet-poor plasma (PPP). The PPP was used to make fibrin smears, ten µL of PPP was pipetted onto coverslip and to induce fibrin formation, five µL of thrombin (South African National Blood Services) was added.

All the samples on coverslips were incubated at room temperature for ten minutes. The samples were placed in a twenty-four well plate and washed with phosphate buffered solution (PBS) 0.075 M (pH = 7.4) for twenty minutes. For primary fixation, samples were fixed in 4% formaldehyde for thirty minutes. Following primary fixation, the smears were then washed three times in PBS for three minutes. For the secondary fixation, the samples were placed in 1% osmium tetraoxide (OsO_4) for fifteen minutes. The samples were again washed three times with PBS for three minutes. The samples were then serially dehydrated in 30%, 50%, 70%, 90% and 100% ethanol for three minutes each. The final dehydration step using 100% ethanol was repeated three times.

The samples were dried using hexamethyldisilazane and coated with carbon before being viewed with a Zeiss Ultra PLUS FEG SEM (Carl Zeiss Microscopy Europe, Germany). All the samples were viewed at the Microscopy and Microanalysis Unit, at the University of Pretoria. The software that was used to analyse fibrin fiber network was STATA.

3.3.3 Statistical Analysis.

The analyses of the fibrin clot network was performed using ImageJ (ImageJ is a public domain, Java-based image processing program developed at the National Institutes of Health (http://rsbweb.nih.gov/ij/) Images of thrombi-activated PPP clot matrices (triplicates or more) were captured at a magnification of twenty-thousand times for twenty-three controls and thirty-two LCHF participants. The captured images were subjected to ImageJ Histogram analysis, which yielded standard deviation and mean gray values. Coefficient of variation values were further derived by dividing the

standard deviation by mean values. Coefficient of variation serves to ensure that any variability in picture properties (like brightness and contrast) do not affect the derived values. These generated and calculated values served as matrices for comparing differences between visual properties of images captured for control and LCHF participants. A Mann-Whitney test was used to compare standard deviation and coefficient of variation values between the control and LCHF groups with significance set at 0.05. The GraphPad Prism software (version 5.03 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com) was used for statistical analyses.

3.4 Results

The dietary composition of LCHF participants were analysed as shown in Table 3.2 to get mean, standard deviation and IQR and the mean intake of fat was high followed by low mean carbohydrates and moderate mean proteins.

Table 3.2: Intake of fat, protein and carbohydrates in LCHF participants in grams, showing mean, SD, median and IQR analysed from percent.

Dietary	Mean ± SD	median	Median (IQR)
Fat	56.16 ± 17.04	60	20 (70-50)
Protein	31.47 ± 12.65	30	5.75 (30-24.25)
Carbohydrates	12.38 ± 8.809	10	9.75 (16-6.25)

Demographic information of the participants is shown in Table 3.3.

Table 3.3 shows demographic data of our study participants distinguishing controls and LCHF participants. Compared to controls, both female and male participants in the LCHF group were older. The mean body weight in kilograms (kg) for the LCHF group was lower in female vs male LCHF participants with a mean (76.5 vs 89.1). The body mass index in kilograms per meter squared (kg/m^2) was insignificantly different for female vs male LCHF participants with a mean of (28.5 vs 28.4). The blood pressure in millimetres of mercury (mm Hg) was within normal range, with a mean of females (125.3 vs 136.3) in males for systolic pressure while female vs male means were (80.6 vs 81.0) for diastolic pressure. The total cholesterol in millimoles per liter

(mmol/L) for LCHF participants was higher than normal with a median of 5.70 mmol/L in females compared to 5.67 for males.

Table 3.3: Demographics of study participants.

Variables		n (%)	Mean ± SE	Median [IQR]	Min, Max
Control Age	Females	16 (50)	33.3 ± 2.8	29 [24.5 to 42]	23, 57
	Males	16 (50)	32.8 ± 2.6	32 [25.5 to 38]	18, 60
LCHF Age	Females	24 (75)	42.6 ± 2.2	45 [34 to 48.5]	17, 64
	Males	8 (25)	49.9 ± 4.5	47.5 [42 to 57]	34, 72
LCHF body weight (kg)	Females	24 (75)	76.5 ± 16.8	74 [63.9 to 87.9]	
	Males	8 (25)	89.1 ± 22.2	84 [77.8 to 96.9]	
LCHF height (cm)	Females	24 (75)	1.64 ± 0.07	1.6 [1.58 to 1.69]	
	Males	8 (25)	1.77 ± 0.08	1.7 [1.75 to 1.83]	
LCHF body mass index kg/m^2	Females	24 (75)	28.5 ± 6.2	28 [23.9 to 31.2]	
	Males	8 (25)	28.4 ± 7.42	26 [24.2 to 29.7]	
LCHF waist circumference (cm)	Females	20 (63)	90.7± 16.9	90 [77.5 to 100.5]	
	Males	8 (25)	92.0 ±16.7	91 [78.8 to 104.8]	
LCHF systolic blood pressure (mm Hg)	Females	22 (69)	125.3 ± 17.9	126 [114 to 135.3]	
	Males	8 (25)	136.3 ± 23.7	127 [123.3 to142.3]	
LCHF diastolic blood pressure (mm Hg)	Females	22 (69)	80.5 ± 10.8	81 [71 to 86.8]	
	Males	8 (25)	81.0 ± 14.6	76 [69.5 to 92.3]	
LCHF pulse	Females	21 (66)	67.4 ± 11.3	66 [71 to 79]	
	Males	7 (22)	75.5 ± 7.72	75 [60.5 to 78.5]	
LCHF total cholesterol mmol/L	Females	22 (69)	5.70 ± 1.36	5.30 [4.85 to 6.23]	
	Males	8 (25)	5.67 ± 0.98	5.72 [5.36 to 6.17]	

SE=Standard Error, IQR= Interquartile Range

The results for SEM analysis were as follows:

3.4.1 SEM of RBCs

The majority of the participants in both groups had normally shaped discocytes as shown in Figure 3.1.

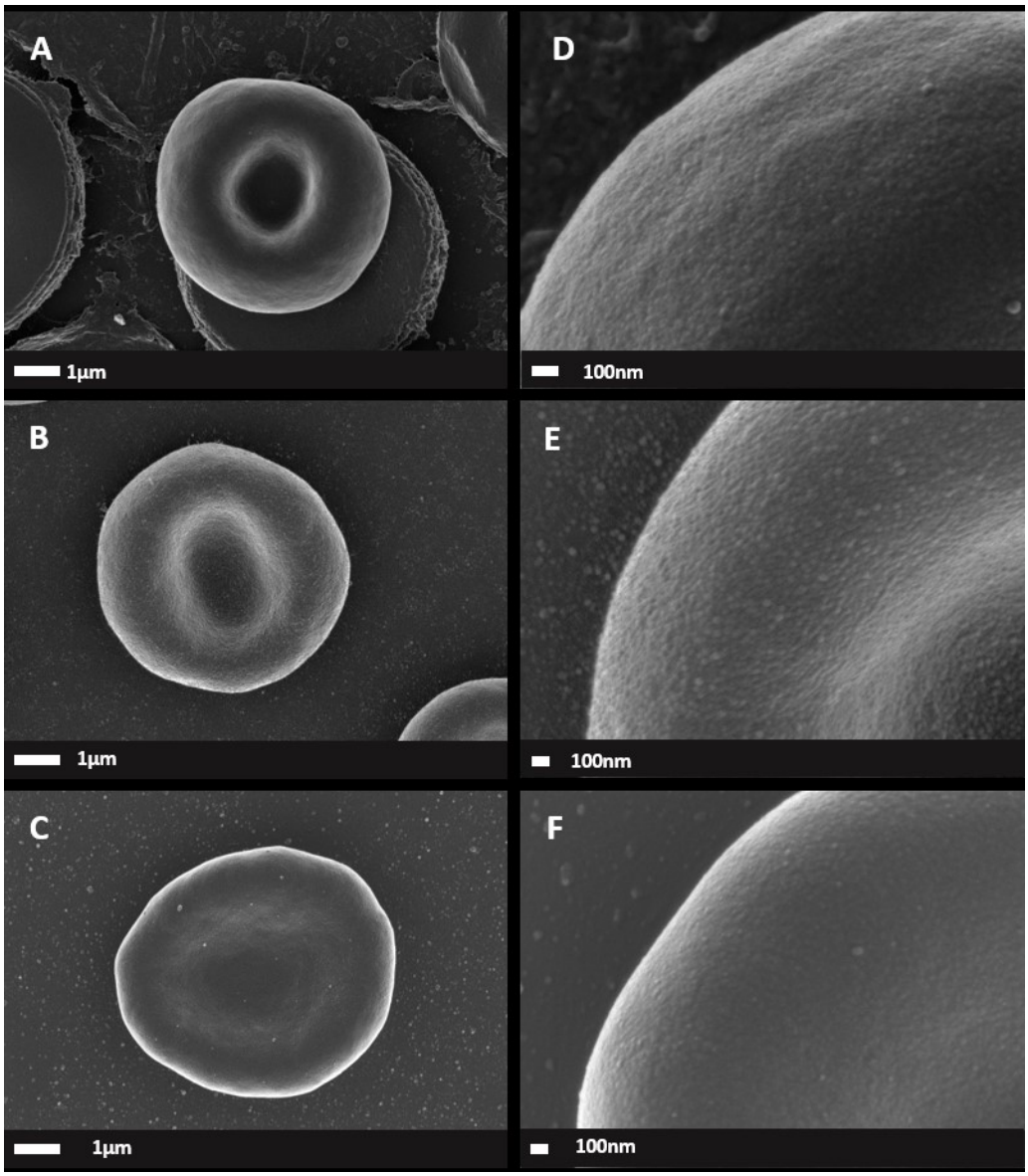

Figure 3.1: SEM images of RBCs in WB smear. Images on the left (A to C) are low magnifications of the same RBCs on the right (100X magnification). (A and B) Individuals on a control diet (C) Individual on an LCHF diet. High resolution images show minimal to no differences in membrane structure of cells and no microparticle budding. (Scale = 1 µm).

A few poikilocytes (primarily echinocytes) were seen among RBCs from controls and these are shown in Figure 3.2.

Other distinct poikilocytes were observed in LCHF participants as shown in Figure 3.3.

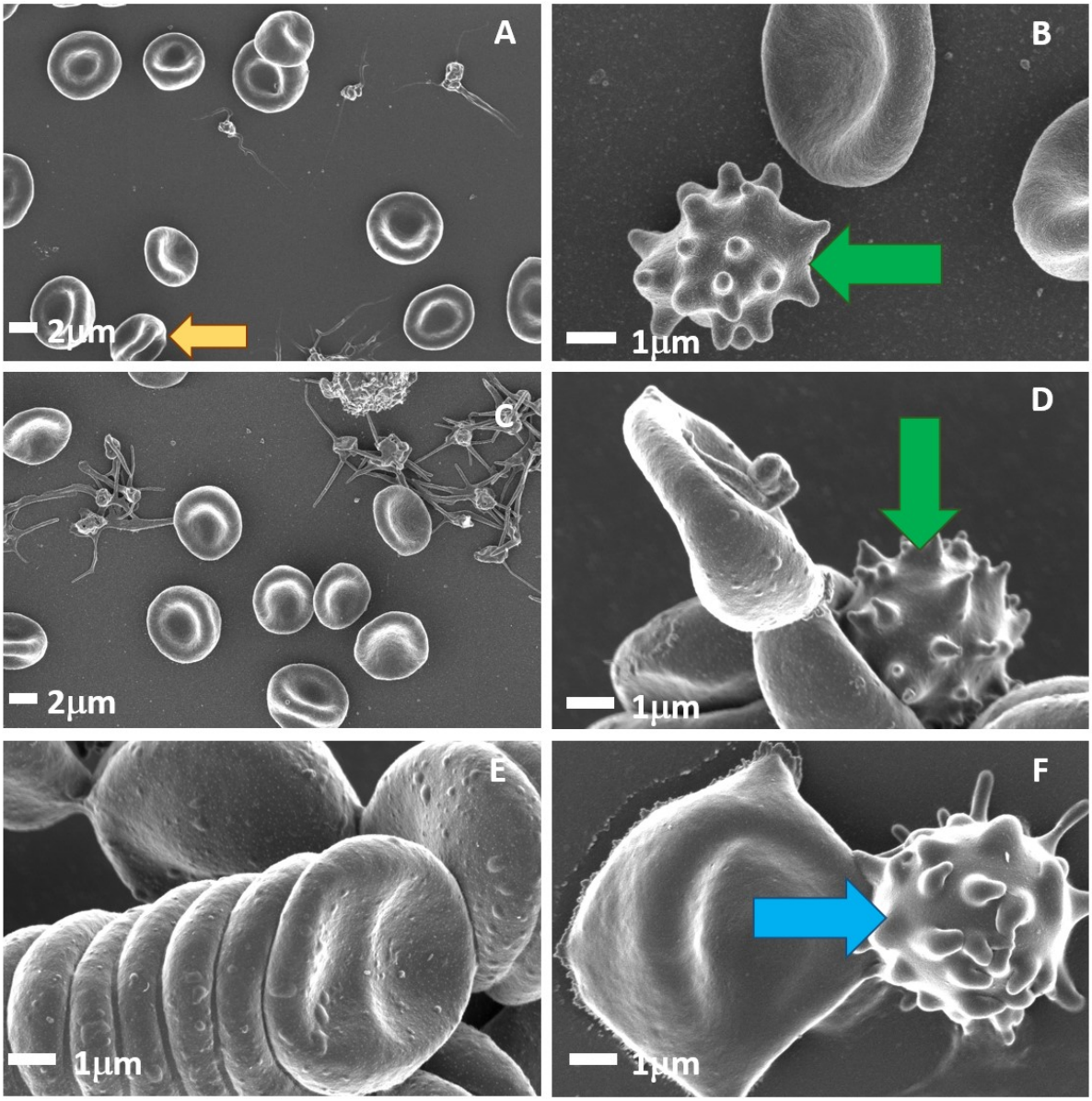

Figure 3.2: SEM in WB smear for controls (A) normally shaped discocytes, knizocyte (orange arrow), (B) Eryptotic echinocytes (burr cells) (green arrow), (C) A micrograph showing mostly discocytes and activated adjacent platelets, (D) Platelet attached to aggregated RBC, one of which is an echinocyte (green arrow), (E) Agglutinated RBCs (rouleaux), (F) Acanthocytes (blue arrow) attached to a boat cell.

