The relationship between blood lipids, fatty acids and plasma clot properties in black South Africans

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To my family and to Josef.

In appreciation of your love, support and belief in me.

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ABSTRACT

Introduction and aim

Cardiovascular disease (CVD) is the foremost cause of mortality worldwide and a major and steadily increasing cause of death among black South Africans. This increase is largely attributed to the nutrition transition the country is undergoing, driven by urbanisation, westernisation and cultural change. The associated dietary changes (including among others a higher total fat and lower ratio of polyunsaturated to saturated fat intake) contribute to the increase in CVD risk, specifically increased serum total cholesterol and low-density lipoprotein cholesterol (LDL-C) and decreased high-density lipoprotein cholesterol (HDL-C). Certain individual plasma phospholipid fatty acids have also been linked to increased or decreased CVD risk. Most CVD events are caused by a blood clot obstructing blood flow to the heart or brain. Numerous factors influence clot properties including plasma fibrinogen concentration (being the structural protein of the clot) as well as certain blood lipids and individual plasma fatty acids. Studies on the relationship between serum lipids, plasma phospholipid fatty acids, fibrinogen and fibrin clot properties and how they affect CVD risk are, however, limited. With the increased CVD prevalence in black South Africans and the associated increase in atherogenic blood lipids in the presence of already high fibrinogen levels, black South Africans are an ideal population in which to investigate these associations. We therefore aimed to investigate the relationship of plasma fibrinogen concentration and fibrin clot properties with different blood lipid fractions and plasma phospholipid fatty acids in black South Africans.

Participants and methods

Data for 1281 participants collected in 2010 for the first follow-up of the Prospective Urban and Rural Epidemiological Study in South Africa (PURE-SA) were included in this cross-sectional study. Participants were apparently healthy black South African men and women aged 35 to 70 years and residing in rural or urban areas in the North-West province. Biochemical analyses included determination of the plasma lipid and phospholipid fatty acid profile, total and γ' fibrinogen (a common fibrinogen splice variant demonstrating altered clot properties) concentration and clot properties (lag time, slope, maximum absorbance and clot lysis time) using a turbidity-based assay.

Results

LDL-C, composite saturated fatty acids (driven by stearic (C18:0) and lignoceric (C24:0) acid) and composite n-3 and n-6 polyunsaturated fatty acids (driven by docosahexaenoic acid (DHA; C22:6n3) and arachidonic acid (C20:4n6), respectively) were associated with a higher fibrinogen concentration and the formation of prothrombotic clots, namely denser clots with a higher rate of lateral aggregation and a lower lysis rate. Despite palmitic acid (C16:0) contributing the largest proportion to the composite saturated fatty acids (27.3% of the total fatty acids), it did not drive the association observed with the composite saturated fatty acids. HDL-C and composite monounsaturated fatty acids (driven by oleic acid (C18:1n9) and palmitoleate (C16:1n7)), on the other hand, were associated with a lower fibrinogen concentration and a profibrinolytic clot phenotype, namely less dense clots with a lower rate of lateral aggregation and a higher lysis rate. Not all individual constituent fatty acids of a composite fatty acid group demonstrated the same relationship with fibrinogen concentration or clot properties as what was reported for their respective composite fatty acid group. For instance, in contrast with associations observed for composite monounsaturated fatty acids, nervonic acid (C24:1n9) was associated with a higher fibrinogen concentration and prothrombotic clots. Similarly, in contrast with associations observed for composite n-3 fatty acids, alpha-linolenic acid (ALA; C18:3n3) was associated with profibrinolytic clots but had no association with fibrinogen concentration. Eicosapentaenoic acid (EPA; C20:5n3) was associated with a lower fibrinogen concentration and profibrinolytic clots, while docosapentaenoic acid (DPA; C22:5n3) showed no associations with fibrinogen concentration or clot properties. The ratio total n-6/n-3 fatty acids was associated with a lower fibrinogen concentration and profibrinolytic clots. In many cases the associations with clot properties were fully or partially mediated by respective higher or lower fibrinogen concentrations. Associations that remained after adjustment for fibrinogen suggest these blood lipids and phospholipid fatty acids have direct effects on clot properties independent from fibrinogen concentration. No associations were found between blood lipids or fatty acids with γ' fibrinogen.

Conclusion

Certain blood lipids and plasma phospholipid fatty acids were associated with a higher fibrinogen concentration and prothrombotic clot properties while others were associated with a lower fibrinogen concentration and profibrinolytic clot properties. Constituent fatty acids often did not have the same relationship with fibrinogen concentration and clot properties as their composite group. Conclusions regarding the relationship of individual fatty acids with fibrinogen concentration or clot properties should therefore not be drawn based on the relationship between composite fatty acids and these factors. The limited number of studies conducted on the topic thus far, along with differences in their study and experimental designs, prohibit the

drawing of firm conclusions. Further investigation is necessary to elucidate the causal relationships and mechanisms involved.

Key words: fibrinogen; fibrinogen γ'; clot properties; blood lipids; plasma phospholipid fatty acids

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LIST OF ABBREVIATIONS

au/s	Absorbance units per second
Acetyl-CoA	Acetyl coenzyme A
CH_3	Acid group
AUTHeR	Africa Unit for Transdisciplinary Health Research
α	Alpha
ALA	Alpha-linolenic acid
AHA	American Heart Association
ANCOVA	Analysis of co-variance
ANOVA	Analysis of variance
β	Beta
BMI	Body mass index
CVD	Cardiovascular disease
cm	Centimetre
CEN	Centre of Excellence for Nutrition
CLT	Clot lysis time
CAD	Coronary artery disease
DEI	Daily energy intake
°C	Degrees Celsius
∆nm	Delta nanometres
DBP	Diastolic blood pressure
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
ELISA	Enzyme-linked immunosorbent assay
≥	Equal or more than
EDTA-coated	Ethylenediaminetetraacetic acid-coated
ELA	Euglobulin lysis area

Factor VIIc	Factor VII coagulant activity
FAMEs	Fatty acid methyl esters
FAO	Food and Agriculture Organization of the United Nations
γ	Gamma
γ’	Gamma prime
GC-EI-MS	Gas chromatography electron ionisation mass spectrometry
Glu	Glutamine
g	Grams
g/day	Grams per day
g/L	Grams per litre
HREC	Health Research Ethics Committee
HDL	High-density lipoprotein
HDL-C	High-density lipoprotein cholesterol
hsCRP	High-sensitivity C-reactive protein
HIV	Human immunodeficiency virus
IDL	Intermediate-density lipoprotein
ILIB	International Lipid Information Bureau
ISAK	International Society for the Advancement of Kinanthropometry
kDa	Kilodalton
kg/m^2	Kilogram per (metre squared)
<	Less than
Lp (a)	Lipoprotein (a)
LpL	Lipoprotein lipase
LDL	Low-density lipoprotein
LDL-C	Low-density lipoprotein cholesterol
Lys	Lysine
COOH	Methyl group
µM	Micrometre
mg/L	Milligrams per litre

mg/ml	Milligrams per millilitre
mmHg	Millimetres of mercury
mmol/L	Millimoles per litre
min	Minutes
mM	Millimolar
MUFA	Monounsaturated fatty acid
ng/mL	Nanogram per millilitre
nm	Nanometre
NHLBI	National Heart, Lung, and Blood Institute
NIH	National Institutes of Health
non-HDL-C	Non-high-density lipoprotein cholesterol
NWU	North-West University
n	Number
n-3	Omega-3
n-6	Omega-6
n-9	Omega-9
r	Pearson correlation coefficient
%	Percent
pM	Picomolar
PAI-1	Plasminogen activator inhibitor type-1
PAI-1 activity	Plasminogen activator inhibitor type-1 activity
PUFA	Polyunsaturated fatty acid
PURE-SA	Prospective Urban and Rural Epidemiological Study in South Africa
p	P-value
SFA	Saturated fatty acid
SEM	Scanning electron microscopy
SAMRC	South African Medical Research Council
SD	Standard deviation
Stats SA	Statistics South Africa

STEMI	ST-elevation myocardial infarction
SBP	Systolic blood pressure
TAFI	Thrombin activatable fibrinolysis inhibitor
TGA	Thrombin generation assay
TEG	Thromboelastogram
TF	Tissue factor
t-PA	Tissue plasminogen activator
THUSA study	Transition and Health during Urbanisation of South Africans study
TB	Tuberculosis
u-PA	Urokinase
VLDL	Very low-density lipoprotein
VLDL-C	Very low-density lipoprotein cholesterol
v/v	Volume/volume
vWF	Von Willebrand factor
WHO	World Health Organization
yrs.	Years

CHAPTER 1 – INTRODUCTION

1.1. Background

Cardiovascular disease (CVD) is the foremost cause of death worldwide (WHO, 2017; Barquera *et al.*, 2015) and a major cause of death in South Africa which is steadily increasing (Vorster, 2002; Connor *et al.*, 2007; Stats SA, 2017). An increase in CVD prevalence has also been seen among black South Africans (Nojilana *et al.*, 2016). This is largely attributed to the nutrition transition the country is undergoing, driven by populations progressively moving to cities and adopting Western lifestyles and cultures (Vorster *et al.*, 2011). Consequently, dietary intakes are changing to, among others, include more fat, as well as a lower ratio of polyunsaturated to saturated fat intake (Vorster *et al.*, 2005). These changes in dietary intakes contribute to the increase in CVD risk factors (specifically increased serum total cholesterol and low-density lipoprotein cholesterol (LDL-C) and plasma fibrinogen) reported in this population (Vorster *et al.*, 2005).

It is well known that blood lipids alter CVD risk, with increased blood levels of triglycerides and LDL-C (with low-density lipoprotein (LDL) being the main cholesterol carrier in the blood) increasing risk (Lusis, 2000; O'Donnell & Elosua, 2008; Harchaoui *et al.*, 2009; Badimon & Vilahur, 2012; Boullart *et al.*, 2012; Buckley & Ramji, 2015). In contrast, high-density lipoprotein cholesterol (HDL-C) offers protection against CVD development as high-density lipoprotein (HDL) removes cholesterol from the arterial wall (Badimon & Vilahur, 2012; Buckley & Ramji, 2015). Certain individual plasma fatty acids have also been linked to CVD risk, such as the saturated fatty acid (SFA) stearic acid (C18:0) which has been linked to increased CVD risk (Khaw *et al.*, 2012), while the n-3 polyunsaturated fatty acid (PUFA) eicosapentaenoic acid (EPA) (C20:5n3) has been linked to decreased CVD risk (Mozaffarian *et al.*, 2013).

Most CVD events are caused by a blood clot or thrombus obstructing blood flow to the heart or brain (Arbustini *et al.*, 1999; Mozaffarian *et al.*, 2015) with several proteins involved in the coagulation cascade. One of the main proteins involved is the glycoprotein fibrinogen (Fibrinogen Studies Collaboration, 2004) for which there are different variants. One of these is the splice variant fibrinogen γ' (Mosesson *et al.*, 2001; Farrell, 2012), which constitutes 10-15% of fibrinogen in the blood (Standeven *et al.*, 2005; Duval & Ariens, 2016) and has, independently of total fibrinogen, been linked to thrombotic diseases (Farrell, 2012). During clot formation, the enzyme thrombin converts fibrinogen to fibrin, which ultimately forms the scaffold of the blood clot (Mosesson *et al.*, 2001; Wolberg, 2007). Increased plasma levels of total and γ' fibrinogen have been shown to produce dense, more stable clots with thinner fibrin fibres that are more

resistant to lysis, consequently increasing CVD risk (Cooper *et al.*, 2003; de Moerloose *et al.*, 2010; Duval & Ariens, 2016).

Apart from these, various other genetic and environmental factors influence clot properties (Weisel & Litvinov, 2013). As certain blood lipids and individual plasma fatty acids also seem to be associated with both fibrinogen concentration and plasma fibrin clot properties, researchers are increasingly focussing on the relationship between haemostatic factors with blood lipids and plasma fatty acids and how the combination of these affect CVD risk. Recent studies on the topic suggest that blood lipids and plasma fatty acids may alter the rate at which a clot forms (Kim *et al.*, 2015; Tanka-Salamon *et al.*, 2016), can incorporate into clots (Rabai *et al.*, 2007; Silvain *et al.*, 2011; Talens *et al.*, 2012a), may alter clot density and firmness (Talens *et al.*, 2013; Zabczyk *et al.*, 2013; Tanka-Salamon *et al.*, 2016) and could even hinder or promote clot lysis (Al-Awadhi & Dunn, 2000; Zabczyk *et al.*, 2013).

In summary, the prevalence of CVD is increasing on a global scale as well as among black South Africans specifically. The combined effect of haemostasis with blood lipids and plasma fatty acids on CVD risk is gaining attention, but as this is still a novel research field, the relationship between total and γ' fibrinogen, plasma clot properties, blood lipids and plasma fatty acids have not yet been investigated in detail. The present study is therefore highly relevant to the current situation both globally and in South Africa.

1.2. Aim and objectives

The aim of this study was to determine the relationship of fibrin clot properties with different blood lipid fractions and plasma phospholipid fatty acids (as detailed in the objectives below) in 1281 black South Africans who participated in the Prospective Urban and Rural Epidemiological Study in South Africa (PURE-SA) in 2010. The objectives of the present study were:

1. To determine the relationship of total and γ' fibrinogen (a common splice variant of fibrinogen) concentration with blood lipids namely total cholesterol, LDL-C, HDL-C, non-HDL-C, triglyceride concentration and the lipid ratio LDL-C/HDL-C. Fibrinogen being the structural protein responsible for fibrin clot formation.

2. To determine the relationship of total and γ' fibrinogen concentration with composite (saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids (including n-3 and n-6 polyunsaturated fatty acids)) and individual plasma phospholipid fatty acids.

3. To determine the relationship of plasma clot properties (namely clot formation, clot structure and clot lysis) with above-mentioned blood lipids and plasma phospholipid fatty acids.

4. To determine the interaction of total and γ' fibrinogen concentration with above-mentioned blood lipids and plasma phospholipid fatty acids in determining clot properties.

1.3. Research team

The PURE-SA study was conducted by a multi-disciplinary team. The research team listed below are the individuals who were involved in this study.

Title, initials and surname	Affiliations	Role in the study
Ms B Kahler	Centre of Excellence for Nutrition (CEN) at North-West University (NWU)	Part-time MSc student. Protocol writing, statistical analysis, interpretation of results, writing up of literature, methodology and data.
Dr Z de Lange	CEN at NWU	Supervisor of MSc mini-dissertation. Provided overall guidance on protocol writing, statistical analysis, the interpretation of results and the writing up of literature and data.
Prof. M. Pieters	CEN at NWU	Co-supervisor of MSc mini-dissertation. Provided guidance (specifically regarding haemostasis) on protocol writing, statistical analysis, the interpretation of results and the writing up of literature and data.
Prof. C.M. Smuts	CEN at NWU	Co-supervisor of MSc mini-dissertation. Provided guidance (specifically regarding blood lipids and plasma phospholipid fatty acids) on protocol writing, statistical analysis, the interpretation of results and the writing up of literature and data.

1.4. Structure of this mini-dissertation

This mini-dissertation is presented in chapter format and consists of five chapters. It was technically edited in the Harvard style as required by NWU. Chapter 1 (the present chapter) introduces the mini-dissertation and provides a brief background to the study, its aim and objectives and the research team and their respective inputs.

Chapter 2 provides an overview of the literature on the study topic. This chapter describes the process of haemostasis from the initiation of coagulation, subsequent clot formation and finally the process by which the body lyses the clot. A brief background on blood lipids and plasma phospholipid fatty acids is further provided including their relationship with CVD risk, as well as a summary of the literature investigating their respective effects on total and γ' fibrinogen and fibrin clot properties.

Chapter 3 describes the methods used to conduct the study. A description of the study design and population is included, as well as detail on the data collection process of the PURE-SA study. Methods used for the measurement of blood lipids, plasma phospholipid fatty acids, total and γ' fibrinogen and clot properties are described. A description of the statistical analyses used in this mini-dissertation is also provided.

In Chapter 4 the results of this study are presented. The typical characteristics of the study population and a sub-sample of the study population for which plasma phospholipid fatty acids were measured, are given. The mean plasma phospholipid fatty acid levels for this sub-sample are also provided. The relationship of total and γ' fibrinogen, firstly with blood lipids and then with plasma phospholipid fatty acids, are presented. Finally, the results of the relationship of clot properties with blood lipids and then with plasma phospholipid fatty acids are provided.

In the final chapter (Chapter 5) these results are discussed focussing on a few key blood lipids and plasma phospholipid fatty acids identified from the results. Firstly, the relationship between these blood lipids with total and γ' fibrinogen and clot properties are discussed, followed by the relationship between the plasma phospholipid fatty acids and those factors. Finally, the limitations of the study are given, a conclusion reached, and suggestions made for future studies.

CHAPTER 2 – LITERATURE REVIEW

2.1. Introduction

Cardiovascular disease (CVD), particularly ischaemic heart disease and stroke, is the leading cause of global deaths (WHO, 2017; Barquera *et al.*, 2015) and a major cause of death in South Africa (Stats SA, 2017), with this figure set to increase in the coming years (Vorster, 2002; Connor *et al.*, 2007).

The rise in CVD among black South Africans (Nojilana *et al.*, 2016) can largely be attributed to the nutrition transition that the country is undergoing – a by-product of rapid urbanisation, modernisation and cultural change (Vorster *et al.*, 2011). In the Transition and Health during Urbanisation of South Africans (THUSA) study, it was observed that those living in urban areas consumed more fat, more cholesterol and a lower ratio of polyunsaturated to saturated fats, than their rural counterparts (Vorster *et al.*, 2005). These dietary changes could explain why it was also observed that the prevalence of abnormal levels of CVD risk factors such as serum total cholesterol and low-density lipoprotein cholesterol (LDL-C), as well as plasma fibrinogen, seem to increase as populations move into urban areas (Vorster *et al.*, 2005).

Most CVD events are the result of an obstruction of blood flow to the heart or brain caused by a clot or thrombus (Arbustini *et al.*, 1999; Mozaffarian *et al.*, 2015). There are many hereditary and environmental factors that can influence clot formation, structure and lysis or breakdown (Weisel & Litvinov, 2013). These factors include certain blood lipids and individual plasma fatty acids which seem to alter clot properties in several ways (Rabai *et al*., 2007; Weisel & Litvinov, 2013). Recent advances in the haemostasis field is increasingly focussing on the relationship between haemostasis and blood lipids and their combined effect or interaction in increasing CVD risk. Current evidence indicates a complicated relationship between blood lipids and coagulation, with lipid levels not only being involved in the regulation thereof, but also binding directly to coagulation proteins, such as fibrinogen and fibrin and thereby altering their function (Talens *et al.*, 2012a). In addition, increased plasma total and γ' fibrinogen levels have been shown to result in clots that are denser and more stable with thinner fibres and are subsequently more resistant to lysis, ultimately increasing CVD risk (Cooper *et al.*, 2003; de Moerloose *et al.*, 2010; Duval & Ariens, 2016).

To provide the necessary background to the current study, this chapter describes the process of haemostasis starting with the initiation of coagulation through injury, the subsequent formation of the clot and ultimately the process by which the clot is lysed. A brief background on blood

lipids and plasma phospholipid fatty acids is provided, as well as a summary of the literature investigating their respective effects on total and γ' fibrinogen as well as fibrin clot properties.

2.2. Clot formation

Haemostasis, or the prevention of blood loss (Hall, 2011; Widmaier *et al.*, 2011), is an important survival mechanism of the body in the case of injury (Davie *et al.*, 1991; Colman *et al.*, 2001). Haemostasis comprises several successive mechanisms, namely vascular constriction, platelet plug formation, blood clot formation through blood coagulation, lysis of the blood clot and eventual fibrous tissue growth (Davie *et al.*, 1991; Hall, 2011).

Numerous factors play a role in blood coagulation – either promoting (procoagulant) or inhibiting (anticoagulant) coagulation. Under normal circumstances, the predominant presence of anticoagulants prevents circulating blood from coagulating (Hall, 2011). However, in the event of injury, procoagulants in the surrounding tissue are successively converted to active enzymes ultimately resulting in blood clot formation (Davie & Ratnoff, 1964; Hoffman & Monroe, 2005; Hall, 2011). Two interrelated pathways (intrinsic and extrinsic) for haemostasis have been proposed for which various factors are initially involved, with both pathways ultimately resulting in the activation of factor X (Marlar *et al.*, 1982; Widmaier *et al.*, 2011). Haemostasis occurs in three overlapping phases, namely initiation, amplification and propagation (Ajjan & Grant, 2006; Monroe & Hoffman, 2006) and these phases are described below:

2.2.1. Initiation

The initiation phase occurs when injury exposes blood to extravascular tissue factor (TF)-releasing cells such as vascular smooth muscle cells, pericytes and adventitial fibroblasts local to the site of injury (Hoffman & Monroe, 2005; Mackman, 2009; Widmaier *et al.*, 2011). Circulating factor VII binds to TF on these cells, although it is speculated that some factor VII is bound to TF even without injury present (Monroe & Hoffman, 2006). TF/factor VIIa complexes initiate the extrinsic pathway by activating factors X (Nemerson, 1966; Jesty & Nemerson, 1974) and IX passing through the tissues. Factor Xa then binds with factor Va, which is secreted by platelets adhering to the site of injury, to form prothrombinase complexes (Davie *et al.*, 1991; Colman *et al.*, 2001; Monroe & Hoffman, 2006).