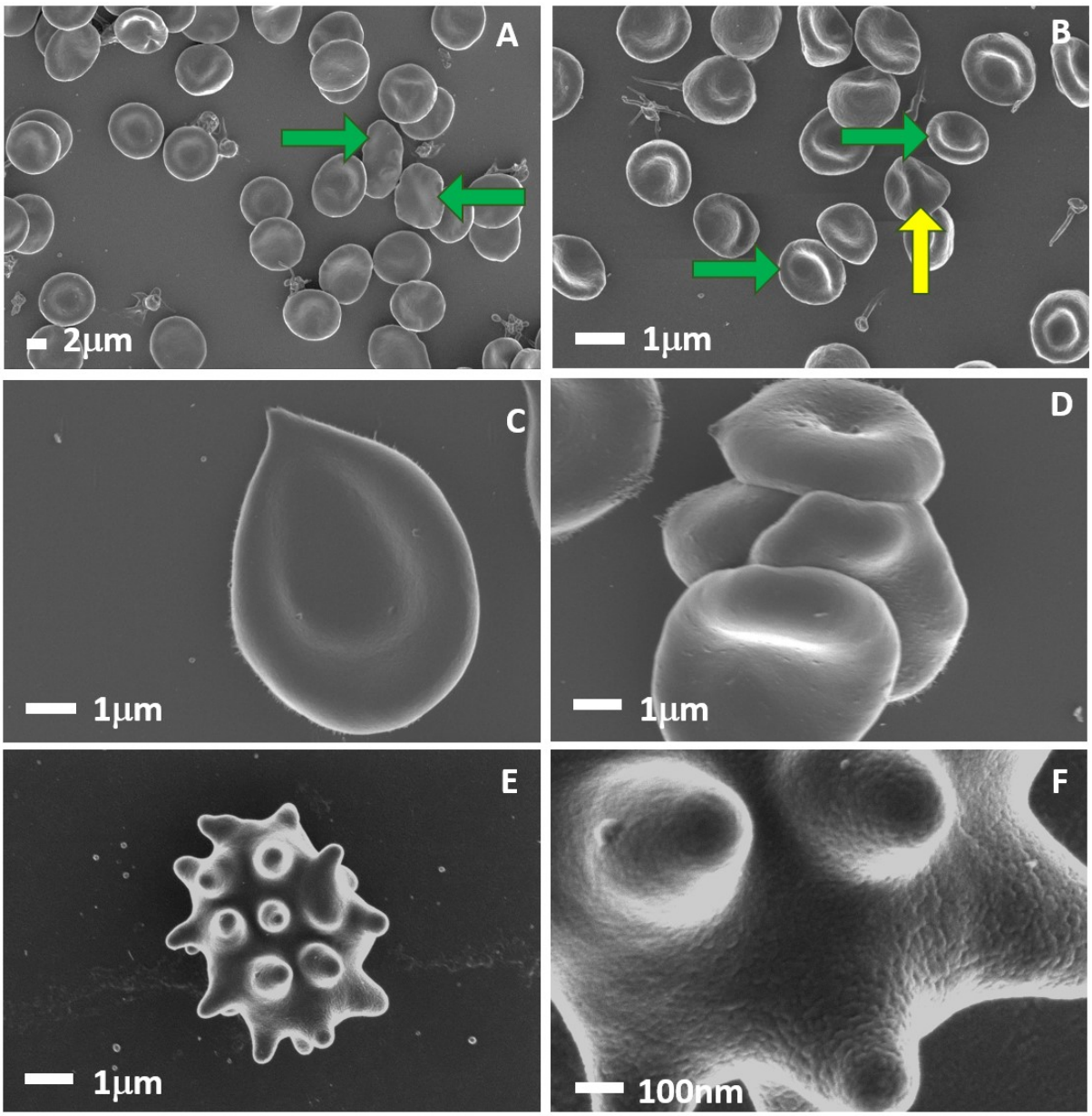

Figure 3.3: SEM of a WB smear from participants on an LCHF diet showing A: Mostly discocytes but there were some leptocytes (green arrows) alongside mildly activated platelets, B: Other distinct poikilocytes identified were the stomatocyte (green arrows) and knizocyte (yellow arrow), C: A micrograph showing a dacrocyte, D: A RBC aggregate, E: An echinocyte or burr cell, F: 100X magnified image of cell in E.

3.4.2 SEM of platelets

For participants on a healthy diet, platelets with some pseudopodia and minimally spread lamellipodia were detected as shown in Figure 3.4.

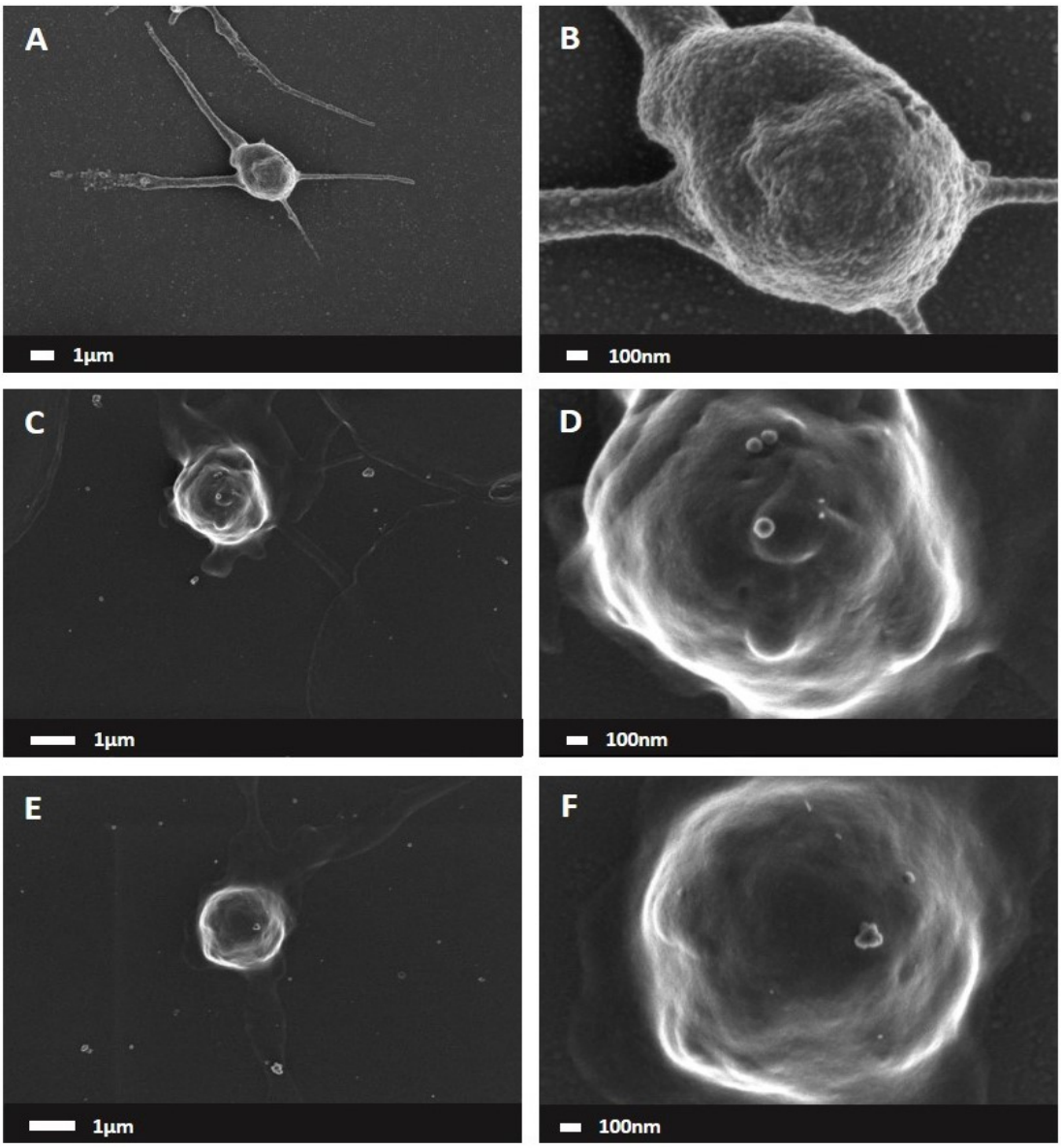

Figure 3.4: SEM images of WB showing platelets from control participants with relatively healthy diet inclusive of carbohydrates (A) Platelet with slightly projected pseudopodia, (B) Platelet in 'A' magnified by 100X, (C) and (E) Platelets with minimally spread lamellopodia, (D) and (F) Platelets in 'C' and 'F' respectively magnified by 100X. Scale = 1 µm.

Individuals on the LCHF diet also had mostly mildly activated platelets with minimally projected filopodia and minimal spreading of lamellipodia as shown in Figure 3.5.

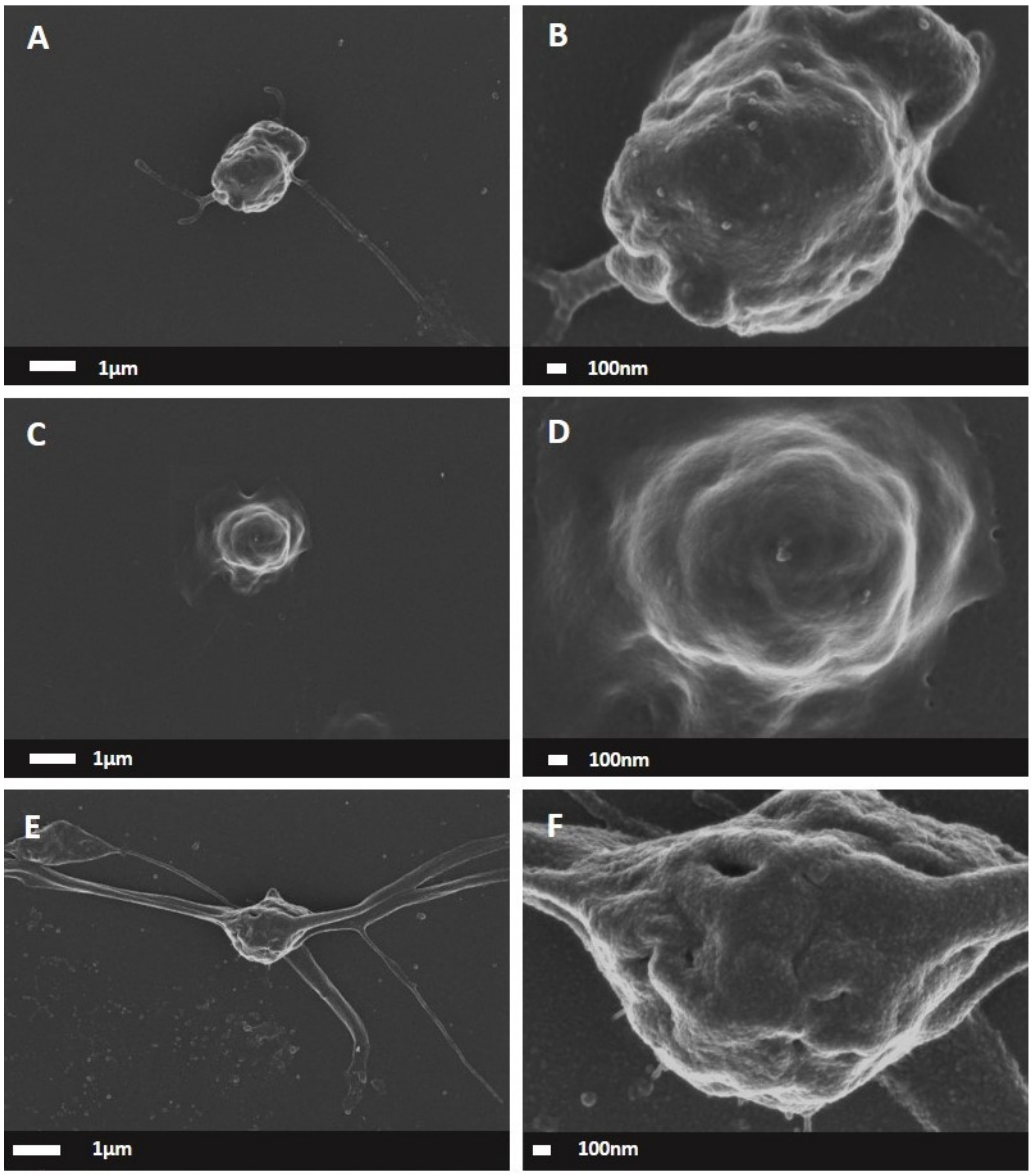

Figure 3.5: SEM images of WB showing platelets from participants on a LCHF: (A) Platelet with minimally projected filopodia (B) Platelet in 'A' magnified by 100X, (C) and (E) Platelets with minimally spread lamellopodia, (D) and (F) Platelets in 'C' and 'F' respectively magnified by 100X. Scale = 1 µm.

Also detected were platelet aggregates that appeared smaller in LCHF participants compared to larger aggregates detected in controls as seen in Figure 3.6 and this was further explored via flow cytometry.

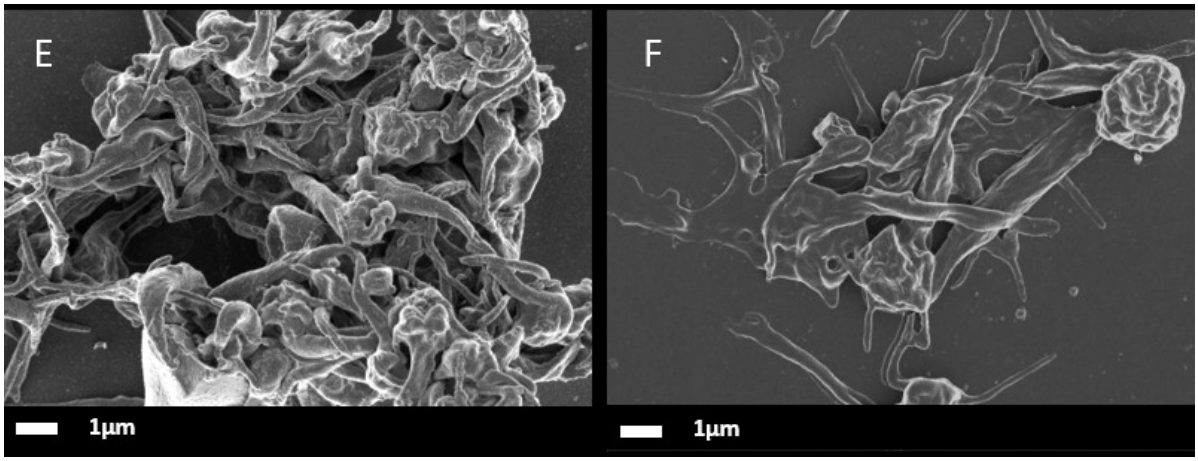

Figure 3.6: SEM of platelets in WB smears, (E) Large platelet aggregate in control smear, (F) Small platelet aggregate in LCHF smear. Scale = 1 µm.

3.4.3 SEM of fibrin fibers

The analyses of visible aspects of the clot complexity using histogram analyses showed statistically significant difference (P = 0.0005) between standard deviation of mean grey values of controls and LCHF participants as shown in Table 3.4. Clots from the LCHF group appeared to have greater complexity, shown in wider standard of deviation of grey values of the images captured.

Table 3.4: Analysis of standard deviation of grey values of fibrin clots in LCHF participants and controls.

Group	Mean ± SD	Median [IQR]	Min, Max
Controls	31.4 ± 10.7	29.8 [24.6 to 36.4]	14, 66
LCHF	33.0 ± 9.7	32 [26.9 to 38.2]	12, 59

When comparing clots from individuals on a control diet (Figure 3.7) with those on the LCHF diet, images captured revealed some differences in the appearances of PPP fibrin networks.

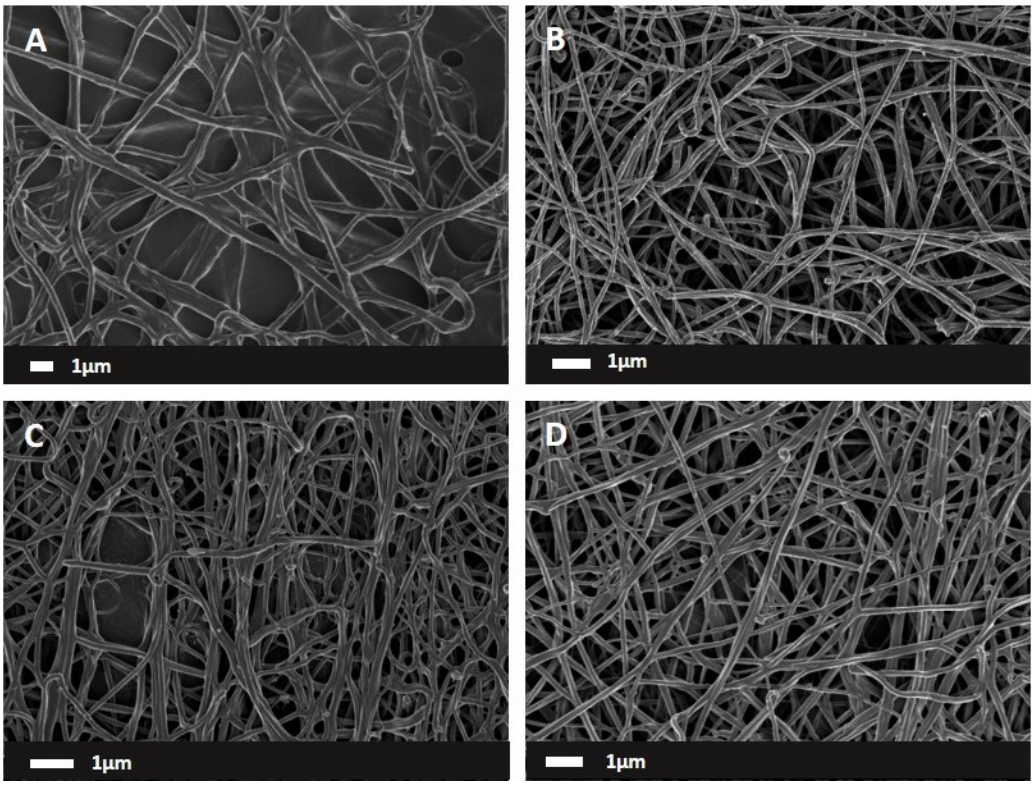

Figure 3.7: SEM images of Platelet-poor plasma clots from participants on the control diet inclusive of carbohydrates: (A) Clot with relatively low fibrin fiber density embedded in matted clot deposits, (B) Clot with relatively high fibrin fiber density but no matted clot deposits, (C) Clot with relatively high fibrin fiber density with some degree of matted clot deposits, (D) Clot with relatively high fiber density and minimal matted deposits. Clot shown in B is closest to what should be detected in healthy individuals. Scale =1 µm.

When examined more thoroughly, there appeared to be some discontinuous fibrin fibers detected in clots, and some clot matted regions for participants on the LCHF diet as shown in Figure 3.8.

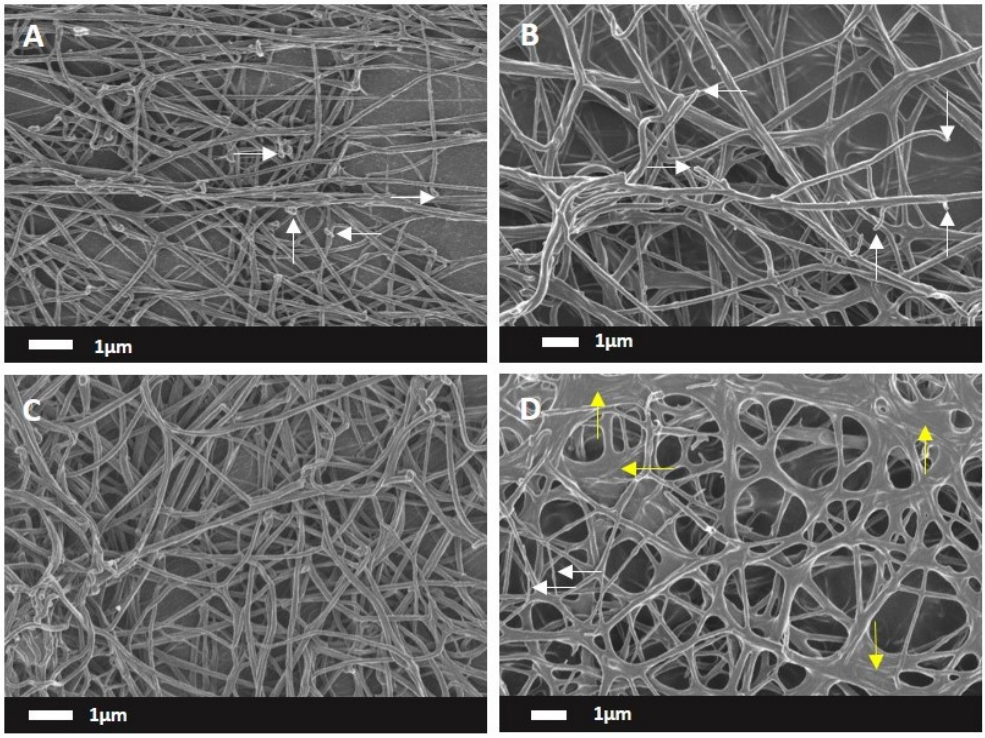

Figure 3.8: SEM images of PPP clots from participants with on a LCHF (A and B) Clot with relatively lower fiber density and showing some discontinuous fibers (white arrows), (C) Clot with relatively normal fiber distribution, (D) Clot with relatively less distinctive (unstable) fibrin fibers (white arrows), some matted clot regions (yellow arrows). Scale =1 µm.

Visual analysis suggests potential differences in the clot from fibrin-generated clots. For this reason, we decided to further investigate in more detail, the nature of the clots formed in WB using TEG to compare both groups.

3.5 DISCUSSION

SEM of RBCS

The study of RBC morphology is very important in hemorheology, since the deformability of RBCs has an impact on rheological properties of blood.[167]

For controls, the following shapes were detected: few knizocytes (appearing pinched in the centre on one of the sides that should be invaginated). RBC agglutination (rouleaux formation), where RBCs clumps together forming a stack. We also observed few acanthocytes that appear as irregularly spaced, blunt-tipped projections, and can form due to altered membrane cholesterol or phospholipids.[15,168] A typical discoid human RBC has a diameter of six to eight µm.[169] The properties of RBCs such as morphology, membrane elasticity, cell volume, protein and lipid composition can serve as biomarkers of different pathophysiological-states.[169] RBCs can undergo deformation changing from a normal discocyte structure to poikilocytes like

echinocytes. In this case, RBCs slowly lose deformability. RBC deformation involves a change in cell curvature or area expansion.[170] The changes of membrane properties which are lipid bilayer and membrane skeleton proteins can thus affect RBC deformability.[171] A model that was proposed by Sheetz and Singer (1974) and Evans, which explains how discocytes transform to echinocytes or stomatocytes.[172-173] The bilayer-coupled hypothesis, explains how convex structures, such as spicules of echinocytes, form when any factor causes the outer membrane leaflet to expand to the inner leaflet.[172-173] The alteration of RBC morphology can serve as diagnosis for diseases.[174] However, agents which induce changes to extracellular conditions like high pH, salinity, and (ATP) depletion are also reported to induce echinocyte formation.[174]

In this study, LCHF participants showed greater number of poikilocytes than controls. Poikilocytes that were identified in LCHF include echinocytes (with regularly spaced short projections or spicules), stomatocytes (cells with cup-shaped appearance), leptocytes (flattened cells with more surface area than volume) were also identified, and these cells are associated with oxidative stress. [154] Also, dacrocytes (teardrop shape) were detected and these cells have been identified in pathological conditions such as hemochromatosis (iron overload), liver disease, and heart disease.[175] In a study recently conducted in mice, it was reported that chronic high fat feeding in mice induced structural, biochemical and functional alterations in RBCs.[152] RBC membrane cholesterol was significantly higher in RBCs from mice on a high fat diet. This suggests a role for pathologically remodelled RBC that may highlight inflammation and vascular dysfunction.[33]

SEM of platelets

Platelets are important for prevention of blood loss.[176] Activation of platelets is the first step to initiate the coagulation cascade. In the process of activation, platelet changes shape, release their granule contents and eventually forms aggregates through adhesion with each other.[114] The activation of platelets occurs in three stages: 1) Initiation phase: this involves rolling, adhesion and spreading of platelets to the extracellular matrix forming an activated platelet monolayer 2) Extension phase: when activated platelets adhere to the site of vascular injury and more platelet recruitment occurs through factors like ADP, TXA2 and there is formation of platelet aggregates. 3) Perturbation phase: where the clot is stabilized.[58,160,177] In the present study, smaller

platelet aggregates made up of fewer platelets were observed in LCHF blood smears as compared to controls as shown in the SEM results. In a healthy person, platelets are mostly spherical with minimal pseudopodia formation due to contact activation.[178] In the present study, platelets detected in smears for some individuals in the control group showed slightly projected pseudopodia with minimally spread lamellipodia. In most other individuals in the control group, larger platelet aggregates with extensively spread pseudopodia were detected, as compared to LCHF participants. In LCHF participants, platelets with minimally projected filopodia and minimally spread lamellipodia were predominant. ADP is a potent platelet-recruiting factor that promotes platelet aggregation via interaction of P2Y12 receptors.[140] It works alongside the main platelet receptor $\alpha_2b\beta_3$ in association with secreted CD63 and CD9 to bind fibrinogen to recruit other platelets.[179] These receptors are important in platelet activation, aggregation and stabilization.[159] They also play a role in amplification of aggregation induced by other platelet activators.[55] Therefore, P2Y12 receptors are very important in platelet recruitment and the coagulation cascade.[180] A recent study done on rats demonstrated that HFD alters platelet aggregation and signalling in platelets.[140] As a result of changes to platelet profiles, we anticipate that the LCHF diet also affects the coagulation process. To investigate the coagulation properties further, we subsequently compared the fibrin clot network.