These prothrombinase complexes, in the presence of adequate amounts of calcium and bound to a phospholipid surface, convert the precursor pro-thrombin to thrombin by means of the proteolytic cleavage of two pro-thrombin peptides (Rosing *et al.*, 1980; Davie *et al.*, 1991; Krishnaswamy, 2013). This results in the generation of a limited amount of thrombin on the surface of these TF-releasing cells (Hoffman & Monroe, 2005).

2.2.2. Amplification

The larger coagulation factors, namely platelets and factor VIII bound to von Willebrand factor (vWF), move into the extravascular space upon rupture of the vascular wall and adhere to the exposed collagen at the site of injury. The binding of platelets to collagen, which is facilitated by vWF (Davie *et al.*, 1991; Ajjan & Grant, 2006), partially activates the platelets (Hoffman & Monroe, 2005). The thrombin generated on the TF-releasing cells further activates the platelets as well as cofactors V and VIII and factor XI on the platelet surface – all leading to increased thrombin production (Monroe & Hoffman, 2006).

2.2.3. Propagation

The propagation phase takes place on the surface of activated platelets aggregated at the site of injury. Factors Va, VIIIa, IXa and XI localise to the platelet surface, where after factor IXa binds to factor VIIIa leading to the intrinsic pathway of coagulation (Hoffman & Monroe, 2005). Factor Xa rapidly binds with factor Va to produce adequate amounts of thrombin required for clotting (Davie & Ratnoff, 1964; Monroe & Hoffman, 2006).

2.2.4. Conversion of fibrinogen to fibrin

Various proteins are involved in the coagulation cascade with the final step in the cascade being the conversion of fibrinogen to fibrin (Mosesson *et al.*, 2001; Wolberg, 2007). Fibrinogen is a large plasma glycoprotein (Standeven *et al.*, 2005) with a molecular length of 45 nm (Mosesson *et al.*, 2001) and a weight of 340 kDa. It is normally present in the blood at a concentration of 2-4 mg/ml or 6-12 µM (Wolberg, 2007). Different variants of fibrinogen have been described in the literature, with one of the most studied being a splice variant of fibrinogen known as fibrinogen γ' (Mosesson *et al.*, 2001; Farrell, 2012) that constitutes 10-15% of the total fibrinogen concentration in the blood (Standeven *et al.*, 2005; Duval & Ariens, 2016). The fibrinogen molecule consists of three pairs of polypeptide chains, namely Aα, Bβ and γ chains (Mosesson *et al.*, 2001; Standeven *et al.*, 2005; Ajjan & Grant, 2006) with fibrinogen γ' having modified γ chains with 20 additional amino acids as a result of alternative splicing (Wolfenstein-Todel & Mosesson, 1981; Standeven *et al.*, 2005).

Thrombin, as described above, converts fibrinogen to fibrin (Figure 2.1.) by binding to and cleaving four fibrinopeptides (A and B) from the central region of each fibrinogen molecule to form one fibrin monomer with exposed knobs and holes (Davie *et al.*, 1991; Weisel, 2005; Ajjan & Grant, 2006). These knobs on fibrin monomers bind with corresponding holes on other monomers resulting in the formation of protofibrils. Protofibrils aggregate laterally to form fibrin fibres and ultimately forms a three-dimensional structure trapping cells, platelets and plasma (Mosesson *et al.*, 2001; Wolberg, 2007; Weisel & Litvinov, 2013). In addition, thrombin

activates factor XIII causing covalent cross-links to form between fibrin molecules by means of transglutaminase reactions (Mosesson *et al.*, 2001) resulting in a more stable clot (Ajjan & Grant, 2006; Weisel & Litvinov, 2013).

Increased circulating levels of total and γ' fibrinogen are associated with increased CVD risk (Lovely *et al.*, 2002; Uitte de Willige *et al.*, 2005; Bridge *et al.*, 2014), which can be partly explained by the fact that total and γ' fibrinogen influence clot structure and lysis. Dense, stiff clots which are resistant to lysis are associated with increased CVD risk (Bridge *et al.*, 2014). Increased fibrinogen concentrations lead to clots with tightly-packed thinner fibrin fibres, while clots produced with fibrinogen γ' (with the negatively charged γ' chain interfering with fibrin polymerisation and protofibril formation) are denser with thinner fibrin fibres and are resistant to lysis (Cooper *et al.*, 2003; de Moerloose *et al.*, 2010; Pieters *et al.*, 2013; Duval & Ariens, 2016).

The clot forms the framework on which wound healing occurs and is lysed during the healing process. However, if the clot is not optimally lysed, the risk of complete or partial occlusion of the blood vessel is increased and could result in a CVD event. In the following section, clot lysis is described.

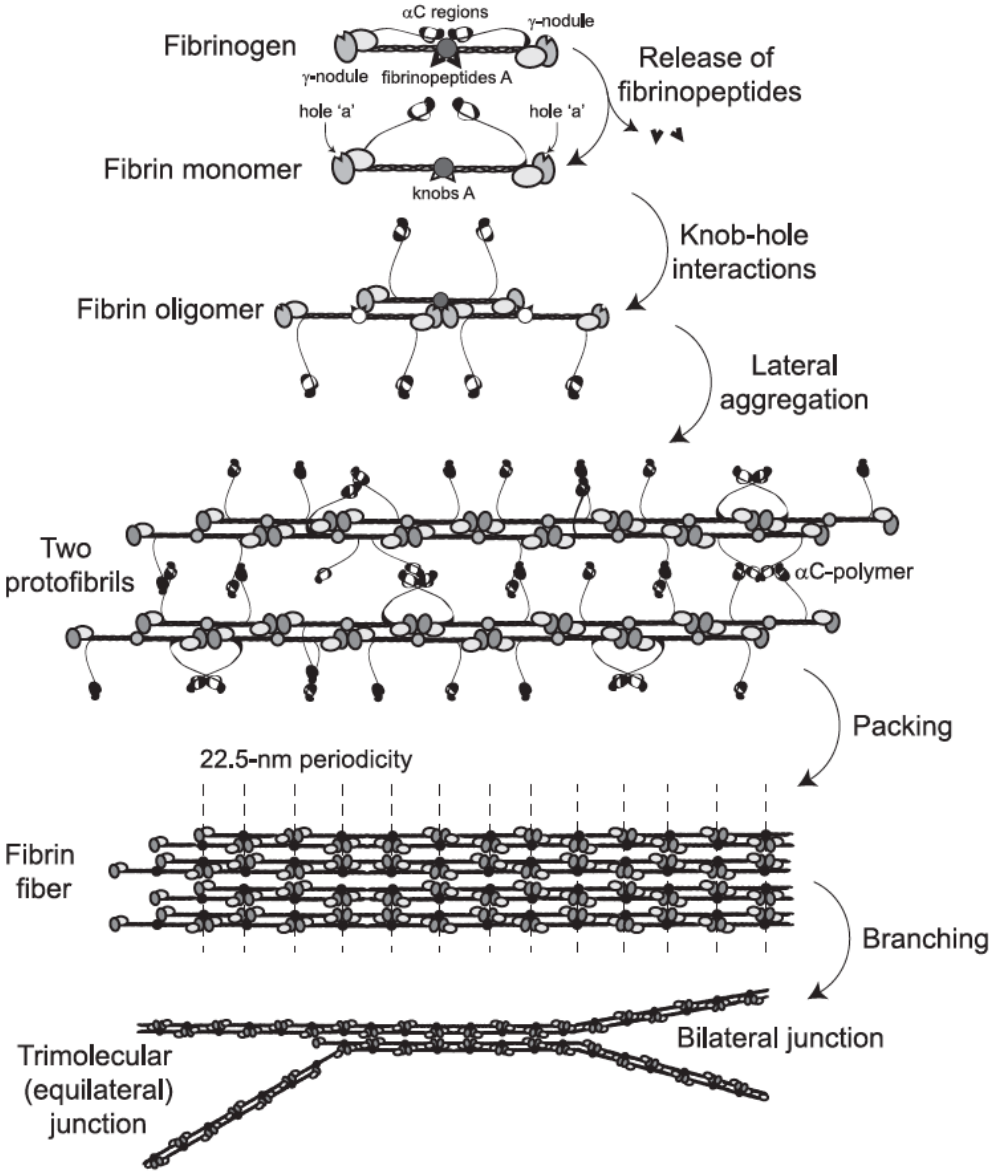


Figure 2.1. Illustration of the conversion of fibrinogen to fibrin (Weisel & Litvinov, 2013)

Weisel, J.W. & Litvinov, R.I. 2013. Mechanisms of fibrin polymerization and clinical implications. *Blood* Online. http://www.bloodjournal.org

2.3. Fibrinolysis

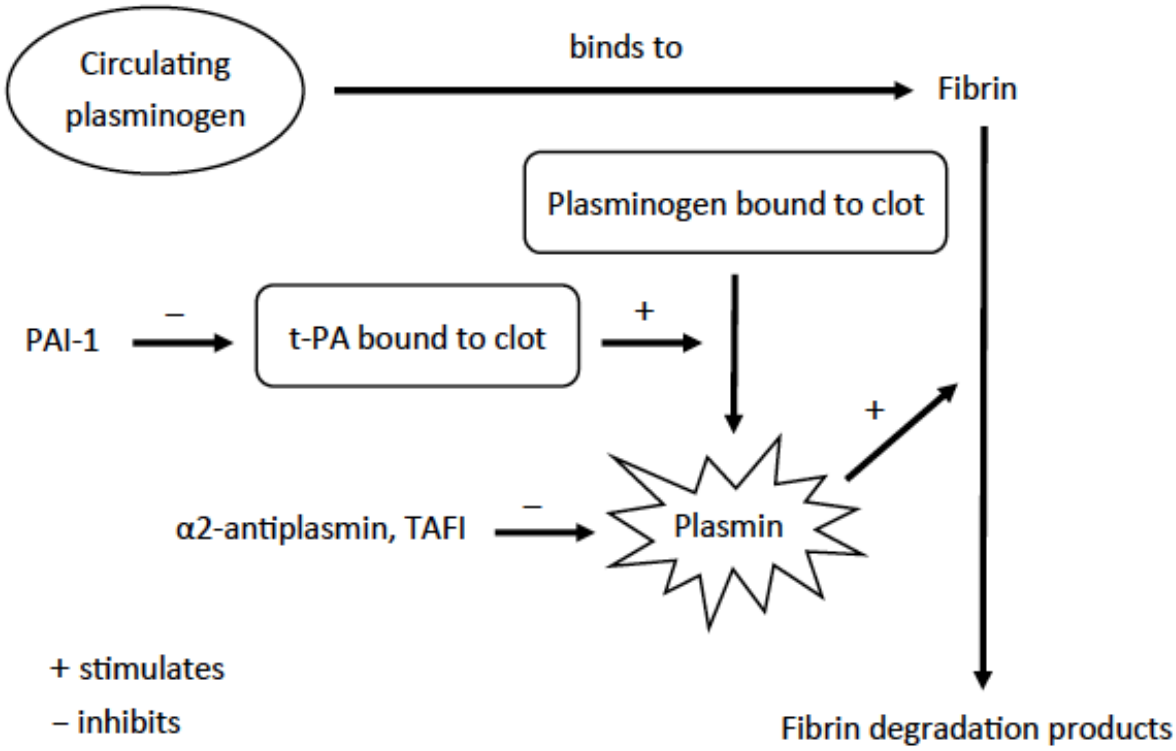


Figure 2.2. Self-compiled illustration of the primary factors and inhibitors involved in fibrinolysis (Colman *et al.*, 2001; Rijken & Sakharov, 2001; Weisel, 2005; Ajjan & Grant, 2006)

TAFI: thrombin activatable fibrinolysis inhibitor; t-PA: tissue plasminogen activator; PAI-1: plasminogen activator inhibitor type-1

The production of fibrin both promotes and inhibits fibrinolysis as it stimulates the conversion of plasminogen to plasmin while also binding α2-antiplasmin and α2-macroglobulin (plasminogen inhibitors) and plasminogen activator inhibitor type-1 (PAI-1) (an inhibitor of tissue plasminogen activator (t-PA) and urokinase (u-PA)) (Rijken & Sakharov, 2001; Standeven *et al.*, 2005; Talens *et al.*, 2012b).

Plasmin is the main enzyme responsible for fibrinolysis and circulates in the blood in its inactive form (plasminogen) becoming trapped in the fibrin network during clot formation and also binding to fibrin (Rijken & Sakharov, 2001; Weisel, 2005; Ajjan & Grant, 2006). t-PA and u-PA, produced by endothelial cells local to the site of injury, also bind to fibrin and in so doing convert plasminogen to plasmin (Ajjan & Grant, 2006; Bansal & Roychoudhury, 2006; Longstaff & Kolev, 2015). In turn, plasmin digests fibrin by cleaving bonds in the polypeptide chain next to

lysine or arginine residues of the αC-domain, followed by cleavage of an N-terminal peptide of the β-chain and the coiled coil connector of the D- and E-regions (Longstaff & Kolev, 2015). It is suggested that plasmin accomplishes this by cleaving Glu-plasminogen molecules, the native circulating form of plasminogen that cluster at specific sites on the fibrin fibre rather than being evenly distributed, to form Lys-plasminogen that is more readily activated (Miles *et al.*, 2003; Longstaff & Kolev, 2015).

There are a number of proteins that inhibit fibrinolysis (Figure 2.2), namely PAI-1 (hinders the conversion of plasminogen to plasmin by binding to t-PA bound to the clot, thus inhibiting it), α2-antiplasmin and α2-macroglobulin (binds to and inhibits plasmin) and thrombin activatable fibrinolysis inhibitor (TAFI) (inhibits plasmin activity by cleaving C-terminal lysine residues from fibrin consequently preventing plasminogen and plasmin from binding to fibrin) (Colman *et al.*, 2001; Rijken & Sakharov, 2001; Weisel, 2005; Ajjan & Grant, 2006). Fibrinolysis is depicted in Figure 2.2.

In addition to these proteins, fibrinolysis can be hindered by clots with altered properties. Clots with thin and densely packed fibrin fibres have been shown to be more resistant to lysis as, although individual thin fibres digest faster than thicker fibres, the action of plasmin is slowed down due to its slow diffusion through these types of clots (Bridge *et al.*, 2014; Longstaff & Kolev, 2015).

Various hereditary and environmental factors can influence clot properties and lysis in different ways. These factors include a number of inherent and developed variations in fibrinogen structure (such as fibrinogen splice variants), the environment in which the clot forms (such as in the presence of certain plasma proteins, blood lipids and individual plasma fatty acids), the interaction of cells (including platelets, leukocytes, fibroblasts and endothelium) trapped and in close vicinity to the clot, as well as the level of blood flow around the clot (Rabai *et al.*, 2007; Weisel & Litvinov, 2013). For the current study, the following paragraphs focus on blood lipids and plasma fatty acids.

2.4. Blood lipids

2.4.1. Lipoproteins

Lipids are transported in the blood bound to proteins. These complexes are called lipoproteins and consist of a non-polar triglyceride and cholesteryl ester core enclosed in a layer of phospholipids, cholesterol and proteins (apolipoproteins). Lipoproteins are classified according to density and include (from least to most dense) chylomicrons, very low-density (VLDL), intermediate-density (IDL), low-density (LDL) and high-density (HDL) lipoproteins (Marshall *et al.*, 2012). The composition of these lipoproteins is presented in Figure 2.3.

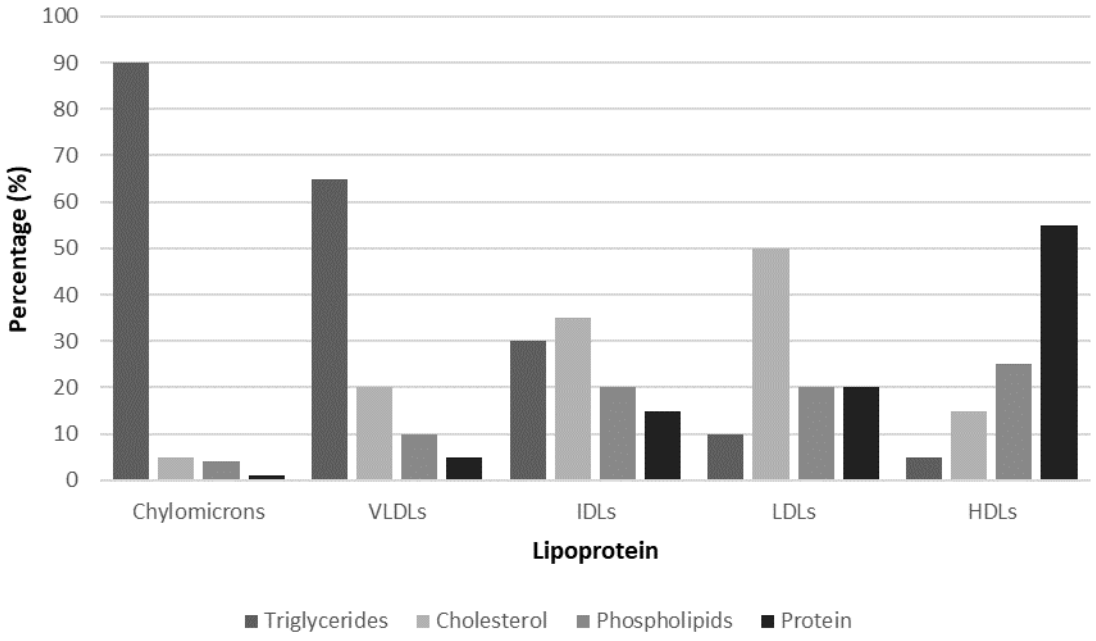


Figure 2.3. Composition of various lipoproteins (Adapted from Marshall *et al.*, 2012)
HDLs: high-density lipoproteins; IDLs: intermediate-density lipoproteins; LDLs: low-density lipoproteins; VLDLs: very low-density lipoproteins

Ingested fat and cholesterol are absorbed by enterocytes, incorporated into chylomicrons, released into the lymphatic system and ultimately finds its way into the circulation through the thoracic duct (Boullart *et al.*, 2012). Triglycerides present in chylomicrons are hydrolysed by the enzyme lipoprotein lipase (LpL) in order to supply fuel (fatty acids) for the heart and skeletal muscles or to be stored within adipose tissue (Boullart *et al.*, 2012).

The liver assembles triglycerides from circulating glycerol and circulating free fatty acids or fatty acids produced by the liver itself and packages these within VLDLs (Rui, 2014). As VLDLs circulate throughout the body, its triglycerides are hydrolysed, gradually resulting in smaller,

denser VLDLs, followed by IDLs (Boullart *et al.*, 2012). Around half of IDLs do not return to the liver and ultimately form LDLs (Boullart *et al.*, 2012), the main transporters of endogenous cholesterol (Widmaier *et al.*, 2011; Marshall *et al.*, 2012). HDLs are also formed by the liver and differ from the other lipoproteins in that it gathers and returns excess cholesterol from tissues back to the liver as well as exchanges cholesterol with LDLs, instead of transporting lipids to cells (Widmaier *et al.*, 2011; Rossouw, 2015). Triglycerides are mainly used to fuel metabolic processes in the body, while other lipids, such as cholesterol, are crucial to the formation of cell membranes (Hall, 2011). Lipid metabolism is illustrated in Figure 2.4.

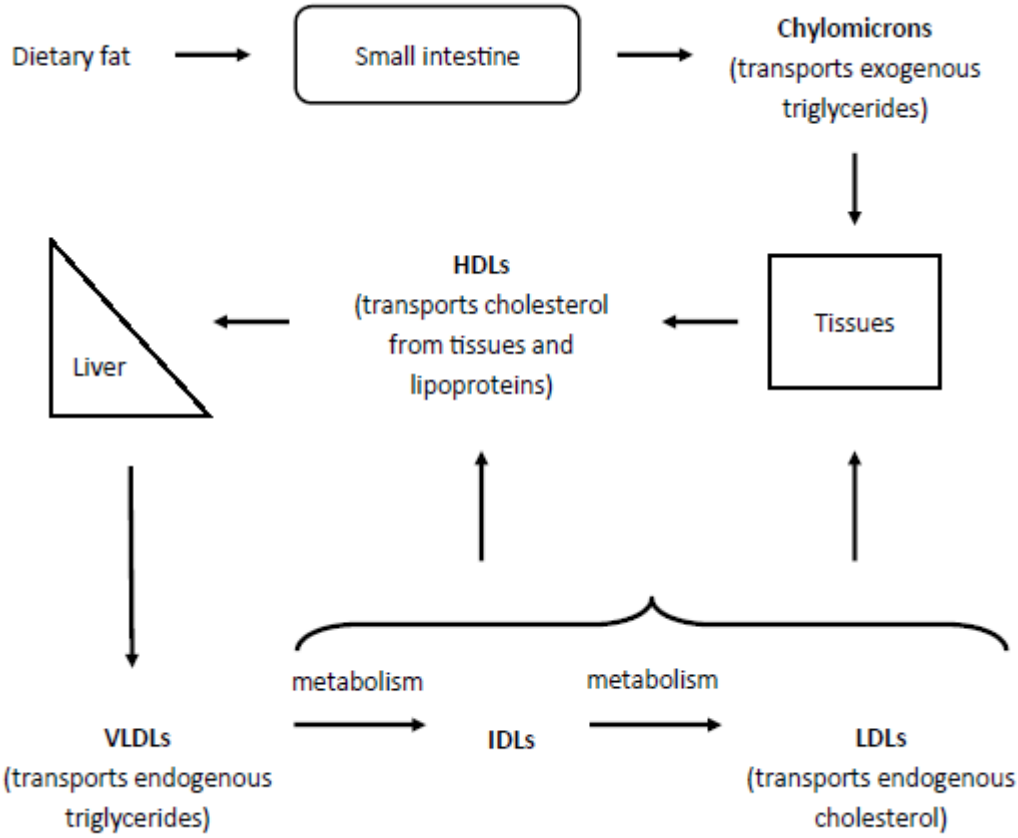


Figure 2.4. Self-compiled illustration of lipid metabolism (Boullart *et al.*, 2012; Marshall *et al.*, 2012; Rossouw, 2015; Rui, 2014; Widmaier *et al.*, 2011)

HDLs: high-density lipoproteins; IDLs: intermediate-density lipoproteins; LDLs: low-density lipoproteins; VLDLs: very low-density lipoproteins

2.4.2. Blood lipids and cardiovascular disease risk

It is well known that increased levels of certain blood lipids are associated with increased CVD risk (Lusis, 2000; Nordestgaard, 2016). Increased serum triglyceride levels, as well as increased levels of the triglyceride-rich lipoproteins, chylomicrons and VLDLs, have been shown to be independently associated with CVD risk (Harchaoui *et al.*, 2009; Boullart *et al.*, 2012). In addition, increased levels of LDL-C, with LDL being the main cholesterol carrier in the blood, is an important risk factor for the development of atherosclerosis owing to oxidised LDL-C contributing to inflammation and the formation of foam cells, eventually resulting in plaque formation (Lusis, 2000; O'Donnell & Elosua, 2008; Badimon & Vilahur, 2012; Buckley & Ramji, 2015). When the atherosclerotic plaque ruptures into the bloodstream, as often happens due to the lack of elasticity of atherosclerotic arteries, the resulting roughened endothelial surface and the exposure of blood vessels to extravascular matter trigger the blood coagulation cascade (Hall, 2011; Buckley & Ramji, 2015). HDL, on the other hand, removes cholesterol from the arterial wall and offers some protection against the development of atherosclerosis (Badimon & Vilahur, 2012; Buckley & Ramji, 2015).