SEM of fibrin fibers

Fibrin fibers of healthy individuals consist of both thick and thin fibers. In the present study, normal fibrin fibers were present with thick and thin fibers for both LCHF and control groups. In the control group an extensive fibrin network with both thick and thin fibers and dense matted deposits in some participants were observed. However, for participants on the LCHF diet, we also observed some discontinuous fiber networks in addition to a few regions with the matted deposits. In majority of individuals in the LCHF group, some long thin discontinuous fibers, masses of thin and thick fibers forming a net like structure and some dense matted regions were detected. An altered fibrin structure can occur in inflammatory diseases such as stroke.[181] In previous research on T2DM, net and matted appearance of fibrin fibers were reported, suggesting hypercoagulability.[182] These outcomes such as dense matted fibrin deposits seen in the mentioned diseases suggest that inflammation induces coagulation.[63]

3.6 Conclusion

Evidence from this study suggests that LCHF diet may increase poikilocytosis, the formation of dense matted clots and discontinuous fibrin fibers and reduce platelet aggregation. These findings may not be adequate for quantitative comparisons and so we decided to further investigate platelet aggregation in participants on the LCHF diet to participants on the control diet. We achieved the quantification of platelet aggregates using flow cytometry as follows in the next chapter.

CHAPTER 4: (FLOW CYTOMETRY)

4.1 Aims and Objectives

The aim of this chapter is to measure platelet activity in WB obtained from participants on the LCHF diet and compare them to those of participants on a normal diet.

To achieve this aim, our objectives are to:

- Apply flow cytometry to examine platelet markers expressed within blood samples obtained from individuals in both groups
- Generate and present findings as quantifiable data using relevant software
- Use statistical software to compare data outcomes from both groups
- Discuss differences detected between the groups so as to draw inferences on how diet may affect the expression of activation, aggregation, and adhesion receptors in platelets.

4.2 Introduction

Flow cytometry is a powerful technique that has been recognized in research and in clinical investigation.[183] Historically, the first established flow cytometry was a single parameter instrument that could only detect the size of cells.[184] Between the 1980s and 1990s the instrument improved and a highly sophisticated instrument was developed.[185] These instruments were able to detect 14 parameters at the same time. It was possible to measure different cell characteristics with their functions using new antibodies and probes. It became easy to apply new clinical and research methods. The markers that are used in flow cytometry may give us valuable information regarding cellular activity and since 1989 flow cytometry has been used as an important tool to examine platelets.[186] It delivers statistical numbers which are both objective and quantitative to measure platelet function.[187]

The principle guiding flow cytometry is that platelets are labelled with fluorescent monoclonal antibodies (MoAbs). The cells are suspended in a fluid which allows them to pass aligned one by one in front of light detectors; at the same time they are illuminated by a focused laser beam that activates bound fluorophores.[188] To identify platelets, the fluorescence is measured. The emitted fluorescence and scattered light are detected so that the intensity of the emitted light is directly proportional to the number of antibodies attached to the platelet receptors or antigens.[189] Thus, it is possible to determine absolute number of antibody-labelled cells using flow cytometer.

The analysis of platelet function in whole blood using flow cytometry has advantages: One is that, within a short amount of time, flow cytometry can measure specific characteristics of a large number of platelets (such as 5000 or 10,000) in each sample.[190] Flow cytometry is a quick, reliable and sensitive technique which can be used to detect genetic and developed platelet disorders.[189] The detection of MoAbs is an important tool to assess platelet function by using flow cytometry as shown in Table 4.1.[188] The membrane surface of platelets contains a number of glycoprotein receptors. The platelets interact with each other via these glycoprotein receptors.

Table 4.1: Platelet parameters used to identify platelet activity.

Platelet Parameters	**Moiety that the probe reacts with**
CD41a	Platelet membrane GPIIb, is a receptor for fibrinogen, von Willebrand factor, fibronectin, and vitronectin which are essential for platelet aggregation intercellular aggregation via fibrinogen.[191]
CD62P	Alpha-granule membrane protein (P-selectin) expressed on the surface of activated platelets. It mediates adhesion of activated platelets to monocytes and neutrophils.[192]
CD63	Member of tetraspanin superfamily and is found on dense and Lysosomal membrane and can be expressed on surface of activated platelet.[179]

Platelets play crucial roles in haemostasis by being a first line of defence against the loss of endothelial integrity.[193] Upon vascular injury, platelets prevent bleeding at the site of injury. However, platelets are also involved in pathological conditions such as inflammation, atherogenesis and atherothrombosis.[194] Platelets respond to endothelium damage through adherence to sub-endothelial proteins via glycoproteins receptors on the platelet surface.[193] Upon activation, platelets secrete adhesion molecules such as P-selectin/CD62P, CD40 ligand and CD63. In the next paragraph is a description of these important platelet proteins namely CD41, CD62P, CD63 and CD40 ligand.

CD41 is a calcium dependent compound of GPIIb/IIIa. It is bound on the membrane surface of the GP component (GPIIb) with a molecular weight of 135-95 kDa, which constitutes a precursor for the fibrinogen receptor.[195] Also present on platelets is CD61 (GPIIIa), and it forms a complex with CD41 resulting to CD41/CD61 (GPIIb/IIIa). Both CD41 and CD61 are expressed on resting platelets.[56] When platelets are in a resting

state, GPIIb/IIIa is unable to bind fibrinogen, as it has a low binding affinity for it. Upon platelet activation, this integrin receptor (GPIIb/IIIa) undergoes a conformational change which then increases its affinity for binding fibrinogen.[56,196] Upon activation, platelets bind to fibrinogen via the activated GPIIb/IIIa (CD41/CD61) receptor complex on the platelet surface. Hence, CD41 is a reliable marker to identify platelets and has been used to differentiate between platelets and other cells.[186]

CD62P or GMP140- also known as P-selectin, is a member of selectin with a mass of 140kDa. P-selectin is stored in the alpha granules of a resting platelet and is expressed on the surface of platelets upon platelet activation.[136,189,197] The MoAb that is specific for CD62P is unable to bind to a resting platelet but can only bind to activated platelets. CD62P acts as an adhesion receptor between platelets and white blood cells or endothelial cells via PSGL and GPIb.[198] The expression of P-selectin is said to be increased on the surface of platelets with patients with coronary artery disease or systemic inflammation.[199]

CD63 also known as GP63 with a mass of 53kDa is a membrane protein of platelet lysosomes and dense granules.[200] It is only expressed on platelet outer surface membrane after platelet activation whereby lysosomal granule fuses with platelet membrane and is secreted. After secretion, it relocates to membrane surface of platelets where it binds with integrin αIIbβ3-CD9 complex.[131] The role of CD63 is to help in platelet adhesion and enables activated platelets to spread over fibrinogen.[179]

CD40L is a transmembrane protein with a similar structure as an inflammatory cytokine (TNF-α).[201] Upon platelet activation, this protein is expressed on platelets. On platelets, CD40L triggers an inflammatory response of endothelial cells by stimulating the cells to secrete chemokines.[132] CD40L stimulates the monocytes expressing TF, an initiator of extrinsic coagulation cascade.[202] It is also present in cells like monocytes, endothelial cells and, where it influences binding of neutrophils to monocytes.[56,133] CD40L also binds to integrin αIIbβ3, a receptor for fibrinogen and vWF thus contributes to stabilization of the thrombi.[203-204] Therefore, it plays a role in both inflammation and thrombosis, crucial in atherosclerosis.[138]

It is indicated that platelets play an essential role in inflammation as well.[205] The presence of inflammation results in an imbalance between procoagulant and anticoagulant properties of the endothelium leading to local stimulation of the

coagulation cascade.[206] TF is a principal initiator of inflammation-induced thrombin generation. In the unstable plaque, TF is expressed by inflammatory cells and may initiate activation of coagulation and the generation of thrombin which then activate platelets and result in the formation of platelet-fibrin thrombus.[47] Another feature of inflammation is the interaction between leukocytes, endothelial cells and platelets. The linkage of thrombosis and inflammation is highlighted at molecular and cellular levels in the endothelium. In the case of tissue injury, platelets adhere to the injured area and by doing so more platelets and leukocytes are recruited. In order to stop bleeding, platelets provide a surface for the binding of leukocyte-derived micro-particles holding TF, an initiator of the coagulation cascade. Upon activation, platelets enhance adhesion of leukocytes to the endothelium and thus promote leukocyte activation through release of chemokines on the endothelium. Leukocytes are now empowered to attach firmly to the vessel wall and to transmigrate into sub-endothelial tissue to form the atherosclerotic plaque.[194,206] Once inside, these cells can turn into “angry” giant cells called foam cells, which inflame the surrounding area and contribute to the build-up of the plaque. Thus, platelets role in chronic inflammatory diseases such as atherosclerosis have been demonstrated.[206]

Therefore, flow cytometry is a tool that can be used to establish both the number of activated and non-activated platelets.[187]

4.3 Materials and Methods

4.3.1 Participants

Thirty-two healthy individuals were recruited as controls for the study. These individuals were non-smokers who were not using any medication for chronic illness and females participants were not on contraceptives. These control participants were recruited from University of Pretoria’s Departments of Physiology, Anatomy and Pharmacology. Thirty-two individuals who were on LCHF diet were recruited from the Banting market in Pretoria, South Africa. Inclusion criteria for these participants were: (a) Individuals older than eighteen years and willing to provide full written informed consent, b) Urine ketones must be present, c) LCHF diet must have been maintained for at least three months or longer. Exclusion criteria were: a) Smoking, b) Excessive alcohol use (more than two glasses of red wine per day), c) Chronic diseases (and medication) such as diabetes, hypertension, d) medicines for acute conditions, such

as antibiotics and chronic medication for diet pills, herbal extracts, etc. Familial hypercholesterolemia was considered a confounder during screening and recruitment, and so, participants with the disorder were excluded from the study.

Informed consent was signed by all participants before any procedures were performed. Once LCHF diet participants were identified, they were asked to fast for at least twelve hours prior to their visit. We provided a questionnaire to obtain information on each participant's diet history, medical history, smoking habits, alcohol intake and use of chronic medication. Height and weight were measured with the participants in a standing position barefoot. Body mass index was calculated as weight in kilograms divided by the square of height in meters. The body mass index was categorized according to WHO cut-offs.[166] The systolic and diastolic blood pressure as well as heart rate were measured. Ethical clearance was obtained for the study from the University of Pretoria's Faculty of Health Sciences Research Ethics Committee (Ethical Clearance number: 366/16).

Blood Sample collection

All procedures were performed on two groups: (1) Healthy volunteers, eating foods without specific restrictions on intake of dietary categories (the control group) and (2) individuals with food intake restrictions limiting carbohydrate intake while increasing fat intake (the LCHF group). Blood samples were collected from all individuals meeting inclusion criteria. Blood was drawn by a qualified doctor upon completion of informed consent form by each participant. Blood samples were collected via venipuncture from all individuals. Two ml of blood was drawn into a citrate tube from each participant. Within thirty minutes of the collection of blood, sample preparation was performed prior to flow cytometry analysis to analyze the platelet specific antigens. The platelet markers used to study platelet function were platelet identifier CD41 and markers of platelet activation and intercellular aggregation, CD62 and CD63.

4.3.2 Flow cytometry

For each blood sample taken, two tubes were prepared. The first tube was stained with five µl of the three fluorescent-conjugated probes: CD41-FITC (Fluorescein Isothiocyanate), CD62P-APC (Allophycocyanin) and CD63-PE (Phycoerythrin). A second tube containing 1 ml of sheath fluid and twenty µl of blood. From the second

tube hundred µl was taken and added to the first tube containing all the different probes. The samples were incubated in the dark for twenty minutes. After twenty minutes six hundred µl of the mixed sample was analysed using a flow cytometer model (Gallios 3 Laser, 10 colour configuration).

Platelets were distinguished from RBCs and white blood cells based on their forward and side-light scatter characteristics by gating. The first gate was set according to platelet size and the second gate was set according to the fluorescence of platelet markers, which identify platelets forming aggregates. The intensity or fluorescence of each antibody was presented on a histogram. The results were expressed using the arbitrary unit, mean fluorescence index (MFI). The software used to analyse the data was STATA.