Depending on additional CVD risk factors, namely advancing age, male gender, hypertension and smoking (Catapano *et al.*, 2016), LDL-C targets are as follows: LDL-C <1.8-2.6 mmol/L for high to very high-risk individuals and <3.0 mmol/L for low to moderate risk individuals. Although there are no targets set, HDL-C levels of >1.0 mmol/L in men and >1.2 mmol/L in women and triglyceride levels of <1.7 mmol/L are associated with a decreased CVD risk (Catapano *et al.*, 2016).

Lipid ratios and non-high-density lipoprotein cholesterol (non-HDL-C) can also be used as markers for CVD risk. Non-HDL-C (total cholesterol minus HDL-C) has been found to be an independent risk factor for CVD and is thought to be more reliable than LDL-C due to it representing the amount of atherogenic particles present, is not affected by calculation biases (such as with LDL-C) and can be measured in a fasting or non-fasting state (Cui *et al.*, 2001; Rossouw, 2015; Wongcharoen *et al.*, 2017). Lipid ratios found to be effective in predicting CVD risk, possibly more so than LDL-C or total cholesterol, are the ratios TC/HDL-C and LDL-C/HDL-C. These lipid ratios may be more useful in determining CVD risk as it takes into account both atherogenic and protective lipoproteins relative to each other, with a lower ratio indicating a higher proportion of the anti-atherogenic lipoprotein HDL-C and thus a reduced CVD risk (Natarajan *et al.*, 2003). Depending on risk level, targets are as follows: non-HDL-C 2.6-3.4 mmol/L (LDL-C target plus 0.8 mmol/L) (Catapano *et al.*, 2016), TC/HDL-C <3.5-4.5 for men and <3.0-4.0 for women and LDL-C/HDL-C <2.5-3.0 for men and <2.0-2.5 for women (ILIB & Gotto, 2000; Genest *et al.*, 2003; Holme *et al.*, 2008; Millan *et al.*, 2009).

2.4.3. Blood lipids and clot properties

Table 2.1. provides a summary of studies in which the influence of different blood lipids on clot properties were investigated using thromboelastography and turbidimetry for investigating clot formation, scanning electron microscopy (SEM) for clot structure and turbidimetry and lysis assays for clot lysis.

2.4.3.1. Clot formation

Higher levels of triglycerides and total cholesterol may shorten clotting time. In a cross-sectional study by Kim *et al.* (2015) (Table 2.1.), a negative correlation between serum triglycerides and total cholesterol levels and clotting time (based on prothrombin time) was observed. Furthermore, serum triglyceride, total cholesterol and LDL-C levels were significantly correlated with several coagulant factors (Kim *et al.*, 2015), and serum triglyceride levels with factor VII coagulant activity (VIIc) in cross-sectional studies by Marckmann *et al.* (1992) and Puccetti *et al.* (2001) on healthy and dyslipidaemic participants, respectively. In the study by Marckmann *et al.* (1992), serum cholesterol levels were positively correlated with PAI-1 activity but not with any of the other haemostatic factors measured, while HDL-C did not significantly associate with any of the measured factors.

Similarly, in a cross-sectional study by Chan *et al.* (1997) including 3066 middle-aged Taiwanese adults, those with hypertriglyceridemia showed higher factor VIIc levels (and higher total cholesterol levels), as well as shortened clotting times based on lower activated partial thromboplastin times, but not higher fibrinogen levels. In an earlier cross-sectional study by Chan *et al.* (1996) on 2158 participants from the same population, participants with hypercholesterolemia had significantly higher levels of fibrinogen and factor VIIc (but also higher triglyceride levels) and shortened clotting times based on lower activated partial thromboplastin and prothrombin times, compared to the control group. Kunz *et al.* (1992) observed that participants with coronary artery disease (CAD) had lower plasma HDL-C and higher plasma triglyceride levels and showed faster clot formation compared to healthy controls, although the authors concluded that the shorter clotting times were unrelated to participants' higher triglyceride levels as no correlation was found between plasma triglyceride levels and clotting times. HDL-C was found to directly and positively correlate with the time taken for the thrombus to form and inversely correlated with fibrinogen concentration in the control group (Table 2.1.).

Al-Awadhi & Dunn (2000) (Table 2.1.) prepared clots *in vitro* in a purified system to determine the effect on the rate of clot formation of adding LDL-C and HDL-C to the system and found that neither had an obvious effect, while Azizova *et al.* (2000) (Table 2.1.) observed that, compared to non-oxidised LDL, highly oxidised LDL significantly reduced the time taken for a clot to form

(measured as prothrombin time) in plasma and whole blood but increased the time taken for a clot to form (measured as thrombin time) in plasma from healthy participants.

2.4.3.2. Clot structure

Lipids have been shown to incorporate into clots and also alter clot density and firmness. Skrzydlewski (1976) (Table 2.1.) found that more LDL-C were incorporated into the clot (in combination with normal to elevated levels of fibrinogen) compared to VLDL-C or HDL-C. Further investigation suggested that LDL-C and fibrin bind by means of ion and hydrogen bonds. In addition, it was found that in a purified system, more LDL-C was incorporated into stabilised (where bindings connecting the monomers are present) and especially denser, clots compared to non-stabilised clots. In blood plasma, however, considerably less LDL-C would bind, suggesting components may be present in plasma that block these interactions (Skrzydlewski, 1976).

Kunz *et al.* (1992) (Table 2.1.) found significantly higher levels of triglyceride and cholesterol incorporated into clots from CAD participants and that these clots were firmer compared to healthy controls, while more recently it was found that 5% of thrombi from myocardial infarction patients were composed of cholesterol crystals (Silvain *et al.*, 2011) (Table 2.1.). However, as Abela *et al.* (2012) pointed out, it is likely that the thrombi in this study contained a higher percentage of cholesterol crystals as the authors used ethanol dehydration (which can dissolve cholesterol crystals) as opposed to vacuum dehydration during tissue preparation. Furthermore, Talens *et al.* (2012a) formed *in vitro* clots from plasma provided by healthy participants in order to determine the composition of proteins bound to the clots (using 2D gel electrophoresis and mass spectrometry) and found that two-thirds of the novel proteins identified are associated with HDL and that it appeared that HDL was directly bound to fibrinogen. The authors found more apolipoprotein A-I (associated with HDL) bound to the clot than apolipoprotein B (associated with LDL) and speculated that the bound apolipoprotein B is likely from Lipoprotein (a) (Lp (a)) and that the bound HDL may only be a sub fraction of HDL. Similarly, Suski *et al.* (2015), using shotgun proteomics, also found HDL-related proteins, including apolipoprotein A-I, present in *ex vivo* clots formed using plasma from four acute myocardial infarction patients.

Zabczyk *et al.* (2013) (Table 2.1.) found that higher serum HDL-C levels were associated with less dense clots. In the study by Skrzydlewski (1976) mentioned above, it was found that increasing LDL-C concentrations led to firmer clots. Azizova *et al.* (2000) (Table 2.1.) found that highly oxidised LDL significantly decreased clot firmness compared to non-oxidised LDL with the authors suggested that oxidised LDL could directly affect fibrin and thus disrupt the clot structure. Talens *et al.* (2013) (Table 2.1.) found oxidised HDL (but not native HDL or oxidised

LDL) decreased clot firmness of *ex vivo* clots formed from blood provided by healthy participants with the exact mechanism undetermined, although likely involving platelets.

Table 2.1. Summary of *in vitro* and *in vivo* studies investigating the effect of blood lipids on clot properties

Study	Aim	Population	Method	Result
Clot formation and structure				
Skrzydlewski (1976)	To determine whether lipoproteins are incorporated into the stabilised fibrin structure formed in blood plasma. (Lipids isolated from plasma and double immunodiffusion used to further examine LDL-C incorporated into the clot as fibrin appeared to bind only LDL-C.)	Patients (unspecified number) with higher fibrinogen or LDL-C levels and higher or lower levels of both; 20 healthy control participants.	TEG	More LDL-C incorporated into the clot compared to VLDL-C and HDL-C; firmer clots observed in the presence of higher LDL-C levels.
Kunz *et al.* (1992)	To determine clotting times and analyse the lipid content of clots, thrombi, plasma and HDL. (Lipid content in clots determined by extraction.)	26 CAD patients; 39 healthy control participants.	TEG	CAD group had lower plasma HDL-C and higher plasma triglycerides, faster clot formation, firmer clots and higher levels of triglycerides and cholesterol in clots. HDL-C directly correlated with the thrombus forming time in the control group.

Table 2.1. ***(continued)***

Study	Aim	Population	Method	Result
Clot formation and structure				
Al-Awadhi & Dunn (2000)	To determine the effect of LDL-C and HDL-C on the rate of clot formation.	*In vitro* clots formed in a purified system using commercially available clotting factors.	Turbidimetry	Neither LDL-C nor HDL-C affected the rate of clot formation.
Azizova *et al.* (2000)	To study the effects of oxidised LDL on coagulation in blood and plasma. (Clotting time measured as prothrombin time.)	Healthy participants (unspecified number); blood or plasma samples diluted with saline as control.	TEG	Highly oxidised LDL significantly reduced time taken for clot to form in whole blood. The addition of non-oxidised LDL increased clot firmness compared to control, while the addition of oxidised LDL-C decreased clot firmness compared to non-oxidised LDL.
Silvain *et al.* (2011)	To analyse the composition of coronary thrombus *in vivo* in STEMI patients.	288 STEMI patients; no control group.	SEM	5% cholesterol crystals present in thrombi.

Table 2.1. ***(continued)***

Study	Aim	Population	Method	Result
Clot formation and structure				
Talens *et al.* (2013)	To investigate the effect of HDL on clot properties.	*Ex vivo* clots formed from blood provided by healthy participants.	TEG	The addition of oxidised HDL decreased clot firmness, while non-oxidised HDL and oxidised LDL had no effect.
Zabczyk *et al.* (2013)	To investigate whether serum HDL-C affects clot properties.	136 apparently healthy adults; no control group.	Turbidity	Higher serum HDL-C was associated with less dense clots.
Kim *et al.* (2015)	To investigate whether blood lipids influence the results of global coagulation tests.	448 healthy middle-aged participants; no control group.	Coagulation assay; TGA	Higher serum triglycerides and total cholesterol associated with slower initiation of clotting, faster clotting and higher thrombin production; higher serum LDL-C associated with slower initiation of clotting and higher thrombin production; higher HDL-C associated with faster initiation of clotting and lower thrombin production.

Table 2.1. ***(continued)***

Study	Aim	Population	Method	Result
Clot lysis				
Mussoni *et al.* (1992)	To evaluate plasma fibrinolytic capacity in hyperlipidaemic patients.	28 hypertriglyceridaemic patients; 27 healthy control participants.	ELA	Comparable ELA in both groups.
Al-Awadhi & Dunn (2000)	To investigate the effect of LDL-C and HDL-C on fibrinolysis of *in vitro* clots.	*In vitro* clots formed in a purified system using commercially available clotting factors.	Turbidity	When added after clot formation, low levels of LDL-C and HDL-C increased lysis, while high levels of both decreased lysis.
Azizova *et al.* (2000)	To study the effects of oxidised LDL on fibrinolysis in blood and plasma.	Healthy participants (unspecified number); blood or plasma samples diluted with saline as control.	Fibrinolytic activity (%) (Method not clearly described.)	Highly oxidised LDL significantly decreased fibrinolytic activity at a longer incubation period compared to non-oxidised LDL.
Meltzer *et al.* (2009)	To investigate increased CLT as a risk factor for myocardial infarction.	560 male survivors of a first myocardial infarction below the age of 70 at the time of event; 646 control participants consisting of men without a history of myocardial infarction.	Turbidity	CLT lengthened with higher total cholesterol and triglyceride concentration (independently associated); CLT shortened with higher HDL-C.

Table 2.1. ***(continued)***

Study	Aim	Population	Method	Result
Clot lysis				
Zabczyk *et al.* (2013)	To investigate whether serum HDL-C affects clot properties.	136 apparently healthy adults; no control group.	Turbidity	Higher serum HDL-C was associated with shorter CLT.

CAD: coronary artery disease; CLT: clot lysis time; ELA: euglobulin lysis area; HDL: high-density lipoprotein; HDL-C: high-density lipoprotein cholesterol; LDL: low-density lipoprotein; LDL-C: low-density lipoprotein cholesterol; STEMI: ST-elevation myocardial infarction; TEG: thromboelastogram; VLDL-C: very low-density lipoprotein cholesterol; TGA: thrombin generation assay

2.4.3.3. Fibrinolysis

Blood lipids were observed to alter clot lysis, with higher levels of total cholesterol, LDL-C and triglycerides possibly inhibiting lysis. Al-Awadhi & Dunn (2000) (Table 2.1.) formed clots *in vitro* in a purified system to determine if adding LDL-C or HDL-C would alter fibrinolysis. Adding LDL-C before the clot formed showed insignificant effects on lysis, with higher concentrations only moderately delaying lysis. When LDL-C was added after clot formation, lysis was significantly increased at lower concentrations, while inhibited at higher concentrations. Similarly, HDL-C had no effect when added before clot formation, but when added after clot formation lower concentrations increased lysis, while the highest concentration delayed lysis (Al-Awadhi & Dunn, 2000). Azizova *et al.* (2000) (Table 2.1.) found that highly oxidised LDL significantly reduced fibrinolytic activity of a clot when incubated for a longer period of an hour compared to non-oxidised LDL.

In the study by Puccetti *et al.* (2001) on 75 Italian participants with dyslipidaemia (but with normal fibrinogen levels) a significant reduction in fibrinolytic activity was found in the presence of high blood levels of triglyceride compared to hypercholesteraemic participants and healthy controls. This observation could be due to the positive correlation observed between triglyceride levels and PAI-1 concentrations (the main inhibitor of fibrinolysis) (Marckmann *et al.*, 1992) and the reduction in t-PA activity (the main activator of fibrinolysis) in the presence of elevated triglyceride levels (Puccetti *et al.*, 2001). This study additionally found higher LDL-C levels to be associated with reduced lysis related to, among others, higher TAFI activity (an inhibitor of lysis) (Puccetti *et al.*, 2001). Participants with isolated low HDL-C levels also had reduced fibrinolytic activity and, among others, increased PAI-1 activity (Puccetti *et al.*, 2001).

Mussoni *et al.* (1992) (Table 2.1.) found comparable fibrinolytic capability between hyperlipidaemic and control participants, although more recent studies by Meltzer *et al.* (2009) (Table 2.1.) and Zabczyk *et al.* (2013) (Table 2.1.) found that higher total cholesterol and triglyceride levels hindered lysis, while higher HDL-C promoted lysis. While Mussoni *et al.* (1992) used the euglobulin clot lysis assay, Meltzer *et al.* (2009) and Zabczyk *et al.* (2013) used global fibrinolytic assays which could have played a role in the difference in results. Kunz *et al.* (1992) suggested that the presence of lipids in clots may make the fibrin fibres less accessible to degradation enzymes and consequently reduce lysis, although additional mechanisms are likely involved.

Lipoprotein (a) (Lp (a)), a pro-atherogenic lipoprotein subclass synthesised by the liver whose physiological function is currently unknown, may additionally reduce lysis by blocking plasminogen binding sites due to apolipoprotein (a) (a protein that forms part of Lp (a)) having a

similar structure to plasminogen (Undas *et al.*, 2006; Maranhao *et al.*, 2014). The study by Zabczyk *et al.* (2013) supports this theory by finding a positive association between Lp (a) serum levels and clot lysis time. The same study also found that higher HDL-C levels were associated with the faster lysis of clots likely due to clots being less dense (Zabczyk *et al.*, 2013).

In summary, the presented studies show that higher levels of total cholesterol and triglycerides may accelerate clot formation, that lipids can incorporate into clots and that LDL-C may increase clot density and firmness, while HDL-C seems to have the opposite effect. In terms of lysis, higher levels of total cholesterol, LDL-C and triglycerides may inhibit lysis, while the effect of HDL-C is unclear. Lastly, lipid oxidation may possibly alter the effects of lipids, as both oxidised LDL and HDL were found to decrease clot firmness, with highly oxidised LDL significantly inhibiting lysis compared to non-oxidised LDL. However, more studies are needed to elucidate the effects of lipid oxidation on clot properties and lysis.

Plasma fatty acids and their influence on clot properties are discussed in the following section.

2.5. Fatty acids

2.5.1. Function and classification of fatty acids

Fatty acids are organic acids (Hall, 2011) mainly found in the blood as part of lipids transported by lipoproteins, although some free (non-esterified) fatty acids bound to albumin are also present (Hall, 2011; Calder, 2015). Fatty acids compose the main lipid portion of triglycerides and phospholipids and while cholesterol does not contain fatty acids, the sterol core of cholesterol is composed of fatty acid fragments (Hall, 2011).

Several general bodily functions are served by fatty acids, with different fatty acids having unique roles. The oxidation of fatty acids provides energy for the metabolic processes of aerobic tissues, especially when limited glucose is available. In addition, phospholipids (largely composed of fatty acids) are essential to the structure and function of cell membranes as they influence membrane mobility and enable components to move in and out of the cell. Fatty acids are furthermore involved in cell signalling (Calder, 2015).

Each fatty acid is structured as a carbon chain with hydrogen atoms attached, with a methyl group (COOH) at one end and an acid group (CH_3) at the other (Whitney & Rolfes, 2011). Numerous fatty acids exist depending on the length of the hydrocarbon chain (which can be up to 24 carbon atoms in length) and the location of double bonds, if present (Garrett & Grisham, 2002). Fatty acids can be grouped according to the level of saturation or number of carbons in its hydrocarbon chain (Table 2.2.). In terms of saturation, fatty acids can either be saturated (only single bonds present between carbon atoms), monounsaturated (one double bond present), or polyunsaturated (two or more double bonds present) (FAO, 2010). In terms of chain length, saturated fatty acids can be classified into four sub-classes, namely short-chain (three to seven carbon atoms), medium-chain (eight to 13 carbon atoms), long-chain (14 to 20 carbon atoms), and very-long-chain (21 or more carbon atoms). Unsaturated fatty acids can be classified into three sub-classes, namely short-chain (19 or fewer carbon atoms), long-chain (20 to 24 carbon atoms), and very-long-chain (25 or more carbon atoms) (FAO, 2010).

Unsaturated fatty acids largely occur in nature with *cis* double bonds (meaning the hydrogen atoms are on the same side of the double bond), whereas with *trans* double bonds (often formed as a result of partial hydrogenation of oils during food processing to turn them into solid fat) the hydrogen atoms are on opposite sides of the double bond (FAO, 2010; Whitney & Rolfes, 2011).

Table 2.2. Common medium and long chain fatty acids

SFAs	MUFAs	PUFAs
Lauric acid (C12:0)	Palmitoleic acid (C16:1n7)	**n-3 family**
Myristic acid (C14:0)	Oleic acid (C18:1n9)	Alpha-linolenic acid (ALA) (C18:3n3)*
Pentadecanoic (C15:0)		Eicosapentaenoic acid (EPA) (C20:5n3)
Palmitic acid (C16:0)		Docosapentaenoic acid (DPA) (C22:5n3)
Margaric (C17:0)		Docosahexaenoic acid (DHA) (C22:6n3)
Stearic acid (C18:0)		**n-6 family**
Arachidic acid (C20:0)		Linoleic acid (C18:2n6)*
Behenic acid (C22:0)		Gamma-linolenic acid (C18:3n6)
Lignoceric acid (C24:0)		Eicosatrienoic acid (C20:3n6)
		Arachidonic acid (C20:4n6)
		Osbond acid (C22:5n6)

*Essential fatty acids

MUFAS: monounsaturated fatty acids; PUFAs: polyunsaturated fatty acids; SFAs: saturated fatty acids

Polyunsaturated fatty acids (PUFAs) are grouped into 12 families, depending on the location of the first double bond from the methyl end of the hydrocarbon chain, of which two families (n-3 and n-6) are most abundant and important for human health (FAO, 2010; Whitney & Rolfes, 2011). The parent fatty acids of these two families, alpha-linolenic acid (ALA) (n-3) and linoleic acid (n-6) (FAO, 2010), are referred to as essential fatty acids (Table 2.2.) as the body cannot produce these and it is therefore necessary to consume them (Garrett & Grisham, 2002; FAO, 2010; Whitney & Rolfes, 2011).

2.5.2. Fatty acid metabolism

Once ingested, dietary fats are hydrolysed in the stomach and small intestine by the enzyme lipase, to yield free fatty acids, along with other components. Short and medium chain fatty acids are readily absorbed into the bloodstream and travel to the liver where they are oxidised. Long chain fatty acids, on the other hand, are absorbed into the cells of the intestinal lining in the form of micelles, converted to triglycerides and repackaged into chylomicrons before entering the bloodstream. Once in the blood, chylomicrons are gradually broken down to provide free fatty acids and glycerol to cells for energy or to be stored as triglycerides. Fatty acids are oxidised by the mitochondria to provide energy for cells, although fatty acids are a less efficient source of energy than carbohydrate. A small amount of free fatty acids in the

blood binds to albumin and is removed from the circulation by the liver. Chylomicrons transport triglycerides to the liver where the liver packages these into VLDLs, which circulates and is hydrolysed to form IDLs and ultimately LDLs, as discussed in Section 2.4.1. (FAO, 2010).

The body can utilise dietary carbohydrate to synthesise SFAs of varying carbon-chain lengths, with the end product being palmitic acid (C16:0) (with acetyl coenzyme A (acetyl-CoA) linking carbohydrate metabolism and fatty acid synthesis) (Figure 2.5.). Palmitic acid can subsequently be elongated to form stearic acid (C18:0) or other SFAs. These SFAs can, in turn, be de-saturated by the body up until the formation of n-9 MUFAs, with the main end product being oleic acid (C18:1n9).

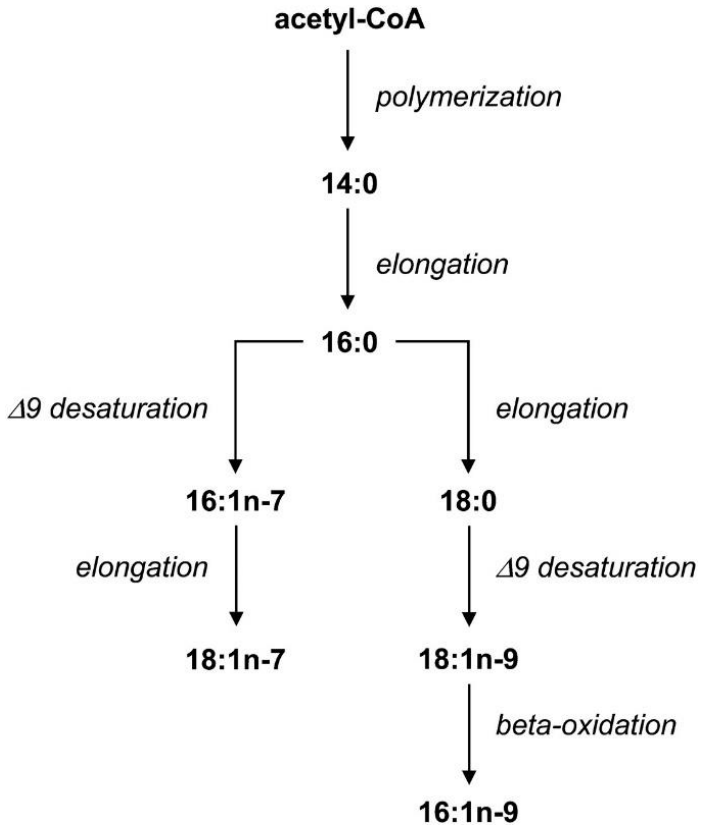


Figure 2.5. Metabolic pathways in the synthesis of SFAs and MUFAs from carbohydrate (Wu *et al.*, 2011)

The body cannot produce the PUFAs ALA (C18:3n3) and linoleic acid (C18:2n6) as it cannot introduce double bonds after the $\Delta 10$ position on the carbon chain and as these fatty acids are required for the production of long chain PUFAs (Figure 2.6.) and pro- and anti-inflammatory lipid mediators (e.g. eicosanoids and docosanoids), it has to be ingested (FAO, 2010).

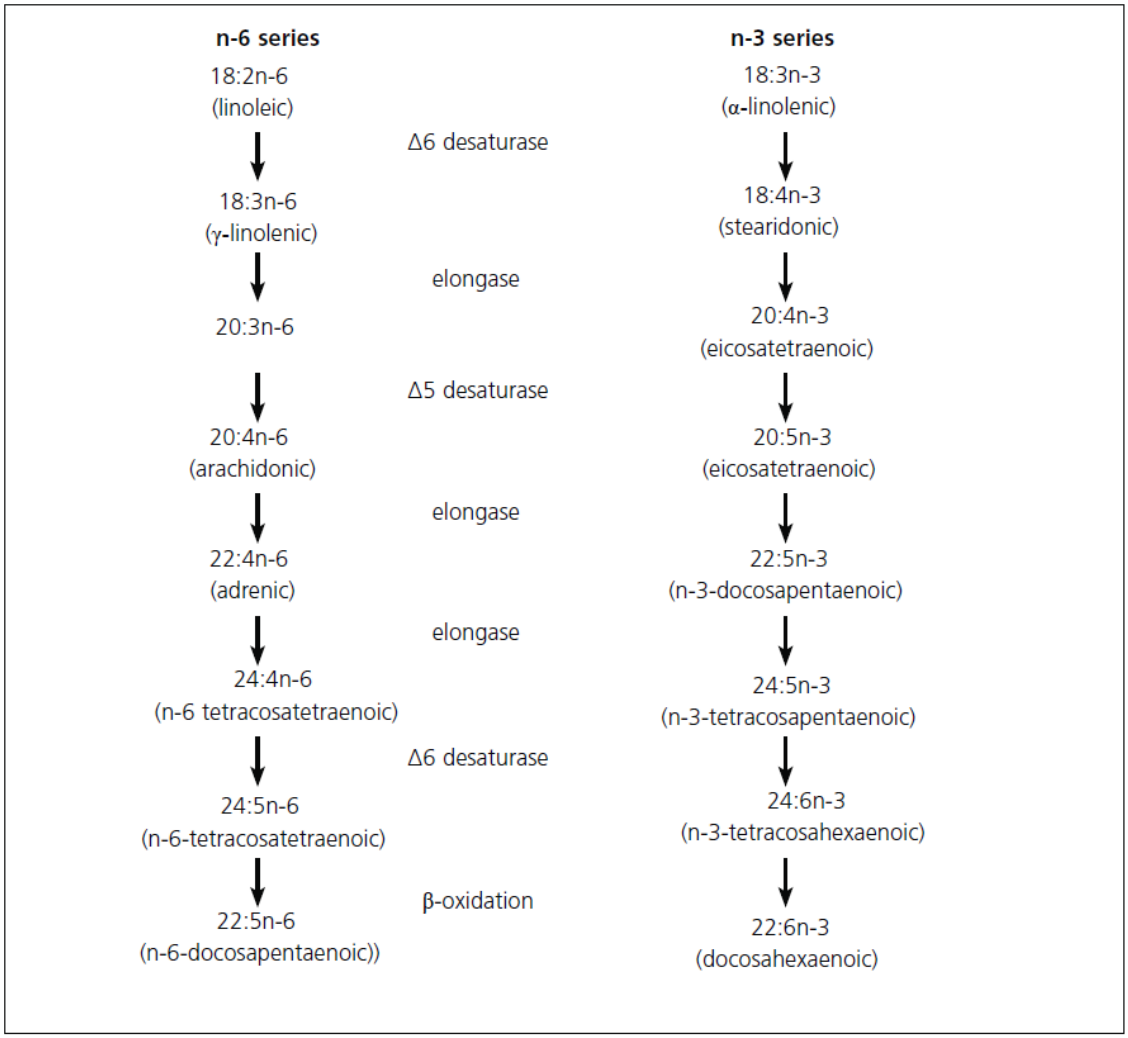


Figure 2.6. Metabolic pathways in the synthesis of long chain PUFAs from ALA and linoleic acid (FAO, 2010)

Research has traditionally focussed on the relationship between dietary fatty acids and CVD risk, whereas research is now shifting to the role of plasma fatty acids. Fatty acid intake cannot be extrapolated to plasma levels as metabolic factors, explained above, along with dietary intake, determine ultimate blood levels. As the current study measured plasma phospholipid fatty acid concentrations, research around these is reviewed.

2.5.3. Plasma phospholipid fatty acids and cardiovascular disease risk

The association of plasma fatty acids with CVD seems complex with several inconsistencies between studies. A significant positive association has been found between plasma concentrations of total even-chain phospholipid SFAs (combined myristic (C14:0), palmitic (C16:0) and stearic (C18:0) acid) and CVD, even after adjusting for cholesterol levels and especially after adjusting for other plasma fatty acids. Individually, stearic acid showed the strongest association in that particular study, followed by palmitic acid (Khaw *et al.*, 2012), while

no association was found for myristic acid in that and other studies (Simon *et al.*, 1995; Wu *et al.*, 2011; Khaw *et al.*, 2012). Additional studies found no association for palmitic acid (Simon *et al.*, 1995; Wu *et al.*, 2011) and a positive to no association for stearic acid (Simon *et al.*, 1995; Wang *et al.*, 2003; Wu *et al.*, 2011). In terms of odd chain phospholipid SFAs, there does not appear to be an association between pentadecanoic acid (C15:0) and CVD risk (Warensjo *et al.*, 2010; Khaw *et al.*, 2012). Both an inverse (Warensjo *et al.*, 2010) and no association (Khaw *et al.*, 2012) have been found for margaric acid (C17:0). Among *trans* fatty acids, phospholipid *trans* 18:2 (particularly *trans/trans* 18:2) has been associated with increased CVD risk (Wang *et al.*, 2014).

Furthermore, there appears to be no association between CVD risk and plasma phospholipid concentrations of the MUFAs palmitoleic (C16:1n7) and oleic (C18:1n9) acid, respectively (Simon *et al.*, 1995; Wang *et al.*, 2003; Wu *et al.*, 2011; Khaw *et al.*, 2012). The PUFA ALA (C18:3n3) has been associated with a decreased CVD risk, although no association was found after adjusting for long chain n-3 fatty acids (Lemaitre *et al.*, 2003), while other studies found no association (Simon *et al.*, 1995; Khaw *et al.*, 2012). However, strong associations between higher levels of total phospholipid long chain n-3 PUFAs (combined eicosapentaenoic (EPA) (C20:5n3), docosapentaenoic (DPA) (C22:5n3) and docosahexaenoic (DHA) (C22:6n3) acid) and lower CVD risk have been found (Hallgren *et al.*, 2001; Mozaffarian *et al.*, 2013). Individually, studies found both inverse (Mozaffarian *et al.*, 2013) to no associations for EPA (Simon *et al.*, 1995; Wang *et al.*, 2003; Khaw *et al.*, 2012), although the lack of associations may be due to limited power in these studies. Mostly inverse associations were found for DPA (Simon *et al.*, 1995; Khaw *et al.*, 2012; Mozaffarian *et al.*, 2013) and strong inverse (Simon *et al.*, 1995; Mozaffarian *et al.*, 2013) to no associations (Khaw *et al.*, 2012) for DHA.

Studies investigating n-6 PUFAs and CVD risk found contradicting results for linoleic acid (C18:2n6) (Simon *et al.*, 1995; Lemaitre *et al.*, 2003; Wang *et al.*, 2003; Khaw *et al.*, 2012), while there does not seem to be an association for gamma-linolenic (C18:3n6) and eicosadienoic (C20:2n6) acid (Simon *et al.*, 1995; Wang *et al.*, 2003; Khaw *et al.*, 2012). Arachidonic acid (C20:4n6) has shown no (Simon *et al.*, 1995) to an inverse association (Wang *et al.*, 2003; Khaw *et al.*, 2012) with CVD risk.

Although much more research is required to reach a conclusion, certain plasma fatty acid ratios may also be important CVD risk factors, such as the palmitic acid/oleic acid ratio – which was found to reliably and independently from blood lipid levels, predict CAD (Sun *et al.*, 2011).

Several mechanisms have been proposed to explain the way fatty acids influence CVD risk, although exact mechanisms have not yet been established. Firstly, as the type and amount of dietary and plasma fatty acids affect specific blood lipid levels, it is proposed that these altered

lipid levels, with fatty acids possibly facilitating the relationship between blood lipids and clot properties, are in part responsible for the associations of specific fatty acids with CVD. Increased SFA intake (particularly myristic (C14:0) and palmitic (C16:0) acid) increases LDL-C levels, whereas replacing dietary SFAs with PUFAs (particularly long chain n-3 PUFAs) or MUFAs decreases LDL-C levels (Chowdhury *et al.*, 2016). Furthermore, in a cross-sectional study by Ebbesson *et al.* (2015) on 758 adult Alaska Natives, positive associations were found between the plasma SFAs myristic, palmitic and stearic (C18:0) acid, as well as the plasma MUFAs palmitoleic (C16:1n7) and oleic (C18:1n9) acid, and triglyceride and LDL-C levels, as well as negative associations between myristic, palmitoleic and oleic acid and HDL-C levels. The plasma PUFAs, ALA (C18:3n3), EPA (C20:5n3), linoleic (C18:2n6) and gamma-linolenic (C18:3n6) acid were positively associated with triglyceride levels, while linoleic and EPA were also positively associated with LDL-C levels, along with arachidonic acid (C20:4n6) and DHA (C22:6n3). ALA was negatively associated with HDL-C levels in this study. Finally, n-3 PUFAs may decrease CVD risk by lowering inflammation, reducing platelet aggregation and by lowering blood pressure (Chowdhury *et al.*, 2016). In the body, EPA is converted to local-acting hormones (eicosanoids) namely the 3-series prostaglandins, thromboxane A3 and leukotriene B5 which lower inflammation and inhibit platelet aggregation (Nagy & Tiuca, 2017). The n-6 fatty acid arachidonic acid, on the other hand, has been shown to be pro-inflammatory and prothrombotic as it is a precursor to three types of pro-inflammatory eicosanoids, namely the prostaglandins 2-series, thromboxane A2 and leukotriene B4 which stimulate platelet adhesion and aggregation as well as clot formation (Bogatcheva et al., 2005; Nagy & Tiuca, 2017).

As it has also been proposed that certain blood fatty acids may alter CVD risk, in part, due to its effect on blood clotting (Simon *et al.*, 1995), the effects of plasma fatty acids on clot properties are now discussed. The limited research on this subject to date highlights the need for the current study.

2.5.4. Plasma fatty acids and clot properties

Table 2.3. provides a summary of studies in which the influence of different plasma fatty acids on clot properties were investigated using thromboelastography and turbidimetry for investigating clot formation, SEM for clot structure and turbidimetry and lysis assays for clot lysis.

2.5.4.1. Clot formation

Researchers have been interested in the effect of fatty acids on clot properties as far back as the fifties, as is seen from a study by Poole (1955) who investigated the effects of fatty acid salts by recalcifying citrate plasma from healthy participants and found that salts from palmitic

(C16:0), stearic (C18:0) and oleic (C18:1n9) acid appeared to shorten the time it took for a clot to fully form.

More recently, Al-Awadhi & Dunn (2000) (Table 2.3.) prepared clots *in vitro* in a purified system to determine what effect adding EPA (C20:5n3) and DHA (C22:6n3) has on clot formation. The authors found that the addition of EPA had no effect on clot formation, while adding DHA inhibited, had no effect, or prevented clot formation depending on the dose. The addition of both EPA and DHA in combination had no effect on the rate of clot formation. Another study by Tanka-Salamon *et al.* (2016) (Table 2.3.) on *in vitro* clots showed that the presence of stearate and oleate salts resulted in slower clot formation.

A randomized, multiple crossover study by Thijssen *et al.* (2005) investigated the effects of diets high in stearic, oleic or linoleic (18:2n6) acid, respectively, on markers of coagulation. That study included 45 healthy participants with slightly elevated cholesterol levels aged between 28 and 66 years who all followed three different diets (namely a diet high in stearic, oleic or linoleic acid) in random order over three five-week periods. Dietary adherence was confirmed by measuring serum phospholipid fatty acid levels. The authors did not find the measured markers of coagulation (factor VII, prothrombin fragments and plasma fibrinogen concentration) or lysis (PAI-1 activity and t-PA/PAI-1 complexes) to differ between the participants following the different diets.

As with blood lipids, fatty acids also seem to incorporate into clots. Rabai *et al.* (2007) analysed surgically removed thrombi from four adults with hyperlipidaemia and atherosclerosis and found that free fatty acids were incorporated into the blood clots in varying amounts, suggesting that fatty acids were incorporated during the formation of these thrombi (Rabai *et al.*, 2007). Kunz *et al.* (1992), however, found no correlation between total plasma free fatty acids and free fatty acids incorporated into *in vitro* clots, and did not find thrombi from CAD patients to contain higher levels of free fatty acids.

2.5.4.2. Clot structure

Gajos *et al.* (2011) conducted a randomised controlled trial on 54 Polish patients aged 30 to 80 years with CAD. Along with standard drug treatment, participants were either assigned a placebo or 1 g n-3 PUFAs (combined EPA and DHA) per day for one month. After the month, supplementation with n-3 PUFAs resulted in larger pores in clots formed *in vitro* from plasma (Gajos *et al.*, 2011).

The more recent study by Tanka-Salamon *et al.* (2016) (Table 2.3.) found that free fatty acids may alter fibre thickness. While the addition of sodium stearate resulted in thicker fibrin fibres,

the opposite was true for sodium oleate which resulted in slightly thinner fibrin fibres. Furthermore, the addition of fatty acids resulted in less stable clots which was measured using rheometry.

2.5.4.3. Fibrinolysis

Individual fatty acids may either promote or hinder lysis. Muraoka & Okuda (1977) found that long chain unsaturated free fatty acids (specifically oleic (C18:1n9), linoleic (C18:2n6) and linolenic (C18:3) acid) had a strong inhibitory effect on plasmin activity (74.5, 60.8, and 70.5 percent, respectively) in a purified system with fibrin as a substrate. That study suggested that these fatty acids may inhibit plasmin activity by binding to the clot thereby distorting its surface and preventing plasmin from acting on the clot. Similarly, Higazi *et al.* (1994) found that free (non-esterified) oleic acid enhanced u-PA activity (which activates plasmin) but inhibited plasmin activity in a dose-dependent manner in a purified system, and it was suggested that oleic acid may stimulate plasmin self-digestion and displace plasmin on the fibrin clot. This study also found that fatty acids of at least 16 carbon chains and one double bond in *cis* configuration inhibited plasmin activity.

In the study by Gajos *et al.* (2011), mentioned in section 2.5.3.2., CAD patients receiving supplementation of combined EPA (C20:5n3) and DHA (C22:6n3), along with standard drug treatment, demonstrated a shorter lysis time for clots formed *in vitro* from plasma. Similarly, Al-Awadhi & Dunn (2000) (Table 2.3.) prepared clots *in vitro* in a purified system to assess the effects of EPA and DHA on fibrinolysis and found that EPA significantly increased lysis when added before clot formation, while the addition of EPA after the clot has formed, delayed lysis. When DHA was added after the clot has formed, the authors found that lysis was statistically inhibited. When a combination of EPA and DHA was added to the system before clot formation, lysis was increased at lower concentrations, but inhibited at higher concentrations.

In a cross-sectional study by Lee *et al.* (2012) on 915 middle-aged Caucasian and Japanese men, it was found that total serum n-6 fatty acids, as well as linoleic (C18:2n6) and arachidonic (C20:4n6) acid individually, were inversely associated with PAI-1. It was further suggested that this inverse association could be due to delayed platelet aggregation. Similarly, in the *in vitro* component of the study by Rabai *et al.* (2007), it was found that, in the presence of oleic acid (C18:1n9), formed fibrin clots were shown to have a higher lysis rate because of the accelerated activation of plasminogen to plasmin and through increased affinity of fibrin for t-PA. However, oleic acid inhibited plasminogen activation in a fluid phase. Most recently, in a cross-sectional study on 716 healthy black African male and female adults, it was found that myristic acid (C14:0) and DHA (C22:6n3) were positively associated with PAI-1 activity, but not with clot lysis time (Richter, 2014).

Table 2.3. Summary of *in vitro* and *in vivo* studies investigating the effect of blood fatty acids on clot properties

Study	Aim	Population	Method	Result
Clot formation and structure				
Al-Awadhi & Dunn (2000)	To investigate the effect of EPA (C20:5n3) and DHA (C22:6n3) on lysis of *in vitro* clots.	*In vitro* clots formed in a purified system using commercially available clotting factors.	Turbidity	EPA had no effect on clot formation, while DHA had a dose-dependent inhibition on clot formation. The addition of combined EPA and DHA had no effect on the rate of clot formation.
Tanka-Salamon *et al.* (2016)	To investigate the effect of free fatty acids (oleic and stearic acid) on clot properties.	*In vitro* clots formed in a purified system using commercially available clotting factors.	Turbidity; SEM	Stearate and oleate increased the clotting time. Fibre thickness increased up to 73% in the presence of sodium stearate and decreased by 20% in the presence of sodium oleate.