4.3.3 Statistical Analysis

For each participant, percentage gated (%Gated) for single platelets and platelet aggregates, as well as MFI was calculated in a large sample of 20 000 platelets per individual. To analyse the data, Wilcoxon rank-sum test (non-parametric statistical test) was used to compare platelet populations of LCHF and control groups. P-value of less than 0.05 was considered significant.

4.4: Results

The percentage gated for single platelets and platelet aggregates was calculated for both controls and LCHF participants. The analyses include: % gated CD41+/62P+, CD41+/63+ and CD63+/62P+ (single) platelets. There was no statistical difference in platelet activation indicated by detected platelet expression of CD62P and CD63 between LCHF and control group as shown in Table 4.2.

Table 4.2: Analysis of participants presented as mean, standard deviation (SD), median of MFI and percentage gated platelets. (n=20 000 platelets total analysed for each participant).

MoAb	Controls n=32	Median	LCHF n=32	median	P-value
% Gated CD41 FITC/CD62P APC (platelets only)	3.28±4.77	1.24	2.55±4.38	1.54	0.640
% Gated CD41 FITC/CD63 PE (platelets only)	0.65±0.51	0.49	0.66±0.88	0.36	0.588
% Gated CD63 PE/CD62P APC (platelets only)	0.36±0.43	0.15	0.48±0.90	0.15	0.963

Results of the flow cytometry analyses show that compared to healthy controls, total CD41+ aggregates MFI was significantly lower for the LCHF group compared to controls, mean (32.31±9.14* vs 38.97±6.94) as shown in Table 4.3. Also calculated for the controls and LCHF groups were % gated CD41+/CD63+, CD41+/CD62P+, CD63+/CD62P+ aggregates. The results showed no statistically significant difference between controls and LCHF participants except when in total CD41+ platelets (detected due to gating by size) and this is shown in Table 4.3.

Table 4.3: Analysis of participants presented as mean, standard deviation (SD), median of MFI and percentage gated platelet aggregates. (n=20 000 platelets total analysed for each participant).
*Statistically significant difference with P < 0.005.

MoAb	Controls n=32	Median	LCHF n=32	median	P-value
% Gated Total CD41+ aggregates	1.61±0.53	1.55	1.71±0.38	1.75	0.345
Total CD41+ aggregates X-median	38.97±6.94	39.05	32.31±9.14*	33.98	0.002*
% Gated CD41 FITC/CD62P APC (aggregates)	0.04±0.05	0.03	0.04±0.06	0.03	0.807
% Gated CD41 FITC/CD63 PE (aggregates	0.02±0.02	0.01	0.02±0.02	0.01	0.662
% Gated CD63 PE/CD62P APC (aggregates)	0.01±0.02	0.01	0.01±0.02	0.01	0.024

A graphical summary illustrating the significant difference identified between numbers of platelet aggregates in control versus LCHF participants is presented in Figure 4.1

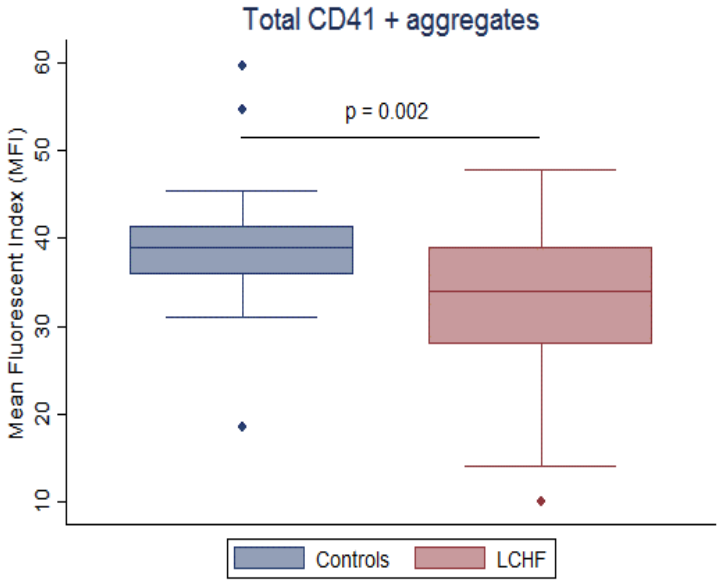

Figure 4.1: Graphical representation of control and LCHF group showing MFI for total CD41+ aggregates. Statistical difference: P-value = 0.0022*

A representative of platelet-leukocytes (CD41/CD62P) and platelet-platelet aggregates (CD41/CD63) is shown in Figure 4.2 and 4.3 for both controls and LCHF group.

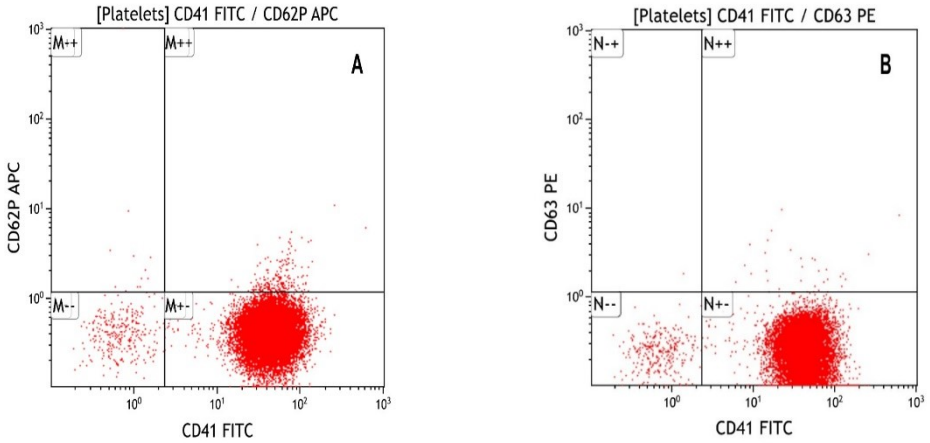

Figure 4.2: Scatterplot showing fraction of detected (A) Platelet-leukocyte aggregates and (B) Platelet-platelet aggregates in a participant on the control diet.

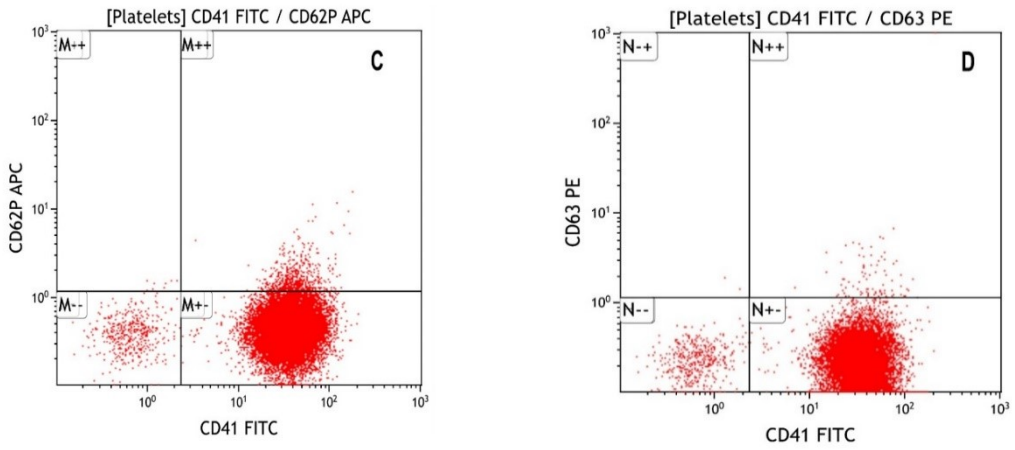

Figure 4.3: Scatterplot showing fraction of detected (C) platelet-leukocyte aggregates and D) platelet-platelet aggregates in a participant on the LCHF diet.

4.5 Discussion

Flow cytometry is an important technique that has been used clinically and in research for immunophenotyping platelet glycoproteins.[207] It provides quantitative assessment of the physical and antigenic properties of platelets. Flow cytometry has been used to study surface expression of receptors, bound ligands, components of granules, interactions of platelets with other platelets via aggregation, and platelets with other blood cells like leukocytes.[189] Studying these surface expressions may help in diagnosis of acquired and inherited diseases. It also helps to study platelet activation pathology associated with diseases such as acute coronary syndromes, cerebrovascular ischemia and peripheral vascular disease.[208]

The markers that were used for this study include CD41 FITC, a platelet identifier, CD62P APC and CD63PE markers that indicates platelet activation.

The flow cytometry results for this study as shown in Figure 4.1, indicates a statistically significant difference between the total number of CD41+ (platelet) aggregates detected in controls compared to the LCHF group with a mean of (38.97±6.94 vs 32.31±9.14*). As shown in Figure 4.1, the graph indicates that the total CD41+platelet aggregates were lower in the LCHF participants compared to controls. Our findings contrast with research that was recently completed on rats fed a HFD, which showed that platelet aggregation was increased prior to the administration of α-tocopherol, which significantly decreased aggregation.[140] In another study that was done on diabetic mice fed high fat diet for four months, platelets showed less aggregation and lower CD61 expression.[197]

It is important to understand platelet activation and aggregation, which are driven by receptor-ligand interactions.[209] Upon vascular damage, activation of platelets occurs. Platelets interact with vWF bound within the sub-endothelial extracellular matrix via their GPIb receptor. This is followed by binding of platelets to collagen via collagen receptor GPVI.[55] Further adhesion of additional platelets is enabled by fibrinogen binding to platelets via activated GPIIb/IIIa (CD41/CD61) integrin complex to form a platelet aggregate.[210].This in turn activates intracellular signalling cascade which leads to synthesis and release of platelet agonists TxA2, ADP and thrombin, which activate GPCR on platelet membrane surface.[55] Among the platelet agonists, thrombin is the most potent activator of platelets. Thrombin assists in the formation of fibrin from fibrinogen, which then contributes to the formation of the haemostatic plug and platelet thrombus growth. The release of TxA2 and ADP strengthens the adherence of platelets and also induces platelet change, release of granule contents and increases expression of pro-inflammatory molecules on the platelet surface including P-selectin. Platelets need to be fully activated and adhered as more platelets aggregate to form a thrombus. ADP and TxA2 then act on nearby platelets by recruiting them into the growing thrombus. ADP nucleotides are secreted from dense granules after platelet activation signal through purinergic receptors on platelet membrane.[211] Once these receptors are activated, they initiate a complex of signalling cascade that eventually result in platelet activation and thrombus formation.[212]

The research study using rats fed HFD that showed that HFD alters platelet aggregation and purinergic signalling in platelets suggests that purinergic signalling may have been altered in our LCHF group through similar mechanisms.[152] Altered receptor function may influence haemostatic profile by reducing platelet aggregation and this may also influence the coagulation cascade.

In this study, participants on a LCHF diet showed that although there was greater expression of CD63 by single platelets in the LCHF group as shown in Table 4.2 and 4.3, the differences from the control group were not significant. However, overall aggregation was found to be significantly lower in the LCHF group. Mild or low platelet activation (detected as slight pseudopodia and aggregate expression) was also confirmed by SEM in platelets of most individuals on the LCHF diet. Multiple pathways involving ADP, TxA2 and thrombin are involved in platelet activation as described above. One of the functions of agonists (ADP and TxA2) is to contribute to the

formation of haemostatic plug and platelet thrombus growth, as well as to strengthen the adherence of platelets which also induces platelet change that initiates release of alpha granule contents by increasing an expression of P-selectin. A study on coronary artery disease patients showed no significant difference in expression of CD63 by their platelets.[213] Comparatively, a study done in diabetic patients showed an increase of CD63 by their activated platelets.[214] However, a study has also confirmed that it is possible for CD63 to be more highly expressed in platelets in the absence of aggregation.[215] CD63 associates with the platelet's main aggregation receptor (GPIIbIIIa) which forms aggregates with other platelets via fibrinogen. It plays a subtle role as a negative regulator of platelet aggregation *in vitro* but has no demonstrable effect on thrombus formation *in vivo*.[216] Increased platelet expression of the CD63 protein in the LCHF group appears to affect platelet signalling variably in different conditions. Hence, the presence of reduced aggregation in the LCHF group may influence their contribution to clot formation.

4.6 Conclusion

LCHF diet not only affects the metabolic profile of individuals but may also impact on their haemostatic profile. The platelet aggregates were significantly lower in LCHF participants compared with controls in spite of greater activation signified by CD63 expression. Upon vascular damage, platelets become important mediators that trigger the coagulation cascade pathway.[217] They facilitate haemostasis through activation, adhesion and aggregation. Platelets adhere to the endothelium and undergo degranulation where they secrete their granule contents.[218] Platelet activation is followed by aggregation. The aggregation of platelets requires an integrin receptor for fibrinogen.[53]

Activation of platelets occurs prior to recruitment of additional platelets, leading to aggregation. The activation is mediated by agonists like ADP which are released by activated platelets and these agonists act on GPCR.[53,55] Aggregation of platelets promotes formation of platelet plug. Once the plug is formed it needs to be stabilized and strengthened by the formation of fibrin network.[55] In order for fibrin network to be formed blood coagulation must be initiated, where tissue factor an initiator of coagulation binds to FVII, eventually generating thrombin. Thrombin binds to circulating fibrinogen and converts it to fibrin network. The formation of fibrin then

strengthens the initial platelet plug.[159-160] Therefore activation of platelets followed by aggregation triggers the coagulation cascade.