Table 2.3. ***(continued)***

Study	Aim	Population	Method	Result
Clot lysis				
Al-Awadhi & Dunn (2000)	To investigate the effect of EPA and DHA on lysis of *in vitro* clots.	*In vitro* clots formed in a purified system using commercially available clotting factors.	Turbidity	EPA increased lysis when added before clot formation, while the addition of EPA after the clot has formed, delayed lysis. DHA inhibited lysis when added after the clot has formed. Combined EPA and DHA added to the system before clot formation, increased lysis at lower concentrations, but inhibited lysis at higher concentrations.
Richter (2014)	To investigate associations of plasma phospholipid fatty acids with PAI-1 and global clot lysis time.	716 healthy black African male and female adults.	CLT	Myristic acid (C14:0) and DHA positively associated with PAI-1 activity, but not with CLT.

CLT: clot lysis time; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; SEM: scanning electron microscopy; PAI-1: plasminogen activator inhibitor-1

In summary, it seems that the effect of plasma fatty acids on fibrin clot formation, structure and lysis is influenced by the study design (studies investigating plasma fatty acid levels *vs*. the *in vitro* addition of fatty acids) and the type and dosage of fatty acids used in the studies and therefore highlights the need for further investigation.

Although the literature is limited, it suggests that certain blood lipids and individual fatty acids alter clot properties, which in turn may increase or decrease CVD risk. CVD is increasing worldwide, with this increase being the result of several factors including the transition to a more Westernised diet experienced by developing countries. CVD is also increasing among black South Africans, but to date, the relationship between total and γ' fibrinogen, plasma clot properties, blood lipids and plasma fatty acids, has not been investigated in detail, although some information is available scattered in amongst papers published on related topics as supporting evidence for the main research focus. The current study is therefore highly relevant to the current situation, both globally and in South Africa.

CHAPTER 3 – METHODS

3.1. Introduction

Currently, available literature suggests that blood lipids and individual fatty acids (which are mainly bound to lipoproteins in the blood as part of blood lipids) alter fibrin clot properties, which in turn increases or decreases cardiovascular disease (CVD) risk. CVD is increasing in black South Africans, but to date the relationship between blood lipids, plasma fatty acids and fibrinogen and plasma clot properties in black South Africans has not been investigated in detail. This study is therefore highly relevant to the current situation in South Africa.

The aim of this study was to determine the relationship of fibrin clot properties with different blood lipid fractions and plasma phospholipid fatty acids (as detailed in the objectives below) in 1281 black South Africans who participated in the Prospective Urban and Rural Epidemiological Study in South Africa (PURE-SA) in 2010. The objectives of the present study were:

1. To determine the relationship of total and γ' fibrinogen (a common splice variant of fibrinogen) concentration with blood lipids namely total cholesterol, LDL-C, HDL-C, non-HDL-C, triglyceride concentration and the lipid ratio LDL-C/HDL-C. Fibrinogen being the structural protein responsible for fibrin clot formation.

2. To determine the relationship of total and γ' fibrinogen concentration with composite (saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids (including n-3 and n-6 polyunsaturated fatty acids)) and individual plasma phospholipid fatty acids.

3. To determine the relationship of plasma clot properties (namely clot formation, clot structure and clot lysis) with above-mentioned blood lipids and plasma phospholipid fatty acids.

4. To determine the interaction of total and γ' fibrinogen with above-mentioned blood lipids and plasma phospholipid fatty acids in determining clot properties.

3.2. Ethical considerations

Ethics approval (NWU-00063-16-A1) was obtained from the Health Research Ethics Committee (HREC) of the North-West University (NWU) Potchefstroom campus (Addendum A). This study is affiliated with the PURE-SA study conducted in North West Province, South Africa.

3.3. Study design

The PURE study tracked lifestyle changes, risk factors and the development of chronic disease over ten years in urban and rural parts of 22 transitional countries, including South Africa, by periodically collecting data in a standardised way (Teo *et al.*, 2009). In South Africa, data was collected for two sites in the North West province in 2005, 2010 and 2015. The present study is cross-sectional in design and used data collected from 1281 participants in 2010.

3.4. Recruitment and study population

Permission to conduct the PURE-SA study was obtained from the North West Province Department of Health. In addition, fully informed goodwill permission was obtained from the Mayors of both Potchefstroom and Ganyesa, as well as from the gatekeepers of each community (these being the tribal chiefs and community leaders). During recruitment, the study was explained to each participant by a trained fieldworker in his or her home language (either Tswana, English, or Afrikaans) to ensure that participants fully understood what would be expected of them if they chose to partake in the study. Participants could ask questions regarding the study at any time before or during the study. Fieldworkers were part of the community but not part of the PURE-SA research team and therefore had no conflict of interest when recruiting participants. Voluntary written informed consent was obtained from each participant. Fieldworkers provided the informed consent forms to potential participants when they visited them at their homes to tell them about the study. The forms were left with the participants who brought the signed informed consent forms with them when they went to the collection sites to participate (Addendum B). If a participant was illiterate, they gave verbal consent and an independent witness signed on their behalf. Participants were given adequate time (one week) to decide if they wanted to participate but were fully informed about the study up to three months prior to the start of the study. Participants were informed that they could withdraw, without consequence, from the study at any stage.

3.5. Inclusion criteria

Participants of both genders were eligible to participate in the study if they were between the ages of 35 and 70 years at baseline measurement in 2005. In addition, participants had to be apparently healthy with no known disease or condition, including lifestyle diseases, tuberculosis (TB), or being human immunodeficiency virus (HIV) positive. Eligible participants had to reside in the approached households. Participants of both urban and rural areas were included in the study. The chosen areas had to have migration stability, meaning that participants were not planning on moving away from the area in the foreseeable future. Urban areas were defined as areas where inhabitants live in established townships and squatter camps and in this case included Ikageng, Zonder water and Extension 11 and 7 in Potchefstroom, North West Province. Rural areas were defined as areas with low development where inhabitants still live under tribal law and included Ganyesa and Tlakgameng, both near Vryburg in North West Province.

3.6. Exclusion criteria

Participants were excluded if they did not meet the inclusion criteria mentioned above or if they used medication for chronic diseases. Participants were also excluded if the head of their household refused permission to partake in the study.

3.7. Quantitative food frequency questionnaire

A quantified food frequency questionnaire (QFFQ) (Addendum C) was conducted by trained fieldworkers to collect data on habitual food intake (foods eaten over the previous 30 days) at individual level. The QFFQ was culturally sensitive, interview-based, standardised and conducted in the participants' language of choice. The QFFQ used has demonstrated good reproducibility (MacIntyre *et al.*, 2001a; Wentzel-Viljoen *et al.*, 2011) and is validated in this population (MacIntyre *et al.*, 2001b). Food portion photographs (Venter *et al.*, 2000), utensils and containers and food examples were used to estimate portion sizes. Standard tables (Langenhoven *et al.*, 1991a) were used to convert portion sizes from household measures to weights. Foods were coded, and the nutrient and food group intake calculated using the South African Food Composition Database System of the South African Medical Research Council (SAMRC) (Langenhoven *et al.*, 1991b; Kruger *et al.*, 1998; Sayed *et al.*, 1999).

3.8. Anthropometric measurements

Anthropometric measurements applicable to the present study were weight and height. Trained fieldworkers (level 1 anthropometrists) performed the weight and height measurements.

3.8.1. Weight

Weight was measured to the nearest 0.1 kilogram on portable electronic scales (Precision Health Scale, A&D Company, Tokyo, Japan). It was ensured that each scale was placed on a stable horizontal floor and that it read "zero". Scales were calibrated daily using standard weights. Weight was measured according to the International Society for the Advancement of Kinanthropometry (ISAK) guidelines. Participants wore minimal clothing, were barefoot and kept their arms hanging freely at their sides for these measurements.

3.8.2. Height

Height was measured according to the ISAK guidelines to the nearest 0.1 centimetre on a calibrated stadiometer (IP 1465, Invicta, London, UK). Participants stood upright with their arms hanging freely at their sides, shoulders relaxed, legs straight and knees together. In addition, participants had to be barefoot and stand with their feet flat and their heels together. Participants' shoulder blades, buttocks and heels had to touch the stadiometer. Each participant's head was held in the Frankfort position, meaning the lowest point of the eye orbit had to be in line with the tragion of the ear. The measurement was taken after the participant had inhaled fully and maintained the erect position without altering the load on the heels or the position of the head.

3.9. Blood sampling

Blood samples were collected by professional registered nurses with experience in collecting blood samples for research purposes and in minimising the risk of harm to participants and themselves. Participants had to be fasting for at least eight hours prior to blood collection. Blood was collected before 11:00 and breakfast was provided after collection. Blood samples were collected from the ante-cubital vein of each participant's right arm using a disposable needle. Each collection tube was filled to its capacity to ensure optimal blood to anti-coagulant ratios. The contents of blood collection tubes were mixed by gently inverting each tube five times. Blood meant for determining lipid profiles was collected in tubes without anti-coagulants and allowed to clot at room temperature. Blood meant for determining total and γ' fibrinogen concentration and clot properties was collected in 3.8% sodium citrate tubes. Blood meant for determining plasma phospholipid fatty acids was collected in ethylenediaminetetraacetic acid (EDTA)-coated, trace-element free evacuated tubes (Becton Dickinson). Within 30 minutes of

collection, blood samples were centrifuged at 2000 × g for 15 minutes at 10°C. In the rural areas, serum and plasma were aliquoted, frozen on dry ice and stored in the field at -18℃. After two to four days, samples were moved to bio-freezers and stored at -82℃ until further analysis. In the urban areas, samples were immediately stored at -82℃ in bio-freezers upon collection and processing.

3.10. Biochemical analysis

Biochemical analyses for the following were performed at the Centre of Excellence for Nutrition (CEN) at NWU:

3.10.1. Lipid profile

3.10.1.1. Total cholesterol, HDL-C and triglyceride concentration

Total cholesterol, HDL-C and triglyceride concentration were measured using the Cobas Integra 400 plus (Roche, Basel, Switzerland). Enzymatic colourimetric methods were used for determining total cholesterol and triglyceride concentrations and a homogeneous enzymatic colourimetric assay used for determining HDL-C concentration.

3.10.1.2. LDL-C (calculated)

LDL-C was calculated with the Friedewald Formula (Friedewald *et al*., 1972), using the measured total cholesterol, HDL-C and triglyceride concentration values:

$$C_{LDL} = \frac{C_{plasma} - C_{HDL} - C_{TG}}{5}$$

3.10.1.3. Non-HDL-C and the ratio LDL-C/HDL-C

Non-HDL-C values were determined for the 2010 PURE-SA participants by subtracting HDL-C from total cholesterol concentrations. Non-HDL-C was included as it is a possibly more biologically relevant marker of CVD risk than LDL-C (Rossouw, 2015). The ratio LDL-C/HDL-C was also determined from the lipid panel results already available as lipid ratios are increasingly being used to investigate CVD risk (Gasevic *et al*., 2014).

3.10.2. Plasma phospholipid fatty acid profile

Plasma fatty acid analysis was conducted as soon as possible after blood collection. Plasma lipids were extracted from plasma with chloroform:methanol (2:1 v/v; containing 0.01% butylated hydroxytoluene) using a modified method of Folch *et al*. (1957). The plasma phospholipid fraction was isolated by thin-layer chromatography (silica gel 60 plates, 10 × 10 cm; Merck) and

transmethylated with methanol:sulphuric acid (95:5 v/v) at 70°C for two hours to yield fatty acid methyl esters (FAMEs). The FAMEs were then analysed using quadruple gas chromatography electron ionisation mass spectrometry (GC-EI-MS) on an Agilent Technologies 7890A GC system equipped with an Agilent Technologies 5975C VL mass selective detector (Agilent Technologies), as described by Baumgartner *et al.* (2012).

FAMEs were quantified using the selected-ion extraction method based on the response of two diagnostic ions. FAME peaks were identified and calibrated against two standard reference mixtures of 26 and eight FAMEs, respectively (Nu-Check-Prep) and three single FAME standards (Larodan Fine Chemicals AB). Data analysis was performed using ChemStation mass spectrometry data software (Agilent G1701EA version E.02.00.493). Relative percentages of fatty acids were calculated taking the concentration of a given fatty acid derivative as a percentage of the total concentration of all fatty acids identified in the sample.

3.10.3. Total fibrinogen concentration

Fibrinogen was measured with a modified Clauss method (Clauss, 1957) using the HemosIL test kit on the ACL-200 coagulation analyser (Instrumentation Laboratories, Milan, Italy). This method is based on prothrombin time with rapid thrombin generation and fibrin formation. Fibrinogen concentration is reported as g/L.

3.10.4. Fibrinogen γ' concentration

Fibrinogen γ' concentration was measured according to the method of Uitte de Willige *et al*. (2005) with an enzyme-linked immunosorbent assay (ELISA). A 2.G2.H9 mouse monoclonal coating antibody (Santa Cruz Biotechnology, Santa Cruz, United States of America) was used against the human γ' sequence for antigen capture and a goat polyclonal horseradish peroxidase–conjugated antibody (Abcam, Cambridge, United States of America) was used against human fibrinogen for development. Fibrinogen γ' is reported as relative concentration (%) of total fibrinogen concentration (reported as γ' ratio).

3.10.5. Clot properties

Clot properties were measured with a turbidity-based assay. The method of Lisman *et al.* (2005) was used and adapted to include both clot formation and lysis. This modified method was validated by Talens *et al.* (2012). Clot formation, structure and lysis were measured by monitoring turbidity changes at A405 nm on a Multiskan Ascent spectrophotometer (Labsystems, Virginia, United States of America) following the addition of the following to the plasma samples: tissue factor (59 pM; Dade Innovin, Siemens Healthcare Diagnostics Inc., Marburg, Germany) to clot the plasma (Duckers *et al*., 2010); $CaCl_2$ (17mM) to counter the

citrate in the collection tubes (as mentioned above plasma was collected in citrate tubes to prevent clotting); tissue plasminogen activator (t-PA) (100 ng/mL; Actilyse, Boehringer Ingelheim, Ingelheim, Germany) to initiate clot lysis; and phospholipids (10µM; Phospholipids-TGT, Rossix, Mölndal, Sweden) to provide a lipid surface for the reactions to take place on. Markers of clot formation include lag time (time for activation of the coagulation cascade and time required for protofibrils to grow sufficiently in length to allow lateral aggregation) and slope (rate of lateral aggregation of protofibrils). A marker of clot structure was also obtained from the turbidity curves, namely maximum absorbance (indication of fibre thickness and clot density). Lastly, clot lysis was determined by calculating clot lysis time (CLT) from these analyses. CLT is defined as the time from clot formation (time from the midpoint of the transition from baseline to maximum turbidity) to clot lysis (to the midpoint in transition from maximum turbidity back to baseline) (Lisman *et al*., 2005).

3.11. Statistical analyses

PURE-SA 2010 data for the present study were analysed using Statistica® software version 13 (Statsoft Inc., Tulsa, United States of America). A p-value of ≤ 0.05 was considered statistically significant. The data set was cleaned by identifying outliers using box plots. Data normality was determined visually using histograms and q-q plots and by using the Shapiro-Wilks test.

Outcome variables following a normal distribution (total and γ' fibrinogen concentration, all clot properties, all blood lipids apart from triglyceride concentration and the LDL-C/HDL-C ratio and all plasma phospholipid fatty acids apart from palmitoleate (C16:1n7), oleic acid (C18:1n9), alpha-linolenic acid (ALA) (C18:3n3) and gamma-linolenic acid (C18:3n6)) are reported as mean ± standard deviation (SD). Outcome variables with a skewed distribution are reported as median (25^{th} percentile; 75^{th} percentile).

The not normally distributed outcome variables were logarithmically transformed to improve normality and Pearson's correlation coefficient (parametric method) performed for all outcome variables to investigate the relationships between total and γ' fibrinogen concentration, plasma clot properties, blood lipids and plasma phospholipid fatty acids. Correlations were also performed for all outcome variables with age, body mass index (BMI) and high-sensitivity C-reactive protein (hsCRP).

Blood lipid and plasma phospholipid fatty acid values were subsequently divided into quartiles and analysis of variance (ANOVA) (parametric method) used to compare quartiles in terms of total and γ' fibrinogen concentration and plasma clot properties. *Post hoc* tests with a multiple comparison correction were performed to determine between which quartiles the statistical

significant differences were (Jellinger *et al*., 2012; Jacobson *et al*., 2014). Analysis of co-variance (ANCOVA) (parametric method) were further performed for clot properties adjusting for potential confounders (namely fibrinogen concentration for all clot properties and plasminogen activator inhibitor-1 (PAI-1) and BMI additionally for CLT). The results are presented in the following chapter.

CHAPTER 4 – RESULTS

4.1. Introduction

This chapter presents the characteristics of the PURE-SA 2010 study population followed by the relationship of blood lipids and plasma phospholipid fatty acids with total and γ′ fibrinogen, as well as with plasma fibrin clot properties which was determined with turbidimetry. Clot properties include lag time (the time for activation of the coagulation cascade and the time required for protofibrils to grow sufficiently in length to allow lateral aggregation), slope (the rate of protofibril lateral aggregation), maximum absorbance (fibrin fibre thickness) and clot lysis time (CLT) (the time taken for the clot to lyse).

4.2. Typical characteristics of study population

Key study participant characteristics are presented in Table 4.1. The PURE-SA 2010 population consisted of 1281 black South African participants. Mean age of participants was 55 years, with the majority (66.1%) being women. Nearly half (45.4%) of participants were classified as being either overweight or obese (body mass index (BMI) ≥25 kg/m^2). Many participants (39.5%) had a hypertensive systolic blood pressure (≥140 mmHg), while 27.3% had a hypertensive diastolic blood pressure (≥90 mmHg). The majority (66.6%) of participants had elevated high-sensitivity C-reactive protein (hsCRP) levels (≥2 mg/L). In terms of blood lipids, 37.4% of participants had elevated total cholesterol levels (≥5.2 mmol/L), 30.0% elevated LDL-C levels (≥3.4 mmol/L), 21.1% elevated triglyceride levels (≥1.7 mmol/L) and 49.1% elevated non-HDL-C levels (≥3.4 mmol/L), while 32.7% of participants had low HDL-C levels (<1 mmol/L for men and <1.2 mmol/L for women). Median fat intakes were 70.6 g/day for total fat, 19.1 g/day for SFAs, 20.6 g/day for MUFAs and 20.9 g/day for PUFAs (24.3%, 6.6%, 7.1%, and 7.2% of median daily energy intake, respectively).

Plasma phospholipid fatty acid measurements were performed for a sub-sample of 899 participants of the PURE-SA 2010 study population. Typical characteristics of the sub-sample did not differ significantly from that of the total study population (Table 4.1). Consequently, the relationship of fibrinogen and clot properties with blood lipids was investigated in the total study population, while the relationship with plasma phospholipid fatty acids was investigated in the sub-sample only.

Table 4.1. Study population general characteristics

	PURE-SA 2010 study population		Plasma phospholipid fatty acid sub-sample	
Sex: women n (%); men n (%)	847 (66.1%); 434 (33.9%)		601 (66.9%); 298 (33.1%)	
		n		*n*
Age, *yrs.*	55.0 (54.4-55.5)	1281	55.5 (54.8-56.2)	899
BMI, *kg/m²**	24.0 (19.8-30.2)	1279	23.9 (19.9-30.3)	896
≥25 - <30 (overweight)	*27.2 (26.1-28.5)*	*260 (20.3%)*	*27.1 (26.0-28.5)*	*177 (19.8%)*
≥30 (obese)	*34.9 (32.1-38.6)*	*321 (25.1%)*	*34.6 (32.0-38.3)*	*229 (25.6%)*
SBP, *mmHg*	136 (135-137)	1199	136 (134-137)	855
≥140 (hypertensive)	*159 (157-160)*	*474 (39.5%)*	*159 (157-161)*	*329 (38.5%)*
DBP, *mmHg*	82.8 (82.1-83.5)	1199	82.1 (81.2-83.0)	855
≥90 (hypertensive)	*99.0 (98.1-99.9)*	*327 (27.3%)*	*99.4 (98.2-101)*	*216 (25.3%)*
Lag time, *min*	4.67 (4.61-4.73)	1245	4.57 (4.52-4.62)	887
Slope, *au/s*	8.65 (8.49-8.81)	1242	8.57 (8.38-8.75)	886
Maximum absorbance, *∆nm*	0.585 (0.575-0.595)	1242	0.585 (0.574-0.597)	886
CLT, *min*	62.3 (61.7-62.8)	1238	62.0 (61.4-62.7)	882
Fibrinogen, *g/L*	3.74 (3.69-3.80)	1252	3.74 (3.68-3.81)	893
Fibrinogen γ', *%*	10.3 (10.1-10.5)	1243	10.3 (10.1-10.5)	887
hsCRP, *mg/L**	3.84 (1.41-8.68)	1253	3.87 (1.39-8.76)	895
≥2.0 (elevated)	*6.41 (3.84-12.4)*	*835 (66.6%)*	*6.39 (3.87-12.2)*	*597 (66.7%)*
Total cholesterol, *mmol/L*	4.91 (4.85-4.98)	1250	4.96 (4.88-5.04)	892
≥5.2 (elevated)	*6.13 (6.06-6.21)*	*467 (37.4%)*	*6.18 (6.09-6.28)*	*343 (38.5%)*
LDL-C, *mmol/L*	2.88 (2.82-2.94)	1247	2.90 (2.82-2.97)	890
≥3.4 (elevated)	*4.20 (4.12-4.27)*	*374 (30.0%)*	*4.23 (4.14-4.32)*	*271 (30.4%)*

Table 4.1. ***(continued)***

	PURE-SA 2010 study population		Plasma phospholipid fatty acid sub-sample	
		n		*n*
Triglyceride, *mmol/L**	1.09 (0.82-1.55)	1251	1.12 (0.82-1.56)	893
≥1.7 (elevated)	*2.17 (1.92-2.88)*	*264 (21.1%)*	*2.23 (1.92-3.04)*	*195 (21.8%)*
HDL-C, *mmol/L*	1.42 (1.39-1.46)	1253	1.43 (1.39-1.47)	895
<1 (men) / <1.2 (women) (low levels)	*0.91 (0.89-0.93)*	*410 (32.7%)*	*0.92 (0.89-0.94)*	*299 (33.4%)*
Non-HDL-C, *mmol/L*	3.49 (3.42-3.56)	1250	3.52 (3.44-3.60)	892
≥3.4 (elevated)	*4.46 (4.39-4.53)*	*614 (49.1%)*	*4.53 (4.44-4.62)*	*437 (49.0%)*
Total fat intake, *g/day** **(% of DEI)**	70.6 (42.9-103) (24.3%)	1278	75.2 (46.0-107) (24.8%)	895
SFA intake, *g/day** **(% of DEI)**	19.1 (11.7-28.6) (6.6%)	1278	20.3 (12.3-29.8) (6.7%)	895
MUFA intake, *g/day** **(% of DEI)**	20.6 (11.8-30.9) (7.1%)	1278	22.0 (12.9-33.6) (7.3%)	895
PUFA intake, *g/day** **(% of DEI)**	20.9 (12.3-30.6) (7.2%)	1278	22.2 (13.2-32.1) (7.3%)	895

BMI: body mass index; CLT: clot lysis time; DBP: diastolic blood pressure; DEI: daily energy intake; hsCRP: high-sensitivity C-reactive protein; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; PURE-SA: Prospective Urban and Rural Epidemiological study; SBP: systolic blood pressure; SFA: saturated fatty acid

Parametric data presented as mean (95% confidence interval); *non-parametric data presented as median (lower quartile-upper quartile)

Cut-off point references: BMI (WHO, 2018); DBP & SBP (AHA, 2018); HDL-C (Catapano *et al.*, 2016); hsCRP (Goff *et al.*, 2013); LDL-C (NIH & NHLBI, 2001); non-HDL-C (Catapano *et al.*, 2016); total cholesterol (NIH & NHLBI, 2001); triglyceride (Catapano *et al.*, 2016)

To determine the relationship of the variables of interest for this study with the general characteristics of the population, we investigated the correlations of total and γ' fibrinogen, clot properties, blood lipids and plasma phospholipid fatty acids, with age, BMI, hsCRP and dietary fat intake. BMI correlated positively with non-HDL-C ($r = 0.35$; $p < 0.001$), the LDL-C/HDL-C ratio ($r = 0.34$; $p < 0.001$) and the fatty acid docosahexaenoic acid (DHA) (C22:6n3) ($r = 0.30$; $p < 0.001$) and inversely with HDL-C ($r = -0.30$; $p < 0.001$), total MUFAs ($r = -0.37$; $p < 0.001$) and oleic acid (C18:1n9) ($r = -0.41$; $p < 0.001$). Nervonic acid (C24:1n9) correlated with hsCRP ($r = 0.30$; $p < 0.001$). As fibrin clot properties are strongly determined by fibrinogen concentration, we additionally investigated the associations between total fibrinogen, fibrinogen γ′ and clot properties. Both slope ($r = 0.64$; $p < 0.001$) and maximum absorbance ($r = 0.81$; $p < 0.001$) correlated with total fibrinogen concentration.

4.3. Plasma phospholipid fatty acid composition of the PURE-SA 2010 participants

The mean plasma phospholipid fatty acid profile of the study population sub-sample (n = 899) is provided in Table 4.2. The table presents all fatty acids analysed in the plasma phospholipid fatty acid profile. The fatty acid profile consisted of 45% SFAs, followed by 42% PUFAs and 13% MUFAs. Palmitic acid (C16:0) contributed the most to the composite SFAs with 27.3%, while oleic acid (C18:1n9) contributed the most to the composite MUFAs (8.65%). In terms of the PUFAs, n-3 fatty acids comprised 5.92% and n-6 fatty acids 35.7%. DHA (C22:6n3) was the principle n-3 fatty acid (3.90%) and linoleic (C18:2n6) and arachidonic (C20:4n6) acid the principle n-6 fatty acids (16.4% and 14.5%, respectively).

Table 4.2. Mean (±SD) plasma phospholipid fatty acid profile of study participants (% of total plasma phospholipid fatty acids)

Phospholipid fatty acid	% of total plasma phospholipid fatty acids
Composite SFAs	**45.0 (±3.83)**
C14:0 Myristic acid	0.28 (±0.09)
C16:0 Palmitic acid	27.3 (±2.85)
C18:0 Stearic acid	14.9 (±2.17)
C20:0 Eicosanoic acid	0.33 (±0.10)
C22:0 Behenic acid	1.13 (±0.39)
C24:0 Lignoceric acid	1.01 (±0.28)
Composite MUFAs	**13.1 (±3.59)**
C16:1n7 Palmitoleate	0.86 (±0.60)

Table 4.2. ***(continued)***

Phospholipid fatty acid	% of total plasma phospholipid fatty acids
C18:1n7 Vaccenic acid	1.47 (±0.42)
C18:1n9 Oleic acid	8.65 (±2.88)
C20:1n9 Eicosenoic acid	0.12 (±0.03)
C22:1n9 Erucic acid	0.04 (±0.02)
C24:1n9 Nervonic acid	1.93 (±0.47)
Composite PUFAs	**41.9 (±4.15)**
Total n-3	5.92 (±1.33)
C18:3n3 Alpha-linolenic acid (ALA)	0.10 (±0.06)
Total long chain n-3	5.82 (±1.33)
C20:5n3 Eicosapentaenoic acid (EPA)	0.52 (±0.35)
C22:5n3 Docosapentaenoic acid (DPA)	1.40 (±0.40)
C22:6n3 Docosahexaenoic acid (DHA)	3.90 (±1.01)
Total n-6	35.7 (±3.81)
C18:2n6 Linoleic acid	16.4 (±3.16)
C18:3n6 Gamma-linolenic acid	0.12 (±0.07)
Total long chain n-6	19.2 (±2.65)
C20:2n6 Eicosadienoic acid	0.38 (±0.08)
C20:3n6 Eicosatrienoic acid	2.89 (±0.67)
C20:4n6 Arachidonic acid	14.5 (±2.35)
C22:2n6 Docosadienoic acid	0.02 (±0.01)
C22:4n6 Adrenic acid	0.70 (±0.18)
C22:5n6 Osbond acid	0.71 (±0.28)
Total n-6/n-3	6.31 (±1.45)
Total long chain n-6/n-3	3.43 (±0.75)
C20:3n9 Mead acid	0.24 (±0.15)

MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; SFA: saturated fatty acid

To investigate the relationship of total and γ' fibrinogen as well as plasma fibrin clot properties with blood lipids (in the total study population) and plasma phospholipid fatty acids (in the sub-sample only), we firstly performed correlations between total and γ' fibrinogen with blood lipids and plasma phospholipid fatty acids, as well as between clot properties with blood lipids and plasma phospholipid fatty acids. As the relationships between these factors were often non-linear, we additionally divided blood lipids and fatty acids into quartiles and performed analysis of variance (ANOVA) to compare quartiles in terms of these outcomes as this approach is more informative in the case of non-linear relationships. We subsequently performed *post hoc* tests with a multiple comparison correction to determine between which quartiles the statistically significant differences were. In the case of clot properties, we further performed analysis of co-variance (ANCOVA) adjusting for potential confounders, namely fibrinogen concentration, PAI-1 activity (PAI-1 activity) and BMI. The results of these investigations are presented in the following paragraphs and tables.

4.4. The relationship of total and γ' fibrinogen with blood lipids

The relationship of total and γ' fibrinogen with blood lipids is presented in Table 4.3. There was no association between any of the blood lipids and γ' fibrinogen. Total fibrinogen, on the other hand, was significantly higher across quartiles of all blood cholesterol fractions, except for HDL-C where significantly lower fibrinogen was observed ($p < 0.0001$). LDL-C particularly had a strong relationship with fibrinogen as fibrinogen was significantly higher across all LDL-C driven fractions (LDL-C, non-HDL-C and LDL-C/HDL-C ratio: $p < 0.0001$ for all). The association of total fibrinogen with total cholesterol was weaker comparatively, with significantly higher total fibrinogen from quartile one to quartile two, after which fibrinogen levels plateaued ($p = 0.002$). This is likely because of the negative association of fibrinogen with HDL-C, a major constituent of total cholesterol. Fibrinogen concentration was significantly lower in the highest HDL-C quartile. There was no association between triglycerides and plasma total fibrinogen concentration.

Table 4.3. The relationship of total and γ′ fibrinogen with blood lipids in total study population

Blood lipid quartiles	Fibrinogen (g/L)	ANOVA p-value (Pearson correlation coefficient; p-value)	Fibrinogen γ′ (%)	ANOVA p-value (Pearson correlation coefficient; p-value)
Total cholesterol (mmol/L)				
Q1 (<4.08)	3.56 (3.46-3.66)$^{*\dagger\bullet}$	0.002 (r = 0.080; p = 0.005)	10.1 (9.76-10.5)	0.563 (r = 0.001; p = 0.963)
Q2 (4.08 < 4.81)	3.79 (3.68-3.91)*		10.2 (9.70-10.7)	
Q3 (4.81 < 5.66)	3.83 (3.71-3.95)†		10.5 (10.1-10.9)	
Q4 (≥5.66)	3.78 (3.68-3.87)$^{\bullet}$		10.2 (9.80-10.6)	
LDL-C (mmol/L)				
Q1 (<2.09)	3.45 (3.35-3.54)*†	<0.0001 (r = 0.191; p < 0.0001)	10.0 (9.65-10.4)	0.206 (r = 0.018; p = 0.533)
Q2 (2.09 < 2.81)	3.68 (3.57-3.80)*†		10.4 (9.89-10.8)	
Q3 (2.81 < 3.62)	3.89 (3.77-4.01)*		10.6 (10.1-11.0)	
Q4 (≥3.62)	3.94 (3.84-4.03)†		10.1 (9.68-10.5)	
Triglyceride (mmol/L)				
Q1 (<0.82)	3.63 (3.53-3.74)	0.076 (r = -0.017; p = 0.565)	10.5 (10.1-10.9)	0.614 (r = -0.024; p = 0.400)
Q2 (0.82 < 1.09)	3.82 (3.70-3.94)		10.2 (9.70-10.7)	
Q3 (1.09 < 1.55)	3.79 (3.68-3.89)		10.1 (9.75-10.5)	
Q4 (≥1.55)	3.71 (3.61-3.82)		10.2 (9.82-10.6)	
HDL-C (mmol/L)				
Q1 (<1.05)	3.78 (3.67-3.89)$^{\bullet}$	<0.0001 (r = -0.161; p < 0.0001)	10.3 (9.83-10.7)	0.915 (r = -0.024; p = 0.408)
Q2 (1.05 < 1.32)	3.89 (3.77-4.00)†		10.2 (9.66-10.7)	
Q3 (1.32 < 1.68)	3.77 (3.66-3.87)*		10.4 (9.98-10.8)	
Q4 (≥1.68)	3.52 (3.41-3.63)$^{*\dagger\bullet}$		10.2 (9.91-10.6)	

Table 4.3. ***(continued)***

Blood lipid quartiles	Fibrinogen (g/L)	ANOVA p-value (Pearson correlation coefficient; p-value)	Fibrinogen γ′ (%)	ANOVA p-value (Pearson correlation coefficient; p-value)
Non-HDL-C (mmol/L)				
Q1 (<2.62)	3.52 (3.41-3.62)*†	<0.0001 (r = 0.156; p < 0.0001)	10.1 (9.71-10.4)	0.412 (r = 0.013; p = 0.662)
Q2 (2.62 < 3.37)	3.67 (3.55-3.78)$^{\bullet\#}$		10.3 (9.82-10.8)	
Q3 (3.37 < 4.26)	3.87 (3.76-3.99)$^{*\bullet}$		10.5 (10.1-11.0)	
Q4 (≥4.26)	3.90 (3.81-3.99)$^{\dagger\#}$		10.2 (9.73-10.6)	
LDL-C/HDL-C				
Q1 (<1.40)	3.44 (3.34-3.55)*†	<0.0001 (r = 0.207; p < 0.0001)	10.1 (9.75-10.4)	0.789 (r = 0.033; p = 0.252)
Q2 (1.40 < 2.17)	3.73 (3.62-3.84)*		10.4 (9.91-10.9)	
Q3 (2.17 < 2.99)	3.80 (3.69-3.90)†		10.3 (9.88-10.7)	
Q4 (≥2.99)	3.99 (3.89-4.09)*		10.3 (9.84-10.7)	

HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol

Data given as mean (95% confidence interval)

$^{*\dagger\bullet\#}$ values with identical symbols differ significantly from each other

4.5. The relationship of total and γ′ fibrinogen with plasma phospholipid fatty acids

The relationship of total and γ' fibrinogen with plasma phospholipid fatty acids is presented in Table 4.4. Individual fatty acids were included in the results either based on the percentage they contributed to the total fatty acids, with the fatty acids contributing the least excluded, or based on their biological importance. There was no relationship between γ' fibrinogen concentration and any of the plasma phospholipid fatty acids. Total fibrinogen concentration demonstrated significant associations with many of the composite and individual fatty acids, although the relationship between the respective composite fatty acids and total fibrinogen concentration did not necessarily reflect the relationship with all its constituent fatty acids. The results are presented according to degree of fatty acid saturation.

4.5.1. Saturated fatty acids

Total fibrinogen concentration tended to be higher across the quartiles of total (composite) SFAs, with the increase reaching significance in quartile 4 ($p = 0.003$). When looking at the relationships of total fibrinogen with the individual SFAs, there seems to be varied relationships. Fibrinogen levels were lower across quartiles of one of the main constituent fatty acids, palmitic acid (C16:0), but were higher across quartiles of stearic (C18:0) and lignoceric (C24:0) acid.

4.5.2. Monounsaturated fatty acids

Total fibrinogen concentration was significantly lower in the 4th quartile of the total MUFAs compared to quartile 1 to 3 ($p < 0.0001$). This relationship is strongly driven by one of the major constituting MUFAs, oleic acid (C18:1n9), which demonstrated an even more pronounced decrease in fibrinogen concentration across the quartiles. A similar pattern was also observed for one of the smaller constituent fatty acids, palmitoleate (C16:1n7), while fibrinogen concentrations were higher across quartiles of nervonic acid (C24:1n9).

4.5.3. Polyunsaturated fatty acids

Total fibrinogen concentration was significantly higher in quartile 2, 3 and 4 compared to quartile 1 of the total PUFAs ($p < 0.0001$). This same pattern was observed for total n-6 PUFAs while for n-3 PUFAs, fibrinogen concentration increased steadily across all 4 quartiles. The total PUFAs comprised of 35.7% n-6 PUFAs and 5.92% n-3 PUFAs, demonstrating the relatively larger contribution of n-6 PUFAs. The steady increase in fibrinogen concentration across the n-3 PUFA quartiles is likely a reflection of the higher fibrinogen seen across quartiles of the long chain DHA (C22:6n3), which is one of the major constituent long chain n-3 PUFAs. There was no association between fibrinogen concentration and docosapentaenoic acid (DPA) (C22:5n3)

and a weak negative association with eicosapentaenoic acid (C20:5n3) with fibrinogen concentrations being lower in the 4th quartile compared to quartiles 1 to 3.

In terms of the n-6 PUFAs, the relationship of fibrinogen with the individual n-6 PUFAs varied with lower fibrinogen concentrations observed across gamma-linolenic acid (C18:3n6) quartiles ($p < 0.0001$), no difference across the linoleic (C18:2n6) or eicosatrienoic (C20:3n6) acid quartiles ($p = 0.058$ and $p = 0.199$, respectively), higher fibrinogen concentrations observed across arachidonic acid (C20:4n6) quartiles ($p < 0.0001$) and a slightly irregular pattern seen across the quartiles of osbond acid (C22:5n6) ($p = 0.006$).

Fibrinogen concentration was lower across the total n-6/n-3 PUFA quartiles ($p = 0.001$), although the underlying fatty acid-fibrinogen associations responsible for this observation would be difficult to identify due to the varied relationship of fibrinogen with the different constituent PUFAs and their relative contribution to total n-6 and n-3 plasma phospholipid PUFAs. There was, however, no association between total fibrinogen and the long chain n-6/n-3 PUFA ratio ($p = 0.134$), suggesting that the association observed for the total n-6/n-3 PUFA, is mediated by medium and/or short chain fatty acids rather than long chain fatty acids.

Table 4.4. The relationship of total and γ′ fibrinogen with plasma phospholipid fatty acids in fatty acid sub-sample

Fatty acid quartiles	Fibrinogen (g/L)	ANOVA p-value (Pearson correlation coefficient; p-value)	Fibrinogen γ′ (%)	ANOVA p-value (Pearson correlation coefficient; p-value)
SFAs (%)				
Q1 (<42.6)	3.61 (3.47-3.74)[*]	0.003 (r = 0.103; p = 0.002)	10.5 (10.1-11.0)	0.416 (r = -0.008; p = 0.823)
Q2 (42.6 < 44.0)	3.72 (3.59-3.85)		10.5 (10.1-10.8)	
Q3 (44.0 < 45.9)	3.70 (3.56-3.83)[†]		10.0 (9.65-10.4)	
Q4 (≥45.9)	3.95 (3.82-4.07)[*†]		10.3 (9.79-10.8)	
C16:0 Palmitic acid (%)				
Q1 (<25.5)	3.88 (3.75-4.01)[*]	0.015 (r = -0.056; p = 0.097)	10.6 (10.1-11.0)	0.399 (r = -0.011; p = 0.736)
Q2 (25.5 < 26.9)	3.82 (3.68-3.96)		10.1 (9.64-10.5)	
Q3 (26.9 < 28.8)	3.64 (3.51-3.77)		10.2 (9.83-10.6)	
Q4 (≥28.8)	3.63 (3.51-3.76)[*]		10.4 (9.96-10.9)	
C18:0 Stearic acid (%)				
Q1 (<13.5)	3.44 (3.31-3.57)[*†]	<0.0001 (r = 0.174; p < 0.0001)	10.5 (10.1-10.9)	0.638 (r = -0.006; p = 0.855)
Q2 (13.5 < 14.7)	3.68 (3.56-3.81)[*]		10.1 (9.76-10.5)	
Q3 (14.7 < 16.2)	3.91 (3.78-4.05)[†]		10.4 (10.0-10.9)	
Q4 (≥16.2)	3.94 (3.81-4.07)[*]		10.2 (9.75-10.7)	
C24:0 Lignoceric acid (%)				
Q1 (<0.82)	3.39 (3.26-3.51)[*†•]	<0.0001 (r = 0.210; p < 0.0001)	10.5 (10.1-10.8)	0.193 (r = 0.034; p = 0.318)
Q2 (0.82 < 1.00)	3.75 (3.62-3.89)[*]		9.97 (9.54-10.4)	
Q3 (1.00 < 1.18)	3.87 (3.74-4.00)[†]		10.6 (10.2-11.0)	
Q4 (≥1.18)	3.96 (3.83-4.08)[•]		10.3 (9.82-10.7)	

Table 4.4 ***(continued)***

Fatty acid quartiles	Fibrinogen (g/L)	ANOVA p-value (Pearson correlation coefficient; p-value)	Fibrinogen γ′ (%)	ANOVA p-value (Pearson correlation coefficient; p-value)
MUFAs (%)				
Q1 (<10.6)	3.86 (3.74-3.98)*	<0.0001 (r = -0.243; p < 0.0001)	10.4 (9.90-10.7)	0.951 (r = 0.014; p = 0.681)
Q2 (10.6 < 12.0)	3.95 (3.82-4.08)†		10.4 (9.96-10.8)	
Q3 (12.0 < 14.7)	3.83 (3.69-3.97)•		10.2 (9.81-10.7)	
Q4 (≥14.7)	3.33 (3.21-3.46)*†•		10.3 (9.91-10.7)	
C16:1n7 Palmitoleate (%)				
Q1 (<0.47)	3.89 (3.77-4.01)*	<0.0001 (r = -0.254; p < 0.0001)	10.8 (10.2-11.3)	0.040 (r = 0.002; p = 0.943)
Q2 (0.47 < 0.65)	3.99 (3.85-4.13)†		10.0 (9.64-10.5)	
Q3 (0.65 < 1.03)	3.81 (3.68-3.94)•		10.0 (9.63-10.4)	
Q4 (≥1.03)	3.29 (3.17-3.41)*†•		10.5 (10.1-10.9)	
C18:1n9 Oleic acid (%)				
Q1 (<6.61)	4.07 (3.94-4.20)*	<0.0001 (r = -0.287; p < 0.001)	10.4 (9.95-10.8)	0.688 (r = 0.008; p = 0.809)
Q2 (6.61 < 7.73)	3.88 (3.75-4.01)†		10.4 (9.96-10.9)	
Q3 (7.73 < 9.99)	3.70 (3.58-3.82)*		10.1 (9.69-10.5)	
Q4 (≥9.99)	3.32 (3.19-3.44)*†		10.4 (9.98-10.8)	
C24:1n9 Nervonic acid (%)				
Q1 (<1.61)	3.42 (3.31-3.53)*	<0.001 (r = 0.309; p < 0.001)	10.4 (9.98-10.9)	0.785 (r = -0.013; p = 0.703)
Q2 (1.61 < 1.87)	3.52 (3.40-3.63)†		10.4 (9.99-10.9)	
Q3 (1.87 < 2.23)	3.84 (3.71-3.96)*†		10.2 (9.82-10.6)	
Q4 (≥2.23)	4.20 (4.04-4.35)*†		10.2 (9.79-10.6)	