As we detected comparatively greater numbers of CD63+ platelets (suggesting higher influence on platelet-leukocyte associations) and fewer CD41+ (platelet-platelet) aggregates in our LCHF group, we decided to investigate if this would affect the overall clot properties in this group using TEG.

CHAPTER 5: THROMBOELASTOGRAPHY (TEG)

5.1 Aims and Objectives

The aim of this chapter is to assess the coagulation parameters in WB from the control group and the LCHF diet group.

To achieve this aim, our objectives are to:

- Apply TEG to examine whole blood samples obtained from individuals in both groups
- Generate and present findings as quantifiable data using relevant software
- Use statistical software to compare data outcomes from both groups
- Discuss differences detected between the groups so as to draw inferences on how diet may affect specific parameters associated with clotting in whole blood.

5.2 Introduction

Platelets, fibrin and coagulation factors play a vital role in balanced haemostasis and are also involved in inflammation.[63] Functional fibrinogen, along with platelets, influence clot formation and stability. Fibrin formation is the final end-product of the intricate coagulation cascade. The strength and expansion of a clot is dependent on fibrinogen being converted to fibrin alongside crosslinking processes.[63] An important step in the conversion of fibrinogen is when fibrin is catalysed by the enzyme thrombin. Upon tissue injury, fibrin forms a loose netlike meshwork which acts as a plug that seals the tissue and protects it as the wound heals.[62] As the clot forms, circulating RBCs, white blood cells and platelets are incorporated into the clot structure. The entrapped RBCs gives a red appearance to the clot with fibrin forming the foundation of the clot. The activated platelets act as a stimulus to initiate conversion of fibrinogen to fibrin. Thrombin also activates FXIII which then facilitates the cross linkage of fibrin polymers to form a stable fibrin clot.[219]

TEG, is a technique that was developed by Hartet in 1948.[220] It gives the whole picture of haemostasis by quantitatively measuring the viscoelastic changes occurring during coagulation.[221-222] It assesses the viscoelastic properties of the evolving clot in patient's WB by giving out information about fibrin formation, platelet activation, and breaking down of the clot. [223] TEG measures the speed of fibrin polymerisation and

the strength of the clot by generating parameters and giving out values that are associated with each step of blood clotting.[224] The values obtained are represented graphically giving information about the start of clot, progress, and maximum clot strength and stability. Coagulation parameters measured by TEG have been shown to predict haemorrhage and mortality after traumatic injury.[225-226] The clotting parameters of the TEG give information about the overall haematological health of a person.[227] These parameters can be used as a tool to measure the degree of pathology in the coagulation status of the individual.[228] TEG is a quick test to be used to assess coagulation in individuals.[229]

The principle behind TEG is that it measures the viscoelastic properties of blood as it clots.[230] TEG consist of a torsion wire, a pendulum, and a cup to hold the sample.[223] The device has two operable channels; for each channel, there is a pin suspended by a torsion wire. The pin is suspended in the cup that can hold citrated whole blood and calcium chloride ($CaCl_2$), which can be heated to 37° C.[231] The $CaCl_2$ is added to detect clotting times for the extrinsic and intrinsic coagulation pathways and fibrinolysis after clot formation. The cup oscillates back and forth constantly at a set speed through an arc of 4° 45′.[230] As the clot begins to form, it attaches to the pin followed by fibrin-platelet interaction.[223] The mechanical energy generated by the clot is converted into an electrical signal. This signal is analysed by a computer, which generates symmetrical tracing whose magnitude corresponds with the clot's strength.[232]

5.3 Materials and Methods

5.3.1 Participants

1) Healthy volunteers, eating foods without specific restrictions on intake of dietary categories, not smoking or on chronic medications or contraceptives (the control group) and (2) individuals with food intake restrictions limiting carbohydrate intake while increasing fat intake (the LCHF group). The inclusion criteria for the LCHF group were: (a) participants older than eighteen years, willing to provide full written informed consent, b) participants who had been on the LCHF diet for at least three months or more. Exclusion criteria for the LCHF group were: a) Smoking, b) excessive alcohol use (more than two glasses of red wine per day), c) Chronic diseases (and medication) such as diabetes, hypertension, d) medicines for acute conditions, such as antibiotics and chronic medication for diet pills, herbal extracts. Familial hypercholesterolemia

was considered a potential confounder during recruitment of participants and so individuals with the disorder were excluded. Ethical clearance was obtained for the study from the University of Pretoria's Faculty of Health Sciences Research Ethics Committee, (Ethical clearance number: 366/16).

Blood sample collection

Upon completion of informed consent, two ml of blood was drawn into sodium citrate tube, from each participant blood was left to stand for thirty minutes before using for TEG. The test was performed to obtain TEG clot parameters which are: Reaction time (R value), kinetic (k-value), Angle, maximal amplitude (MA), maximum rate of thrombus generation (MRTG), time till maximum rate of thrombus generation (TMRTG), and total of thrombus generation (TTG).

5.3.2 Clot properties using TEG

To study TEG clot parameters, only WB was used. A small sample of three hundred and forty µL of WB was pippeted in a TEG cup followed by addition of twenty µL of 0.2 M $CaCl_2$ to initiate clot formation, this was heated to 37˚C. The $CaCl_2$ is needed to reverse the effect of the sodium citrate that acts as an anticoagulant to prevent coagulation. The samples were placed into a hemostasis analyser system (TEG 5000 Hemostasis Analyzer System by Haemonetics) for analysis. The different parameters that were studied for the study for each person are explained in Table 5.1. The software that was used to analyse the data was STATA.

Table 5.1: TEG clot parameters studied using WB adapted from (Pretorius et al 2017).[227]

Parameters	Explanation
R value: reaction time measured in minutes	Time of latency from start of test to initial fibrin formation (amplitude of two mm); i.e. initiation time
K: kinetics measured in minutes	Time taken to achieve a certain level of clot strength (amplitude of twenty mm); i.e. amplification
A (Alpha): Angle (slope between the traces represented by R and K) Angle is measured in degrees	The angle measures the speed at which fibrin build up and cross linking takes place, hence assesses the rate of clot formation; i.e. thrombin burst
MA: Maximal Amplitude measured in mm	Maximum strength/stiffness of clot. Reflects the ultimate strength of the fibrin clot, i.e. overall stability of the clot
Maximum rate of thrombus generation (MRTG) measured in Dyn $cm^{-2}s^{-1}$	The maximum velocity of clot growth observed or maximum rate of thrombus generation using G, where G is the elastic modulus strength of the thrombus in dynes per cm^{-2}
Time to maximum rate of thrombus generation (TMRTG) measured in minutes	The time interval observed before the maximum speed of the clot growth
Total thrombus generation (TTG) measured in $Dyn.cm^{-2}$	The clot strength: the amount of total resistance (to movement of the cup and pin) generated during clot formation. This is the total area under the velocity curve during clot growth, representing the amount of clot strength generated during clot growth

5.3.3 Statistical Analysis

To determine the viscoelastic clots properties and differences between the control group and LCHF diet group, statistical analysis was performed. The two-sample Wilcoxon rank-sum (Mann-Whitney) test was used to analyse TEG parameters using GraphPad Prism version 5.03 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com; a P-value of less than 0.05 was considered statistically significant.

5.4 Results

The following parameters showed a significance difference between LCHF diet group and controls:

The R value, which represent the start of the clot was significantly shorter in participants on the LCHF diet as compared to controls (P=0.0334). Alpha angle was significantly higher in LCHF participants compared to controls (p<0.0001). The

TMRTG which is time to maximum rate of thrombus generation, was significantly lower in LCHF groups as compared to controls (p=0.0025) as shown in Tables 5.3 and 5.4.

The following parameters showed no significant difference when comparing between LCHF participants and controls:

The K value representing time taken to achieve clot strength, was shorter in LCHF participants compared to controls. MA which represents the clot stability was lower in LCHF participants as compared to controls. MRTG representing the maximum rate of clot formation, was higher in LCHF compared to controls And TTG which is the total thrombus formation was lower in LCHF as compared to controls.

Table 5.2: Clot parameters for control participants as obtained using TEG.

	Mean ± SD	**Median [IQR]**	**Min, Max**	**95% Confidence Interval**
R	7.6 ± 2.1	7.3 [6.2 to 8.2]	4.5, 12.7	[6.8, 8.4]
K	4 ± 1.5	3.9 [3 to 4.7]	1.7, 8.5	[3.4, 4.6]
Angle	47.9 ± 12.42	48.5 [37.9 to 57]	28.3, 71.6	[43.4, 52.5]
MA	48.6 ± 9	49.1 [41.1 to 55.3]	28.2, 67.9	[45.2, 52]
MRTG	3.9 ± 1.9	3.5 [2.7 to 4.6]	1.4, 9.6	[3.3, 4.6]
TMRTG	10.4 ± 3.6	9.0 [8 to 12.5]	6.4, 20.2	[9.1, 11.8]
TTG	504.9 ± 192	482.1 [348.6 to 621.6]	196.1, 1060	[433.3, 576.6]

R = clot initiation time, K = time till change in clot formation speed, Angle = rate of clot formation, MA = clot stability, MRTG = maximum rate of clot formation, TMRTG = time till maximum clot growth, TTG = total clot strength

Table 5.3: Clot parameters for participants on LCHF diet as obtained via TEG

	Mean ± SD	Median [IQR]	Min, Max	95% Confidence Interval	P-value
R	6.4 ± 1.6	6.2 [5.6 to 7.5]	2.2, 10.5	[5.7, 7]	0.0334 *
K	4.2 ± 1.7	3.8 [3.2 to 5.2]	1.5, 9.8	[3.5, 4.8]	0.7430
Angle	63.2 ± 6.9	63.8 [59 to 67.9]	42.3, 77.6	[60.5, 66]	<0.0001 **
MA	48.6 ± 9	49.1 [41.1 to 55.3]	28.2, 67.9	[40, 48]	0.0829
MRTG	3.6 ± 1.2	3.5 [2.8 to 4]	1.8, 8.1	[3.1, 4.1]	0.7013
TMRTG	8 ± 2.6	9 [8 to 12.5]	6.4, 20.2	[6.9, 9]	0.0025 **
TTG	427.4 ± 181.8	388.4 [295.9 to 570]	161.9, 823.9	[355.5, 499.3]	0.0918

R = clot initiation time, K = time till change in clot formation speed, Angle = rate of clot formation, MA = clot stability, MRTG = maximum rate of clot formation, TMRTG = time till maximum clot growth, TTG= total clot strength. * = P-value < 0.05, ** = P-value < 0.005.

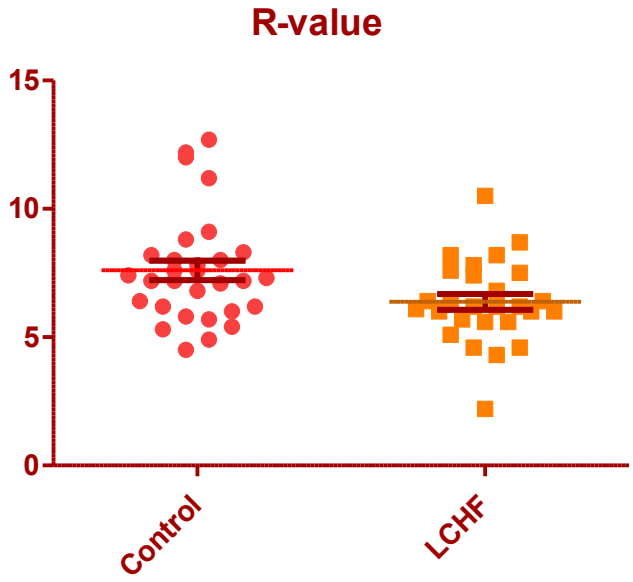

Figure 5.1: Median R-value was significantly shorter for LCHF participants than for the control participants (6.2 vs 7.3 minutes, P=0.0334).

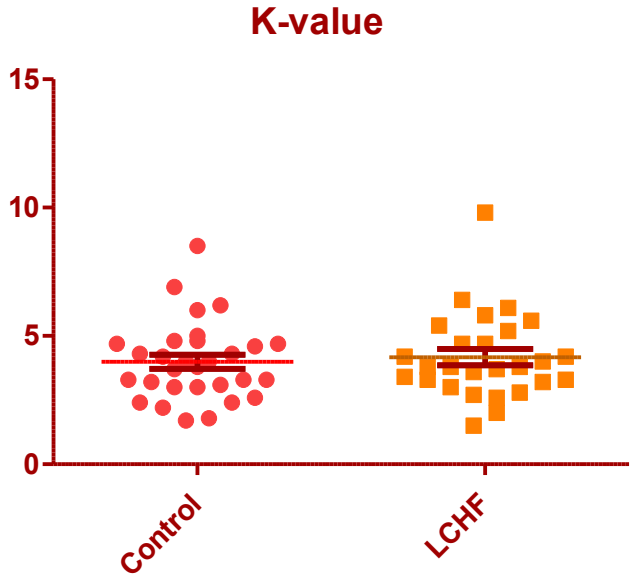

Figure 5.2: Median K-value was not significantly greater in LCHF than for control participants (3.8 vs 3.9 minutes, P=0.7430).