Table 4.4. ***(continued)***

Fatty acid quartiles	Fibrinogen (g/L)	ANOVA p-value (Pearson correlation coefficient; p-value)	Fibrinogen γ′ (%)	ANOVA p-value (Pearson correlation coefficient; p-value)
PUFAs (%)				
Q1 (<39.3)	3.45 (3.33-3.57)$^{*\dagger\bullet}$	<0.0001 (r = 0.116; p = 0.001)	10.5 (10.1-11.0)	0.297 (r = -0.005; p = 0.880)
Q2 (39.3 < 42.8)	3.83 (3.69-3.97)*		10.1 (9.64-10.5)	
Q3 (42.8 < 45.2)	3.79 (3.67-3.91)†		10.2 (9.80-10.5)	
Q4 (≥45.2)	3.91 (3.77-4.04)$^{\bullet}$		10.5 (10.1-11.0)	
Total n-3 (%)				
Q1 (<5.03)	3.54 (3.40-3.68)*†	0.0002 (r = 0.131; p < 0.0001)	10.2 (9.85-10.6)	0.636 (r = -0.011; p = 0.755)
Q2 (5.03 < 5.83)	3.67 (3.56-3.78)$^{\bullet}$		10.5 (10.1-11.0)	
Q3 (5.83 < 6.67)	3.83 (3.70-3.96)*		10.3 (9.91-10.8)	
Q4 (≥6.67)	3.93 (3.79-4.07)$^{\dagger\bullet}$		10.2 (9.72-10.6)	
Total long chain n-3 (%)				
Q1 (<4.93)	3.53 (3.39-3.67)*†	0.0002 (r = 0.133; p < 0.0001)	10.3 (9.88-10.6)	0.653 (r = -0.010; p = 0.760)
Q2 (4.93 < 5.73)	3.69 (3.58-3.80)$^{\bullet}$		10.6 (10.1-11.0)	
Q3 (5.73 < 6.57)	3.81 (3.68-3.94)*		10.2 (9.83-10.6)	
Q4 (≥6.57)	3.94 (3.80-4.08)$^{\dagger\bullet}$		10.2 (9.77-10.7)	
C18:3n3 ALA (%)				
Q1 (<0.06)	3.83 (3.68-3.99)	0.127 (r = -0.056; p = 0.097)	10.3 (9.85-10.8)	0.851 (r = -0.017; p = 0.616)
Q2 (0.06 < 0.09)	3.81 (3.69-3.94)		10.4 (9.98-10.7)	
Q3 (0.09 < 0.12)	3.68 (3.55-3.82)		10.4 (10.0-10.9)	
Q4 (≥0.12)	3.65 (3.53-3.77)		10.2 (9.75-10.6)	

Table 4.4. ***(continued)***

Fatty acid quartiles	Fibrinogen (g/L)	ANOVA p-value (Pearson correlation coefficient; p-value)	Fibrinogen γ′ (%)	ANOVA p-value (Pearson correlation coefficient; p-value)
C20:5n3 EPA (%)				
Q1 (<0.32)	3.78 (3.65-3.91)	0.010 (r = -0.087; p = 0.010)	10.3 (9.89-10.7)	0.794 (r = 0.007; p = 0.825)
Q2 (0.32 < 0.44)	3.83 (3.68-3.97)*		10.5 (10.0-10.9)	
Q3 (0.44 < 0.63)	3.82 (3.68-3.96)†		10.4 (9.95-10.8)	
Q4 (≥0.63)	3.55 (3.44-3.66)*†		10.2 (9.70-10.6)	
C22:5n3 DPA (%)				
Q1 (<1.12)	3.74 (3.59-3.88)	0.993 (r = 0.001; p = 0.975)	10.3 (9.85-10.7)	0.809 (r = 0.001; p = 0.977)
Q2 (1.12 < 1.35)	3.76 (3.63-3.88)		10.3 (9.94-10.7)	
Q3 (1.35 < 1.61)	3.73 (3.60-3.86)		10.5 (10.0-10.9)	
Q4 (≥1.61)	3.74 (3.62-3.87)		10.2 (9.75-10.7)	
C22:6n3 DHA (%)				
Q1 (<3.19)	3.49 (3.36-3.61)*	<0.0001 (r = 0.210; p < 0.0001)	10.3 (9.95-10.7)	0.638 (r = -0.012; p = 0.714)
Q2 (3.19 < 3.86)	3.63 (3.51-3.76)†		10.5 (10.1-11.0)	
Q3 (3.86 < 4.54)	3.79 (3.68-3.91)*		10.2 (9.82-10.5)	
Q4 (≥4.54)	4.06 (3.92-4.21)*†		10.2 (9.75-10.7)	
Total n-6 (%)				
Q1 (<33.2)	3.49 (3.36-3.61)*†•	<0.0001 (r = 0.0861; p = 0.010)	10.5 (10.1-11.0)	0.053 (r = -0.0011; p = 0.974)
Q2 (33.2 < 36.4)	3.83 (3.69-3.96)*		9.87 (9.44-10.3)	
Q3 (36.4 < 38.6)	3.90 (3.75-4.05)†		10.2 (9.86-10.6)	
Q4 (≥38.6)	3.76 (3.65-3.87)•		10.6 (10.2-11.1)	

Table 4.4. ***(continued)***

Fatty acid quartiles	Fibrinogen (g/L)	ANOVA p-value (Pearson correlation coefficient; p-value)	Fibrinogen γ′ (%)	ANOVA p-value (Pearson correlation coefficient; p-value)
Total long chain n-6 (%)				
Q1 (<17.5)	3.53 (3.40-3.66)*†	0.0002 (r = 0.152; p < 0.0001)	10.6 (10.1-11.1)	0.266 (r = -0.037; p = 0.272)
Q2 (17.5 < 19.2)	3.70 (3.57-3.82)		10.2 (9.82-10.6)	
Q3 (19.2 < 20.9)	3.83 (3.70-3.96)*		10.1 (9.68-10.4)	
Q4 (≥20.9)	3.92 (3.78-4.05)†		10.4 (9.94-10.8)	
C18:2n6 Linoleic acid (%)				
Q1 (<14.2)	3.68 (3.55-3.81)	0.058 (r = -0.018; p = 0.598)	10.3 (9.85-10.7)	0.344 (r = 0.029; p = 0.394)
Q2 (14.2 < 16.4)	3.90 (3.76-4.04)		10.0 (9.57-10.5)	
Q3 (16.4 < 18.6)	3.71 (3.57-3.85)		10.5 (10.1-10.8)	
Q4 (≥18.6)	3.68 (3.57-3.80)		10.5 (10.1-11.0)	
C18:3n6 Gamma-linolenic acid (%)				
Q1 (<0.07)	4.01 (3.88-4.14)*	<0.0001 (r = -0.221; p < 0.0001)	10.3 (9.80-10.8)	0.670 (r = 0.017; p = 0.607)
Q2 (0.07 < 0.10)	3.81 (3.68-3.93)†		10.4 (9.91-10.8)	
Q3 (0.10 < 0.15)	3.76 (3.62-3.89)*		10.2 (9.81-10.5)	
Q4 (≥0.15)	3.39 (3.27-3.51)*†		10.5 (10.1-11.0)	
C20:3n6 Eicosatrienoic acid (%)				
Q1 (<2.46)	3.68 (3.55-3.82)	0.199 (r = 0.048; p = 0.149)	10.6 (10.2-11.1)	0.409 (r = -0.003; p = 0.937)
Q2 (2.46 < 2.81)	3.72 (3.59-3.86)		10.2 (9.74-10.6)	
Q3 (2.81 < 3.26)	3.70 (3.58-3.82)		10.2 (9.83-10.7)	
Q4 (≥3.26)	3.87 (3.73-4.00)		10.2 (9.85-10.6)	

Table 4.4. ***(continued)***

Fatty acid quartiles	Fibrinogen (g/L)	ANOVA p-value (Pearson correlation coefficient; p-value)	Fibrinogen γ′ (%)	ANOVA p-value (Pearson correlation coefficient; p-value)
C20:4n6 Arachidonic acid (%)				
Q1 (<13.0)	3.50 (3.37-3.63)*†	<0.0001 (r = 0.171; p < 0.0001)	10.6 (10.2-11.1)	0.256 (r = -0.045; p = 0.179)
Q2 (13.0 < 14.5)	3.69 (3.57-3.82)$^{\bullet}$		10.2 (9.81-10.6)	
Q3 (14.5 < 16.1)	3.84 (3.72-3.96)*		10.0 (9.66-10.4)	
Q4 (≥16.1)	3.94 (3.80-4.08)$^{\dagger\bullet}$		10.4 (9.94-10.8)	
C22:5n6 Osbond acid (%)				
Q1 (<0.52)	3.72 (3.59-3.85)	0.006 (r = -0.053; p = 0.116)	10.3 (9.78-10.8)	0.945 (r = 0.011; p = 0.750)
Q2 (0.52 < 0.65)	3.94 (3.80-4.09)*†		10.3 (9.89-10.7)	
Q3 (0.65 < 0.87)	3.63 (3.51-3.75)*		10.4 (10.0-10.8)	
Q4 (≥0.87)	3.68 (3.55-3.81)†		10.3 (9.88-10.6)	
Total n-6/n-3				
Q1 (<5.28)	3.82 (3.69-3.95)*	0.001 (r = -0.111; p = 0.001)	10.2 (9.75-10.7)	0.440 (r = -0.004; p = 0.906)
Q2 (5.28 < 6.18)	3.91 (3.78-4.05)†		10.2 (9.74-10.6)	
Q3 (6.18 < 7.16)	3.69 (3.57-3.81)		10.6 (10.2-11.0)	
Q4 (≥7.16)	3.55 (3.42-3.68)*†		10.3 (9.88-10.7)	

Table 4.4. ***(continued)***

Fatty acid quartiles	Fibrinogen (g/L)	ANOVA p-value (Pearson correlation coefficient; p-value)	Fibrinogen γ′ (%)	ANOVA p-value (Pearson correlation coefficient; p-value)
Total long chain n-6/n-3				
Q1 (<2.30)	3.60 (3.33-3.87)		11.0 (9.66-12.4)	
Q2 (2.30 < 3.36)	3.83 (3.73-3.93)	0.134	10.2 (9.85-10.5)	0.369
Q3 (3.36 < 3.87)	3.67 (3.55-3.79)	(r = -0.065; p = 0.054)	10.4 (10.0-10.8)	(r = -0.023; p = 0.501)
Q4 (≥3.87)	3.69 (3.55-3.83)		10.3 (9.93-10.8)	

ALA: alpha-linolenic acid; DHA: docosahexaenoic acid; DPA: docosapentaenoic acid; EPA: eicosapentaenoic acid; MUFAs: monounsaturated fatty acids; PUFAs: polyunsaturated fatty acids; SFAs: saturated fatty acids

Data given as mean (95% confidence interval)

*†• values with the same symbol differed significantly from each other

4.6. The relationship of plasma fibrin clot properties with blood lipids

The relationship of plasma fibrin clot properties with blood lipids is presented in Table 4.5. Adjustment for total fibrinogen was made for all lipids due to the observed relationship between total fibrinogen and the blood lipids. No association was observed between lag time and any of the blood lipids. Slope and maximum absorbance was significantly higher across quartiles of all lipids except HDL-C for which they were significantly lower ($p < 0.0001$ for both) and triglyceride concentration with which they had no association. After adjustment for fibrinogen, significance remained for LDL-C, the LDL-C/HDL-C ratio ($p < 0.0001$ for both) and HDL-C (slope only), while triglyceride concentration showed a borderline significant association with maximum absorbance. We observed significantly longer CLTs across quartiles for total cholesterol ($p < 0.0001$), LDL-C ($p < 0.001$), non-HDL-C ($p < 0.001$), triglyceride concentration ($p<0.001$) and the LDL-C/HDL-C ratio ($p < 0.001$). CLT was significantly lower across HDL-C quartiles ($p < 0.001$). These significances remained after adjustment for several factors associated with CLT and/or blood lipids, namely fibrinogen concentration, fibrinogen and PAI-1 activity combined and BMI or PAI-1 activity separately (please refer to the footnote of Table 4.5 for clarification on the different co-variables and combinations thereof adjusted for).

Table 4.5. The relationship of plasma fibrin clot properties with blood lipids in total study population

Blood lipid quartiles	Lag time (min)	ANOVA p-value	Slope (au/s)	ANOVA p-value	Maximum absorbance (Δnm)	ANOVA p-value	Clot lysis time (min)	ANOVA p-value
Total cholesterol (mmol/L)								
Q1 (<4.08)	4.74 (4.64-4.85)	0.637	8.26 (7.94-8.58)*	0.013 0.451^{a}	0.550 (0.530-0.570)*†	0.0006 0.243^{a}	59.4 (58.3-60.6)*†	<0.0001 <0.0001^{a} 0.032^{b} <0.0001^{c}
Q2 (4.08 < 4.81)	4.65 (4.55-4.75)		8.59 (8.28-8.91)		0.585 (0.566-0.605)		60.8 (59.8-61.9)†	
Q3 (4.81 < 5.66)	4.67 (4.50-4.84)		9.00 (8.67-9.33)*		0.606 (0.585-0.626)*		63.1 (62.0-64.1)*†	
Q4 (≥5.66)	4.64 (4.55-4.74)		8.67 (8.37-8.97)		0.595 (0.576-0.613)†		65.8 (64.8-66.9)*	
	(r = -0.042; p = 0.142)		(r = 0.068; p = 0.018)		(r = 0.099; p = 0.001)		(r = 0.266; p < 0.001)	
LDL-C (mmol/L)								
Q1 (<2.09)	4.75 (4.65-4.85)	0.611	7.83 (7.53-8.13)*†	<0.0001 0.020^{a}	0.530 (0.510-0.549)*†	<0.0001 0.003^{a}	58.7 (57.4-60.0)*	<0.001 <0.0001^{a} <0.0001^{b} <0.0001^{c}
Q2 (2.09 < 2.81)	4.67 (4.56-4.77)		8.41 (8.10-8.73)*†		0.570 (0.550-0.589)*†		60.5 (59.5-61.5)†	
Q3 (2.81 < 3.62)	4.65 (4.48-4.82)		9.04 (8.72-9.35)*		0.607 (0.587-0.627)*		63.7 (62.7-64.7)*†	
Q4 (≥3.62)	4.65 (4.55-4.74)		9.25 (8.94-9.56)†		0.630 (0.612-0.648)†		66.4 (65.4-67.3)*†	
	(r = -0.053; p = 0.065)		(r = 0.185; p < 0.0001)		(r = 0.214; p < 0.0001)		(r = 0.313; p < 0.001)	
HDL-C (mmol/L)								
Q1 (<1.05)	4.69 (4.60-4.79)	0.976	8.82 (8.49-9.16)*	<0.0001 0.024^{a}	0.584 (0.563-0.604)*	<0.0001 0.239^{a}	64.5 (63.5-65.6)*	<0.001 <0.0001^{a} <0.0001^{b} <0.0001^{c}
Q2 (1.05 < 1.32)	4.66 (4.55-4.76)		8.99 (8.65-9.32)†		0.609 (0.589-0.630)†		64.1 (62.9-65.2)†	
Q3 (1.32 < 1.68)	4.68 (4.51-4.85)		8.85 (8.55-9.15)$^{\bullet}$		0.597 (0.578-0.615)$^{\bullet}$		62.9 (61.9-63.9)$^{\bullet}$	
Q4 (≥1.68)	4.68 (4.58-4.77)		7.90 (7.62-8.18)$^{*\dagger\bullet}$		0.546 (0.528-0.565)$^{*\dagger\bullet}$		57.7 (56.7-58.8)$^{*\dagger\bullet}$	
	(r = 0.025; p = 0.383)		(r = -0.158; p < 0.0001)		(r = -0.136; p < 0.0001)		(r = -0.256; p < 0.001)	

Table 4.5. ***(continued)***

Blood lipid quartiles	Lag time (min)	ANOVA p-value	Slope (au/s)	ANOVA p-value	Maximum absorbance (∆nm)	ANOVA p-value	Clot lysis time (min)	ANOVA p-value
Non-HDL-C (mmol/L)								
Q1 (<2.62)	4.74 (4.64-4.84)	0.684	8.06 (7.75-8.37)*†	<0.0001 0.136[a]	0.545 (0.525-0.564)*†	<0.0001 0.078[a]	57.3 (56.2-58.4)*	<0.001 <0.0001[a] <0.0001[b] <0.0001[c]
Q2 (2.62 < 3.37)	4.68 (4.58-4.78)		8.36 (8.05-8.68)$^{\bullet\#}$		0.568 (0.548-0.589)$^{\bullet}$		60.7 (59.7-61.6)*	
Q3 (3.37 < 4.26)	4.66 (4.48-4.83)		8.98 (8.67-9.29)$^{*\bullet}$		0.604 (0.584-0.623)*		63.9 (62.9-64.9)*	
Q4 (≥4.26)	4.64 (4.55-4.73)		9.13 (8.82-9.45)$^{\dagger\#}$		0.620 (0.601-0.638)$^{\dagger\bullet}$		67.3 (66.3-68.3)*	
	(r = -0.054; p = 0.060)		(r = 0.143; p < 0.0001)		(r = 0.163; p < 0.0001)		(r = 0.386; p < 0.001)	
Triglyceride (mmol/L)								
Q1 (<0.82)	4.71 (4.60-4.81)	0.856	8.48 (8.17-8.79)	0.530 0.952[a]	0.573 (0.554-0.592)	0.064 0.041[a]	57.7 (56.7-58.7)*	<0.001 <0.0001[a] <0.001[b] <0.0001[c] <0.001[d]
Q2 (0.82 < 1.09)	4.67 (4.57-4.77)		8.77 (8.47-9.08)		0.602 (0.582-0.622)		61.0 (60.0-62.0)*	
Q3 (1.09 < 1.55)	4.64 (4.54-4.73)		8.74 (8.43-9.04)		0.592 (0.572-0.611)		63.2 (62.3-64.2)*	
Q4 (≥1.55)	4.69 (4.52-4.87)		8.57 (8.22-8.92)		0.569 (0.549-0.590)		67.2 (66.1-68.4)*	
	(r = -0.001; p = 0.971)		(r = -0.052; p = 0.070)		(r = -0.062; p = 0.032)		(r = 0.303; p < 0.001)	

Table 4.5. ***(continued)***

Blood lipid quartiles	Lag time (min)	ANOVA p-value	Slope (au/s)	ANOVA p-value	Maximum absorbance (∆nm)	ANOVA p-value	Clot lysis time (min)	ANOVA p-value
LDL-C/HDL-C								
Q1 (<1.40)	4.70 (4.60-4.80)	0.518	7.89 (7.58-8.20)*	<0.0001	0.533 (0.513-0.553)*	<0.0001	57.6 (56.4-58.8)*	<0.001
Q2 (1.40 < 2.17)	4.68 (4.59-4.78)		8.43 (8.13-8.73)†	0.001[a]	0.582 (0.563-0.602)*	0.035[a]	61.1 (60.1-62.0)*	<0.0001[a]
Q3 (2.17 < 2.99)	4.72 (4.55-4.90)		8.67 (8.39-8.95)*		0.585 (0.568-0.603)†		63.4 (62.4-64.3)*	<0.0001[b]
Q4 (≥2.99)	4.60 (4.51-4.70)		9.56 (9.22-9.91)*†		0.636 (0.616-0.657)*†		67.2 (66.1-68.2)*	<0.0001[c]
	(r = -0.046; p = 0.108)		(r = 0.193; p < 0.0001)		(r = 0.204; p < 0.0001)		(r = 0.321; p < 0.001)	

HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol

Data given as mean (95% confidence interval)

*†•# values with the same symbol differed significantly from each other

[a] p-values adjusted for fibrinogen; [b] p-values adjusted for fibrinogen and plasminogen activator inhibitor type-1 activity (PAI-1 activity); [c] p-values adjusted for body mass index (BMI); [d] p-values adjusted for PAI-1 activity

(Pearson correlation coefficient; correlation p-value)

4.7. The relationship of plasma fibrin clot properties with plasma phospholipid fatty acids

The relationship of clot properties with plasma phospholipid fatty acids is presented in Table 4.6. Lag time only associated with the n-3 fatty acid ALA (C18:3n3) ($p = 0.0007$) for which a significantly lower lag time was seen in quartile 1 compared to quartiles 2 and 4. Significant associations were found for the remaining clot properties with many of the composite and individual fatty acids, even after adjusting for relevant factors, namely fibrinogen concentration for slope, maximum absorbance, and CLT, as well as additional factors for CLT namely fibrinogen and PAI-1 activity combined and BMI or PAI-1 activity separately (please refer to the footnote of Table 4.6 for clarification on the different co-variables and combinations thereof adjusted for). The individual constituent fatty acids did not all follow the same pattern as the composite fatty acids with regard to the respective clot properties.