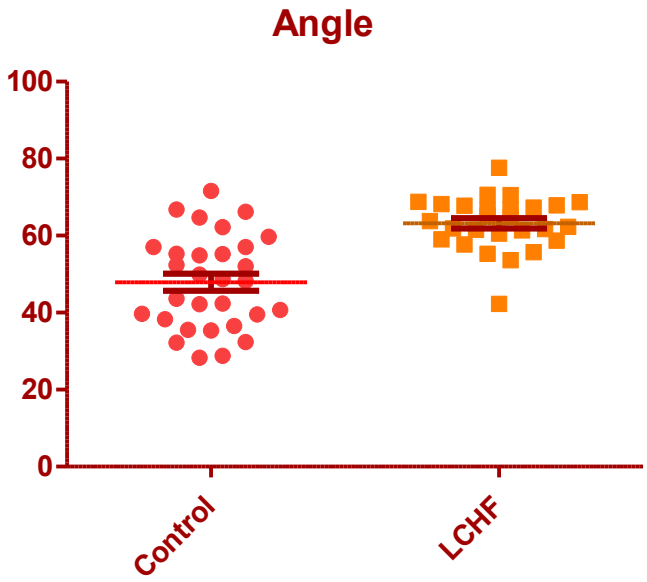

Figure 5.3: Median alpha angle was significantly greater in LCHF participants compared to controls (63.8 vs 48.5 degrees), P<0.0001.

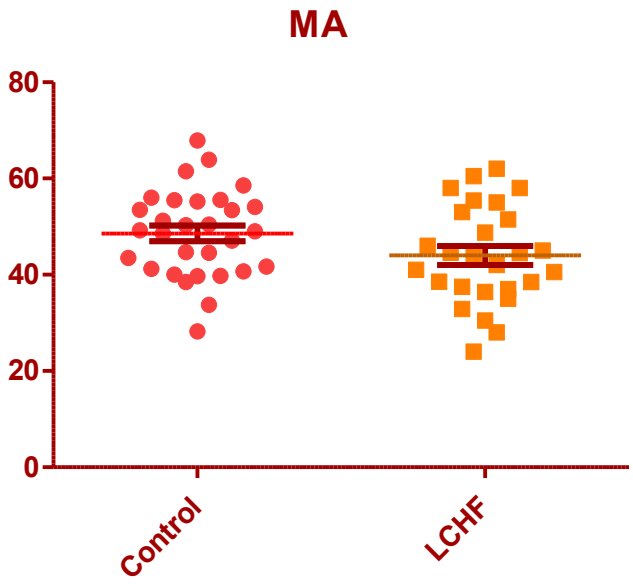

Figure 5.4: Median MA was not significantly lower in LCHF participants than in controls (49.1 vs 49.1 millimeters), P=0.0829.

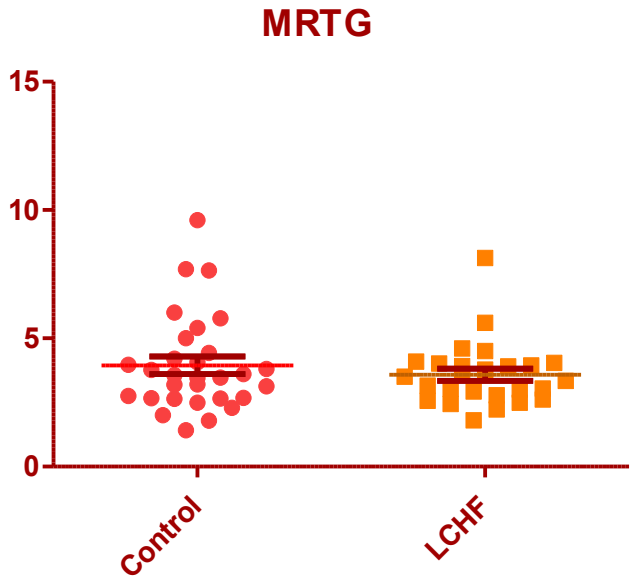

Figure 5.5: Median MRTG was not significantly different between LCHF and control participants (3.5 vs 3.5 Dyn cm^{-1} s^{-1}, P=0.7013).

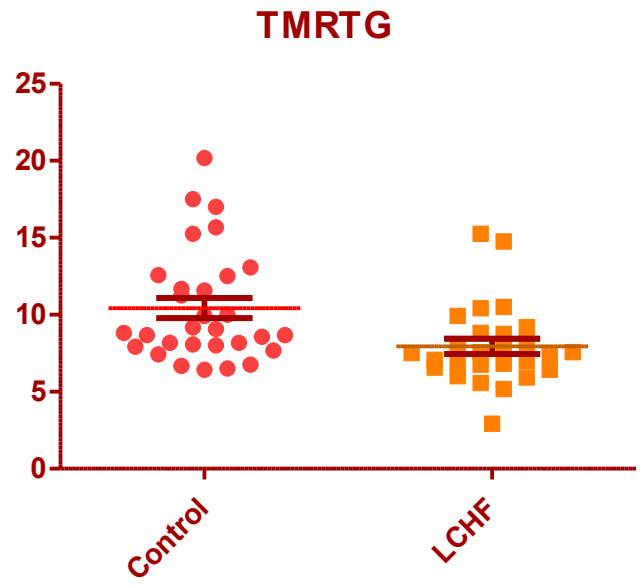

Figure 5.6: TMRTG was significantly shorter in LCHF participants compared to controls (8 vs 10.4 minutes, P=0.0025).

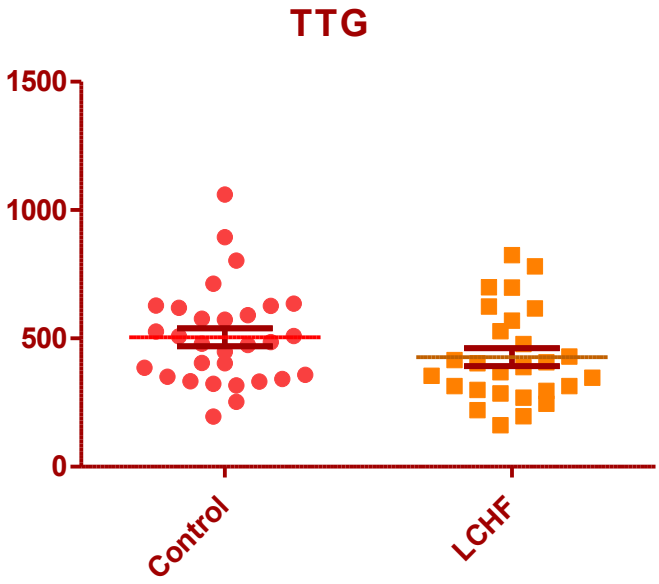

Figure 5.7: TGG was not significantly lower in LCHF than in control participants for TGG (388.4 vs 482.1 Dyn cm^{-2}, P=0.0918).

5.5 Discussion

TEG is an important tool that is used to measure the stability of the clot and it gives clinically valuable information about the entire coagulation process. It is known as a point- of- care method as well as a research technique.[227] TEG can be used to detect

hypercoagulable and hypocoagulable states. The following clotting parameters were measured in the study: R, K, MA, Alpha angle, MRTG, TMRTG, TGG and these parameters are able to give information about the haemostatic activities within a person.[17,63] The clotting parameters such as R value, K value and MA are also used to measure pathology of the clots formed.[227] TEG allows continuous monitoring of the clotting process from its steps of initiation, amplification, propagation, and termination by, generating parameters and producing values related to each step.[233-234] In the sample, the following parameters showed significance in LCHF participants compared to healthy individuals:

- Reaction time (R value) is the time taken from start of the test to initial fibrin formation.
- Alpha or angle representing, the speed in time for fibrin build up and cross-linking of fibrinogen fibers.
- Time to maximum rate of thrombin generation (TMRTG) a time observed before maximum velocity of clot growth.

Using TEG we looked at the coagulation of whole blood of LCHF participants and controls. We suggest that LCHF diet may affect coagulation, as the initial clot formation (R value) was significantly shorter and TMRTG was also shorter. An increase in alpha or angle indicates that the fibrin build -up occurs faster and cross-linking of fibrinogen fibers is increased. Also decrease in MA though, it was non-significantly indicates that the clot is weaker, yet stable. The shorter clot formation and a shorter time to maximum thrombus generation have been reported to show hypercoagulability or an increased coagulability.[149] Hypercoagulability is a feature of inflammation. The results indicate an increased rate of clot initiation and growth in LCHF participants although the formed clot may be insignificantly weaker, yet stable. We also suggest that hypercoagulation may be due to increased TF, which is also a cytokine. In the study that was done in normal human plasma with the addition of TF, the results demonstrated a significant decrease in clot initiation.[235] Also, patients with atherosclerosis have been reported to have higher concentration of TF. TEG-based measures of clot initiation and speed of clot formation have confirmed hypercoagulability and increase risk of thromboembolic events to such patients.[235]

FXIII, platelets and fibrinogen are known to be key players in coagulation and WB clot strength.[236] An increase in FXIII is associated with increased cross-linking of fibrin fibers forming denser clots, which are relatively more difficult to break down.[237] Platelet function influences the progression of the coagulation cascade, while increase in fibrinogen levels were found to facilitate atherosclerosis in mouse models of CVD.[67] An increase in FXIII has been linked to metabolic syndrome, possibly indicating a link between FXIII and CVD.[72] .

In a study that was done on mice which were fed a HFD, plasma levels of the following were measured; fibrinogen, thrombin, FVII, FVIII, FIX, FXI and FXII. In two weeks', time, the plasma levels of fibrinogen, FII and FVIII were increased, and this continued throughout sixteen weeks of HFD feeding.[238] FVII, FIX, FXI and FXII showed an increase at two weeks. FXII has been linked to clot compactness.[239] An increased formation of fibrin clots composed of compact, highly-branched networks with thin fibers has also been observed in patients with venous or arterial thrombosis.[240]

In this study, with the help of SEM, we have observed fibrin fibers in individuals on the LCHF diet where some clots were comprised of thin discontinuous fibers while others show matted regions. The DMDs are usually seen in patients with inflammatory conditions. This suggests that the LCHF diet may be inducing an inflammatory state, which then promotes hypercoagulability. Future studies may ascertain whether the nature of the clots formed in individuals on this diet change over time.

5.6 Conclusion

Clot formation occurs faster in LCHF participants compared to controls. A shorter reaction time usually occurs in hypercoagulability and this a feature of inflammation. The speed of the clot increased in LCHF participants although the strength of the clot may be slightly weaker in LCHF participants. The procoagulable feature may be due to changed presence of fibrinogen as well as increased platelet activation detected via flow cytometry. The slightly weaker clot formed may also be due to reduced platelet aggregation detected via flow cytometry. Future studies may verify these contributions of plasma components like fibrin and platelets to less stable clots as detected in this study.

CHAPTER 6: DISCUSSION

The objectives of this study were to investigate the morphological and physiological effects of the LCHF diet on blood components and coagulation parameters using different techniques. Based on the results obtained from this study, using flow cytometry we showed lower platelet aggregation in LCHF individuals. Using TEG, it was shown that the clot formation was faster, but weaker and less stable in LCHF participants. Using SEM, the morphological analysis of RBCs has shown that in LCHF individuals, more poikilocytes were detected. We also observed less prominent platelet aggregation and the fibrin fibers for LCHF participants were shown to be discontinuous with some regions of DMDs.

We thus suggest that LCHF diet may reduce the expression of platelet receptors which influence platelet aggregation. This was confirmed by using TEG where the clot formation in LCHF participants although formed faster, was weaker and less stable. Pathophysiology of RBCs, platelets and fibrin fibers all contribute to impaired coagulation. The results observed in this study, using SEM, shows changes to RBC morphology of LCHF participants and this alteration was confirmed by an increased number of poikilocytes such as leptocytes, echinocytes and stomatocytes. In another study by Ciccoli et al (2012), leptocytes which were prominent in our LCHF group were found to be induced by oxidative stress.[154] The latter is an important feature of inflammation. An increase in the lipid distribution in the RBC's outer layer has been found to induce echinocyte shapes.[174] This may be applicable to our LCHF group. On the other hand, the stomatocyte transformation was found to occur after phosphorylation of intracellular spectrin and depletion of plasma cholesterol.[241-242] Both inflammation and oxidative stress can affect RBC deformability, aggregation and blood viscosity. SEM findings of platelet aggregation was quantified using flow cytometry and confirmed lower aggregation in the LCHF diet group. Fibrin fibers in some LCHF participants were discontinuous and in addition, clots in some participants showed regions of DMDs. The DMDs have been identified in many other inflammatory conditions such as diabetes, RA, hemochromatosis and Alzheimer's.[80,175,243-244] It is also important to note that all male and most female participants on the LCHF diet had borderline high or high cholesterol levels. These changes to plasma composition may also have increased inflammatory signalling.

As concluding remarks, we suggest that the LCHF diet induces an inflammatory response, which then can lead to changes in overall RBC and whole blood morphology. There, however, appears to be a change in the expression of receptors on platelets, which lowers platelet aggregation, potentially influencing clot properties in individuals on this diet. The morphological abnormalities visualized and described in RBCs, platelets and fibrin fibers in participants on a LCHF diet are novel findings.

6.1 Future perspectives and study limitations

Due to financial constraints, the study sample was confined to thirty-two LCHF participants. It is also noteworthy that strict adherence to the diet could not be ascertained leading to a wider distribution of data outcomes for the LCHF group in this study. A larger sample size within a more controlled setting may have reduced the spread of data and potentially more statistically significant results.

There are distinct morphological changes in the ultrastructure of RBCs, platelets and fibrin fibers caused by a LCHF diet. Therefore, sophisticated techniques that provide much more detailed information such as atomic force microscopy, could be employed, in addition to SEM to quantify the degree of change in the biophysical properties of the RBCs. Measurement of platelet aggregation markers using assays such as ELISA may add significant information to collaborate findings obtained with flow cytometry and TEG. Furthermore, additional tests measuring oxidative stress with ROS markers will be useful. Studies that investigate platelet signalling pathways may also provide substantial information on influences of diet on platelet activity.

More importantly, there appears to be a dearth of research regarding the long-term effects of a LCHF diet on haematology and haemostasis. Taking into consideration findings on the coagulation parameters obtained in this study, it can be postulated that results obtained from a long-term study will impact significantly on the outcome of clinical medicine, in particular cardiovascular diseases.

7. Appendix

Appendix 1: Informed Consent for Controls and LCHF group

Information leaflet and Informed consent form for study participants

[Control Group]

Ethics No: 366/16

Title of the Project: The morphological and physiological effects of a low-carbohydrate high-fat diet on coagulation parameters

Principal Investigator: Babalwa Jobela, Department of Physiology University of Pretoria (under the supervision of Dr P Soma, who will perform all the clinical work)

Introduction (invitation to participate in a Research study)

You are invited to volunteer for the above research study. You must be eighteen years or older to participate in this study. This information leaflet is to help you to decide if you would like to participate. The researcher will explain the reason for the drawing of blood. Blood will be drawn to view your cells in the blood under the microscope. ten ml of blood will be drawn (four ml for SEM and two ml for flow cytometry and four ml for TEG which will be used for microscopic work. Your blood will be used for this study only, remaining blood will be stored in the -80˚C freezer in case the experiments needs to be repeated. If you have any questions, which are not fully explained in this leaflet, do not hesitate to ask me. You should not agree to take part unless you are completely happy about all the procedures involved.

Purpose of the study?

The purpose of the study is to investigate the effect of a low-carbohydrate high-fat diet on the structure of red blood cells, platelets and fibrin (cells within your blood) and measuring your cholesterol. Low-carbohydrate, high-fat diet is the eating of fewer carbohydrates with a higher proportion of fat. High-fat diet is known to disturb red blood cell function and lead to increase platelet activity which can both lead to narrowing of

your blood vessels. In order to do the study, blood samples will be needed. The blood will be analyzed via three procedures: (1) SEM (looking under a microscope that looks at the structure of the cells in your blood),
(2) TEG which is a method of testing the efficiency of blood clotting and (3) Flow cytometry, which measures functions of the platelets.

Who will draw the blood?

Dr Prashilla Soma will draw the blood.

Description of your involvement

If you agree to be part of the research study, I will ask you to provide personal details like your name, surname gender and your age. I will also ask about your health e.g. are you on chronic medication, smoking and alcohol intake. You will be asked to confirm that you not on Banting diet**.**

Has the study received ethical approval?

The Faculty of Health Sciences Research Ethics Committee, University of Pretoria, approved this protocol (366/16) and written approval has been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki (last update: October 2013), which deals with the recommendations guiding doctors in biomedical research involving human/participants. A copy of the Declaration may be obtained from the investigator should you wish to review it.

Risks and Discomforts of Participation

There may be some risks or discomfort from your participation in this research. The risks of taking blood include pain, a bruise at the site where the blood is taken, redness and swelling of the vein and infection, and a rare risk of fainting and bleeding from the site. For your protection, the procedures will be performed under sterile conditions by a qualified medical doctor.

What are your rights as a patient/participant in this study?

Participating in this study is completely voluntary. Even if you decide to participate now, you may change your mind and stop at any time without stating any reason. Even if you decide to withdraw before the study is completed, it will be acceptable. The investigator retains the right to withdraw you from the study if it is considered to be in your best interest.

Confidentiality

I plan to publish the results of this study in medical journals. I will not include any information that would identify you. Your privacy will be protected, and your research records will be confidential. Once we have analysed the information no one will be able to identify you. Research reports and articles in scientific journals will not include any information that may identify you.

Contact information for the study

For the duration of the study, should you have any questions during the study, please do not hesitate to contact me. My telephone number is 078 025 2326 through which you can reach me or contact REC secretary on 012-356 3085)

Informed Consent

I hereby confirm that I have been informed by my doctor about the nature, conduct, benefits and risks of this study. I have also received, read and understood the above written information (Patient Information Leaflet and Informed Consent) regarding the study. By signing this document, you are agreeing to be in the study. I am aware that the results of the study, including personal details regarding gender, age, date of birth, initials and diagnosis will be anonymously processed into a research 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 trial.

Patient's name……

(Please print)

Patient's signature…….Date…….

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

Investigator's name (Babalwa Jobela)

Investigator's signature

…….Date……

Information filled by the Investigator

Age	Gender
Education/ occupation	
Height	Weight BMI …… Blood pressure (BP)….
Do the patient smoke?	**Y / N**
Any other chronic condition?	**Y / N**
Are you taking any medication?	**Y / N**
Contact details	

Twenty-four-hour dietary data of which two will be weekdays and one a weekend

Day	Details of diet (What is your diet consists of :)
1. Weekday	
2. Weekday	
3. Weekends	

Information leaflet and Informed consent form for study participants

[LCHF Group]

Ethics number: 366/16

Title of the Project: The morphological and physiological effects of a low-carbohydrate high-fat diet on coagulation parameters

Principal Investigator: Babalwa Jobela Department of Physiology, University of Pretoria (under the supervision of Dr P Soma, who will perform all the clinical work)

Introduction (invitation to participate in a Research study)

You are invited to volunteer for the above research study. You must be eighteen years or older to participate in this study. This information leaflet is to help you to decide if you would like to participate. I will firstly check whether you are on a high fat diet or not. This will be done by checking the breaking down of fat levels on your urine using urine testing strips. The researcher will explain the reason for the drawing of blood. Blood will be drawn to measure your cholesterol and to view your cells in the blood under the microscopy. Fasting blood will be taken for lipid profiles, ten ml of blood will be drawn (two ml will be used for total cholesterol, as to measure your blood Cholesterol, four ml for SEM and flow cytometry and four ml for Thromboelastography (TEG) which will be used for microscopic work. Your blood will be used for this study only, but remaining blood will be stored in the -80˚C freezer in case the experiments needs to be repeated. If you have any questions, which are not fully explained in this leaflet, do not hesitate to ask me. You should not agree to take part unless you are completely happy about all the procedures involved.

Purpose of the study?

The purpose of the study is to investigate the effect of a high-fat diet on the structure of red blood cells, platelets and fibrin (cells within your blood) and measuring your cholesterol. Low-carbohydrate, high-fat diet is the eating of fewer carbohydrates with a higher proportion of fat. High-fat diet is known to disturb red blood cell function and lead to increase platelet activity which can both lead to narrowing of your blood vessels. In order to do the study, blood samples will be needed. The blood will be

analyzed via three procedures: (1) SEM (looking under a microscope that looks at the structure of the cells in your blood), (2) Thromboelastography which is a method of testing the efficiency of blood clotting and (3) Flow cytometry, which measures functions of the platelets. A blood sample will also be sent to National Health Laboratory Service (NHLS) where your serum cholesterol will be measured.

Who will draw the blood?

Dr Soma will draw the blood.

Description of your involvement

If you agree to be part of the research study, I will ask you to provide personal details like your name, surname gender and your age. I will also ask about your health e.g. are you on chronic medication, smoking and alcohol intake. You will be asked to confirm your high-fat diet (questions on diet) or confirm your participation in the Banting diet.

Has the study received ethical approval?

The Faculty of Health Sciences Research Ethics Committee, University of Pretoria, approved this protocol (366/16) and written approval has been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki (last updated: October 2013), which deals with the recommendations guiding doctors in biomedical research involving human/participants. A copy of the Declaration may be obtained from the investigator should you wish to review it.

Risks and Discomforts of Participation.

There may be some risks or discomfort from your participation in this research. The risks of taking blood include pain, a bruise at the site where the blood is taken, redness and swelling of the vein and infection, and a rare risk of fainting and bleeding from the site. For your protection, the procedures will be performed under sterile conditions by a qualified medical doctor.

What are your rights as a patient/participant in this study?

Participating in this study is completely voluntary. Even if you decide to participate now, you may change your mind and stop at any time without stating any reason. Even if you decide to withdraw before the study is completed, it will be acceptable. The investigator retains the right to withdraw you from the study if it is considered to be in your best interest.

Confidentiality

I plan to publish the results of this study in medical journals. I will not include any information that would identify you. Your privacy will be protected, and your research records will be confidential. Once we have analysed the information no one will be able to identify you. Research reports and articles in scientific journals will not include any information that may identify you.

Contact information for the study

For the duration of the study, should you have any questions during the study, please do not hesitate to contact me. My telephone number is 078025 2326 through which you can reach me or contact the REC Secretary 012-356 3085.

Informed Consent

I hereby confirm that I have been informed by my doctor about the nature, conduct, benefits and risks of this study. I have also received, read and understood the above written information (Patient Information Leaflet and Informed Consent) regarding the study. By signing this document, you are agreeing to be in the study. I am aware that the results of the study, including personal details regarding gender, age, date of birth, initials and diagnosis will be anonymously processed into a research 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 trial

Patient's name…….

(Please print)

Patient's signature…….Date……

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

Investigator's name (Babalwa Jobela)

Investigator's signature

……Date……..

Information filled by the investigator

Age	
Gender	
Education/ occupation	
Does the patient take alcohol?	**Y / N**
Are you on acute medicines?	**Y / N**
(Contact Details)	

Smoking	**Y / N**
Blood pressure (mmHg)	
Height /weight……. BMI (kg/m^2)	
how long have you been on a diet	

Questionnaire for Self-reported diet

Should be on Banting diet at least 3 months **Y** **N**

Eggs **1 or more**

Animal Protein **sausages** **g / day**

Grilled fish, grilled fish .. ☐ **g / day**

Fats

Meat Free range, grass fed, Biltong, steak. ☐ **g / d**

Cheese and yoghurt ☐ **g / day**

Dairy full cream ☐ **g/day**

Broccoli, tomatoes, mushrooms, onions, avocado ☐

Vegetables green leafy veggie ☐ **g /day**

Macadamia, walnuts ☐ **g/ day**

Nuts almonds ☐ **g / day**

Apples, oranges ☐ **g/day**

Fruit /Snack …… ☐ **Greek yoghurt** ☐ **g/ day**

Water ☐ **cups** **Drinks** **coffee, soft drinks** ☐ **cups**

Three, twenty-four-hour dietary data of which two will be weekdays and one a weekend

Day	Details of diet
1. Weekday	
2. Weekday	
3. Weekends	

Disclosure statement: (Regarding consumption of high energy food like Carbohydrates or deviate from Banting (non-Banting moments).

……

……

……

……

Appendix 2: Ethics approval letter

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 28 August 2018.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

13/10/2016

Approval Certificate
New Application

Ethics Reference No.: 366/2016

Title: The effect of a high-fat diet on the morphology of the coagulation profile and on the lipogram

Dear Babalwa Jobela

The **New Application** as supported by documents specified in your cover letter dated 12/10/2016 for your research received on the 12/10/2016, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 12/10/2016.

Please note the following about your ethics approval:
- Ethics Approval is valid for 1 year
- Please remember to use your protocol number (**366/2016**) 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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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

28/04/2017

Approval Certificate
Amendment
(to be read in conjunction with the main approval certificate)

Ethics Reference No.: 366/2016

Title: The morphological and physiological effects of a low-carbohydrate high-fat diet on coagulation parameters

Dear Babalwa Jobela

The **Amendment** as described in your documents specified in your cover letter dated 31/03/2017 received on 31/03/2017 was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 26/04/2017.

Please note the following about your ethics amendment:
- Please remember to use your protocol number (**366/2016**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committe may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics amendment 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
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Faculty of Health Sciences Research Ethics Committee

29/03/2018

Babalwa Jobela
Department of Physiology
University of Pretoria

Dear Babalwa Jobela

RE.: 366/2016 ~ Letter dated 22 February 2018

366/2016 Jobela	
Protocol Title	The effect of a high-fat diet on the morphology of the coagulation profile and on the lipogram.
Principal Investigator	Babalwa Jobela Tel: 078 025 2326 Email: babalwajobela@gmail.com Dept: Physiology

We hereby acknowledge receipt of the following document:

- Extension of study until the end of November 2018

which has been approved at 28 March 2018 meeting.

With regards

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

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