4.7.1. Saturated fatty acids

Slope was higher across total SFA quartiles, with this increase likely driven by higher slopes observed across quartiles for stearic (C18:0) and lignoceric (C24:0) acid ($p < 0.0001$ for both). The higher fibrinogen concentration observed across these fatty acid quartiles only partly explained this association as significance remained after adjustment for fibrinogen. Maximum absorbance was significantly higher across the composite SFA quartiles, as well as across quartiles of stearic (C18:0) and lignoceric (C24:0) acid but was unexpectedly lower across quartiles of the most abundant SFA, palmitic acid (C16:0). After adjustment for fibrinogen concentration, the only significant association that remained was with lignoceric acid (C24:0) ($p = 0.001$). CLTs lengthened across quartiles of total SFAs, again likely driven by stearic (C18:0) and lignoceric (C24:0) acid ($p < 0.0001$ for all), with significance remaining after adjusting for fibrinogen concentration, PAI-1 activity and BMI. An opposite relationship was observed with palmitic acid (C16:0) with a trend of shorter CLTs across quartiles, like the patterns observed for slope and maximum absorbance.

4.7.2. Monounsaturated fatty acids

As with fibrinogen concentration, slope and maximum absorbance were significantly lower in the 4th quartile of the total MUFAs compared to quartile 1 to 3 ($p < 0.0001$ for both), while CLTs were significantly shorter in quartile 3 already ($p < 0.001$). The lower slope and maximum absorbance observed for total MUFAs is mirrored in their relationship with the constituent oleic acid (C18:1n9; $p < 0.0001$ and $p < 0.001$, respectively) and palmitoleate (C16:1n7; $p < 0.0001$ and $p < 0.001$, respectively), while an opposite association was observed with nervonic acid (C24:1n9) where both slope and maximum absorbance were higher across quartiles ($p <$

0.0001 and p < 0.001, respectively). These relationships remained after adjusting for fibrinogen concentration. Significantly shorter CLTs were observed for oleic acid (C18:1n9; p < 0.001) and palmitoleate (C16:1n7; p < 0.001) across quartiles before and after adjusting for fibrinogen concentration, PAI-1 activity and BMI. There was no association between CLT and nervonic acid (C24:1n9).

4.7.3. Polyunsaturated fatty acids

There was no association between lag time and the composite or any of the individual PUFAs, except for a longer lag time in quartile 4 of ALA (C18:3n3; p = 0.0007) which remained after adjustment for fibrinogen concentration. Slope and maximum absorbance were significantly higher and CLT significantly longer from quartile 2 across the total PUFA quartiles (p = 0.001, p < 0.0001 and p < 0.0001, respectively). Slope and maximum absorbance was also significantly higher across quartiles of total (p = 0.001 and p < 0.0001, respectively) and long chain (p = 0.0012 and p < 0.0001, respectively) n-3 PUFAs. These associations are largely explained by the association with docosahexaenoic acid (C22:6n3) as this was the only n-3 PUFA across which quartiles slope and maximum absorbance were higher (p < 0.0001 for both). Both slope and maximum absorbance were lower across ALA (C18:3n3) quartiles (p < 0.0001 and p = 0.0009, respectively) and was also significantly lower in quartile 4 of EPA (C20:5n3) (p = 0.009 and p = 0.001, respectively). All the above associations of slope disappeared after adjustment for total fibrinogen, but the associations of maximum absorbance remained. There was no association with DPA (C22:5n3) (p = 0.565 and p = 0.596, respectively). CLT was significantly shorter in quartile 1 of the total and long chain n-3 PUFAs than the other quartiles (p < 0.0001 for both). This was again likely reflecting the association with DHA (C22:6n3; p < 0.0001) as there was a negative association with ALA (C18:3n3; p = 0.032) and no association with EPA (C20:5n3; p = 0.727) or DPA (C22:5n3; p = 0.628). The association with ALA (C18:3n3) disappeared after adjusting for fibrinogen concentration.

In terms of n-6 PUFAs, slope and maximum absorbance were higher and CLT longer from quartile 2 of total n-6 PUFAs (p = 0.0327, p < 0.0001 and p = 0.0004, respectively) and from quartile 3 of the composite long chain n-6 PUFAs (p < 0.0001 for all). These associations are largely explained by the association with arachidonic acid (C20:4n6; p < 0.0001 for all) which followed the same pattern as the composite long chain n-6 PUFAs. The association with the composite long chain n-6 PUFAs and arachidonic acid (C20:4n6) remained after adjustment for total fibrinogen. For CLT the association also remained after additional adjustment for PAI-1 activity but was nullified after adjustment for BMI. None of the other individual fatty acids demonstrated a relationship with either slope or maximum absorbance. CLT was longer in quartile 4 of eicosatrienoic acid (C20:3n6) (p < 0.0001) with significance disappearing after adjustment for BMI. Slope and maximum absorbance were significantly lower and CLT

significantly shorter in quartile 4 of the total n-6/n-3 ratio ($p = 0.0003$, $p = 0.0001$ and $p = 0.0001$, respectively), with CLT also found to be shorter in quartile 4 of the long chain n-6/n-3 ratio ($p = 0.017$), although the underlying associations responsible for these observations would be difficult to identify due to the varied relationship of the fibrin clot properties with the different constituent PUFAs and their relative contribution to total n-6 and n-3 plasma phospholipid PUFAs. The associations with maximum absorbance and CLT remained after adjustment.

Table 4.6. The relationship of plasma fibrin clot properties with plasma phospholipid fatty acids in fatty acid sub-sample

Fatty acid quartiles	Lag time (min)	ANOVA p-value	Slope (au/s)	ANOVA p-value	Maximum absorbance (Δnm)	ANOVA p-value	Clot lysis time (min)	ANOVA p-value
SFAs (%)								
Q1 (<42.6)	4.57 (4.47-4.67)	0.909	7.97 (7.62-8.32)*	<0.0001 0.002^{a}	0.563 (0.539-0.586)*	0.0157 0.558^{a}	59.6 (58.4-60.9)*†	<0.0001 <0.0001^{a} <0.0001^{b} 0.0001^{c}
Q2 (42.6 < 44.0)	4.57 (4.46-4.69)		8.38 (8.02-8.75)†		0.576 (0.553-0.599)		60.4 (59.2-61.6)$^{\bullet\#}$	
Q3 (44.0 < 45.9)	4.54 (4.43-4.65)		8.62 (8.24-9.01)		0.588 (0.564-0.611)		64.0 (62.8-65.3)$^{*\bullet}$	
Q4 (≥45.9)	4.60 (4.49-4.71)		9.30 (8.92-9.68)*†		0.615 (0.592-0.637)*		64.1 (62.9-65.3)$^{\dagger\#}$	
	(r = -0.006; p = 0.854)		(r = 0.158; p < 0.0001)		(r = 0.086; p = 0.009)		(r = 0.160; p < 0.0001)	
C16:0 Palmitic acid (%)								
Q1 (<25.5)	4.55 (4.44-4.65)	0.743	8.69 (8.36-9.02)	0.334 0.801^{a}	0.614 (0.591-0.637)*†	0.0003 0.061^{a}	63.4 (62.3-64.5)	0.051 0.150^{a} 0.371^{b} 0.453^{c}
Q2 (25.5 < 26.9)	4.54 (4.43-4.65)		8.79 (8.36-9.22)		0.607 (0.583-0.632)$^{\bullet}$		62.2 (61.2-63.2)	
Q3 (26.9 < 28.8)	4.58 (4.46-4.70)		8.42 (8.04-8.80)		0.566 (0.544-0.588)*		61.2 (59.7-62.7)	
Q4 (≥28.8)	4.61 (4.51-4.72)		8.37 (8.02-8.72)		0.555 (0.532-0.577)$^{\dagger\bullet}$		61.3 (59.9-62.7)	
	(r = 0.028; p = 0.415)		(r = -0.007; p = 0.846)		(r = -0.087; p = 0.010)		(r = -0.070; p = 0.040)	
C18:0 Stearic acid (%)								
Q1 (<13.5)	4.63 (4.52-4.75)	0.474	7.59 (7.26-7.93)$^{*\dagger\bullet}$	<0.0001 0.006^{a}	0.529 (0.507-0.551)*†	<0.0001 0.078^{a}	57.8 (56.4-59.1)*†	<0.0001 <0.0001^{a} <0.0001^{b} <0.0001^{c}
Q2 (13.5 < 14.7)	4.51 (4.41-4.62)		8.59 (8.20-8.97)*		0.574 (0.553-0.595)*		62.0 (60.7-63.2)*	
Q3 (14.7 < 16.2)	4.58 (4.47-4.68)		8.93 (8.55-9.30)†		0.613 (0.589-0.637)†		63.3 (62.3-64.4)†	
Q4 (≥16.2)	4.56 (4.44-4.67)		9.18 (8.80-9.55)$^{\bullet}$		0.626 (0.602-0.649)*		65.1 (63.9-66.2)*	
	(r = -0.033; p = 0.323)		(r = 0.196; p < 0.0001)		(r = 0.183; p < 0.0001)		(r = 0.272; p < 0.0001)	

Table 4.6. ***(continued)***

Fatty acid quartiles	Lag time (min)	ANOVA p-value	Slope (au/s)	ANOVA p-value	Maximum absorbance (∆nm)	ANOVA p-value	Clot lysis time (min)	ANOVA p-value
C24:0 Lignoceric acid (%)								
Q1 (<0.82)	4.58 (4.47-4.69)	0.989	7.53 (7.19-7.86)*†	<0.0001 0.002[a]	0.513 (0.491-0.535)*†•	<0.0001 0.001[a]	59.2 (57.8-60.7)*†	<0.0001 <0.0001[a] <0.0001[b] 0.027[c]
Q2 (0.82 < 1.00)	4.55 (4.44-4.67)		8.50 (8.15-8.85)*		0.592 (0.569-0.614)*		61.6 (60.3-62.8)*	
Q3 (1.00 < 1.18)	4.57 (4.46-4.68)		9.05 (8.64-9.47)†		0.609 (0.586-0.633)†		63.2 (62.0-64.4)†	
Q4 (≥1.18)	4.58 (4.48-4.68)		9.18 (8.82-9.54)*		0.626 (0.604-0.648)•		64.0 (63.0-65.1)*	
	(r = -0.018; p = 0.605)		(r = 0.240; p < 0.0001)		(r = 0.224; p < 0.0001)		(r = 0.199; p < 0.0001)	
MUFAs (%)								
Q1 (<10.6)	4.56 (4.45-4.67)	0.709	8.96 (8.60-9.32)*	<0.0001 <0.0001[a]	0.601 (0.580-0.623)*	<0.0001 <0.001[a]	65.2 (64.1-66.4)*	<0.001 <0.0001[a] <0.0001[b] <0.0001[c]
Q2 (10.6 < 12.0)	4.54 (4.44-4.65)		9.30 (8.90-9.70)†		0.632 (0.610-0.655)†		64.6 (63.4-65.8)†	
Q3 (12.0 < 14.7)	4.56 (4.45-4.66)		8.71 (8.35-9.07)•		0.604 (0.580-0.629)•		61.8 (60.7-63.00)*†	
Q4 (≥14.7)	4.62 (4.51-4.74)		7.32 (6.99-7.66)*†•		0.505 (0.483-0.526)*†•		56.5 (55.3-57.7)*†	
	(r = 0.050; p = 0.139)		(r = -0.263; p < 0.0001)		(r = -0.268; p < 0.0001)		(r = -0.375; p < 0.001)	
C16:1n7 Palmitoleate (%)								
Q1 (<0.47)	4.55 (4.44-4.65)	0.200	8.97 (8.62-9.32)*	<0.0001 0.0007[a]	0.611 (0.589-0.632)*	<0.001 <0.0001[a]	63.7 (62.6-64.9)*	<0.001 <0.0001[a] <0.0001[b] <0.0001[c]
Q2 (0.47 < 0.65)	4.64 (4.53-4.75)		9.22 (8.83-9.62)†		0.640 (0.616-0.664)†		64.4 (63.3-65.5)†	
Q3 (0.65 < 1.03)	4.48 (4.37-4.59)		8.84 (8.45-9.23)•		0.594 (0.571-0.616)†		63.1 (61.7-64.4)•	
Q4 (≥1.03)	4.61 (4.50-4.72)		7.25 (6.94-7.57)*†•		0.498 (0.478-0.518)*†		57.0 (55.7-58.2)*†•	
	(r = 0.028; p = 0.407)		(r = -0.262; p < 0.0001)		(r = -0.283; p < 0.0001)		(r = -0.281; p < 0.0001)	

Table 4.6. ***(continued)***

Fatty acid quartiles	Lag time (min)	ANOVA p-value	Slope (au/s)	ANOVA p-value	Maximum absorbance (Δnm)	ANOVA p-value	Clot lysis time (min)	ANOVA p-value
C18:1n9 Oleic acid (%)								
Q1 (<6.61)	4.51 (4.40-4.61)	0.362	9.43 (9.07-9.79)*	<0.0001 <0.0001[a]	0.642 (0.620-0.664)*	<0.001 0.0001[a]	66.0 (64.8-67.1)*†	<0.001 <0.0001[a] <0.0001[b] <0.0001[c]
Q2 (6.61 < 7.73)	4.62 (4.51-4.72)		9.17 (8.76-9.58)†		0.620 (0.595-0.644)†		63.5 (62.5-64.6)*	
Q3 (7.73 < 9.99)	4.54 (4.43-4.65)		8.36 (8.03-8.69)*†		0.580 (0.559-0.601)*		61.6 (60.3-62.8)†	
Q4 (≥9.99)	4.62 (4.51-4.73)		7.32 (6.98-7.65)*†		0.500 (0.479-0.521)*†		57.1 (55.8-58.3)*†	
	(r = 0.053; p = 0.118)		(r = -0.305; p < 0.001)		(r = -0.318; p < 0.001)		(r = -0.363; p < 0.001)	
C24:1n9 Nervonic acid (%)								
Q1 (<1.61)	4.60 (4.49-4.70)	0.668	7.53 (7.21-7.84)*	<0.0001 0.0006[a]	0.514 (0.494-0.534)*	<0.001 <0.0001[a]	62.0 (60.7-63.3)	0.690 0.754[a] 0.804[b] 0.430[c]
Q2 (1.61 < 1.87)	4.61 (4.51-4.72)		8.17 (7.82-8.52)†		0.538 (0.518-0.558)†		61.4 (60.1-62.7)	
Q3 (1.87 < 2.23)	4.54 (4.43-4.65)		8.82 (8.47-9.16)*		0.608 (0.587-0.629)*†		62.2 (61.0-63.4)	
Q4 (≥2.23)	4.53 (4.42-4.64)		9.75 (9.33-10.2)*†		0.681 (0.656-0.706)*†		62.4 (61.2-63.7)	
	(r = -0.057; p = 0.094)		(r = 0.288; p < 0.0001)		(r = 0.373; p < 0.001)		(r = 0.010; p = 0.776)	
PUFAs (%)								
Q1 (<39.3)	4.62 (4.51-4.74)	0.382	7.92 (7.58-8.27)$^{*\dagger\bullet}$	0.001 0.781[a]	0.523 (0.501-0.545)$^{*\dagger\bullet}$	<0.0001 0.011[a]	58.9 (57.7-60.2)$^{*\dagger\bullet}$	<0.0001 <0.0001[a] <0.0001[b] 0.030[c]
Q2 (39.3 < 42.8)	4.54 (4.43-4.65)		8.73 (8.32-9.14)*		0.602 (0.577-0.626)*		62.2 (60.8-63.6)*	
Q3 (42.8 < 45.2)	4.61 (4.50-4.72)		8.75 (8.36-9.14)†		0.601 (0.579-0.622)†		62.9 (61.8-64.0)†	
Q4 (≥45.2)	4.51 (4.41-4.60)		8.87 (8.53-9.22)$^{\bullet}$		0.616 (0.593-0.639)$^{\bullet}$		64.1 (62.9-65.2)$^{\bullet}$	
	(r = -0.038; p = 0.264)		(r = 0.083; p = 0.013)		(r = 0.153; p < 0.0001)		(r = 0.179; p < 0.0001)	

Table 4.6. ***(continued)***

Fatty acid quartiles	Lag time (min)	ANOVA p-value	Slope (au/s)	ANOVA p-value	Maximum absorbance (∆nm)	ANOVA p-value	Clot lysis time (min)	ANOVA p-value
Total n-3 (%)								
Q1 (<5.03)	4.65 (4.54-4.77)	0.374	8.08 (7.70-8.46)*†	0.002 0.452[a]	0.543 (0.520-0.566)*†	<0.0001 0.020[a]	58.6 (57.2-59.9)*†•	<0.0001 <0.0001[a] <0.0001[b] 0.0006[c]
Q2 (5.03 < 5.83)	4.55 (4.45-4.65)		8.34 (8.00-8.68)		0.576 (0.555-0.597)		62.5 (61.2-63.8)*	
Q3 (5.83 < 6.67)	4.52 (4.42-4.63)		8.89 (8.51-9.26)*		0.612 (0.588-0.636)*		62.8 (61.7-64.0)†	
Q4 (≥6.67)	4.55 (4.45-4.66)		8.97 (8.57-9.37)†		0.611 (0.587-0.635)†		64.2 (63.0-65.3)•	
	(r = -0.033; p = 0.328)		(r = 0.130; p < 0.0001)		(r = 0.132; p < 0.0001)		(r = 0.222; p < 0.0001)	
Total long chain n-3 (%)								
Q1 (<4.93)	4.66 (4.54-4.77)	0.321	8.04 (7.66-8.42)*†	0.0012 0.534[a]	0.541 (0.518-0.563)*†	<0.0001 0.018[a]	58.5 (57.1-59.8)*†•	<0.0001 <0.0001[a] <0.0001[b] 0.0004[c]
Q2 (4.93 < 5.73)	4.54 (4.44-4.64)		8.41 (8.07-8.75)		0.579 (0.558-0.601)		62.4 (61.1-63.7)*	
Q3 (5.73 < 6.57)	4.53 (4.42-4.63)		8.79 (8.42-9.16)*		0.607 (0.583-0.632)*		62.9 (61.8-64.1)†	
Q4 (≥6.57)	4.55 (4.44-4.67)		9.04 (8.63-9.44)†		0.614 (0.590-0.638)†		64.2 (63.1-65.4)•	
	(r = -0.037; p = 0.268)		(r = 0.134; p < 0.0001)		(r = 0.136; p < 0.0001)		(r = 0.226; p < 0.0001)	
C18:3n3 ALA (%)								
Q1 (<0.06)	4.39 (4.28-4.51)*†	0.0007 0.0008[a]	9.43 (8.99-9.87)*†•	<0.0001 <0.0001[a]	0.626 (0.597-0.655)*†	0.0009 0.0009[a]	63.3 (61.9-64.7)*	0.032 0.069[a] 0.0004[b] 0.713[c] 0.0001[d]
Q2 (0.06 < 0.09)	4.61 (4.51-4.71)*		8.64 (8.31-8.98)*		0.588 (0.568-0.608)		62.1 (60.9-63.2)	
Q3 (0.09 < 0.12)	4.52 (4.39-4.64)		8.29 (7.92-8.66)†		0.576 (0.553-0.600)*		62.4 (61.1-63.7)	
Q4 (≥0.12)	4.71 (4.61-4.80)†		8.04 (7.69-8.38)•		0.558 (0.537-0.579)†		60.6 (59.5-61.8)*	
	(r = 0.111; p = 0.001)		(r = -0.149; p < 0.0001)		(r = -0.117; p = 0.001)		(r = -0.089; p = 0.009)	

Table 4.6. ***(continued)***

Fatty acid quartiles	Lag time (min)	ANOVA p-value	Slope (au/s)	ANOVA p-value	Maximum absorbance (∆nm)	ANOVA p-value	Clot lysis time (min)	ANOVA p-value
C20:5n3 EPA (%)								
Q1 (<0.32)	4.60 (4.49-4.71)	0.217	8.82 (8.44-9.21)*	0.009 0.047^{a}	0.604 (0.582-0.627)*	0.001 0.032^{a}	62.1 (60.8-63.5)	0.727 0.962^{a} 0.426^{b} 0.938^{c} 0.142^{d}
Q2 (0.32 < 0.44)	4.64 (4.52-4.75)		8.46 (8.10-8.82)		0.590 (0.565-0.614)†		62.4 (61.2-63.6)	
Q3 (0.44 < 0.63)	4.48 (4.38-4.58)		8.89 (8.49-9.30)†		0.601 (0.576-0.625)$^{\bullet}$		62.2 (60.9-63.4)	
Q4 (≥0.63)	4.56 (4.46-4.67)		8.08 (7.74-8.42)*†		0.546 (0.526-0.566)$^{*\dagger\bullet}$		61.4 (60.1-62.7)	
	(r = 0.016; p = 0.634)		(r = -0.057; p = 0.090)		(r = -0.102; p = 0.003)		(r = -0.004; p = 0.904)	
C22:5n3 DPA (%)								
Q1 (<1.12)	4.64 (4.53-4.75)	0.481	8.37 (7.99-8.76)	0.565 0.468^{a}	0.586 (0.561-0.611)	0.596 0.109^{a}	61.4 (60.0-62.8)	0.628 0.645^{a} 0.637^{b} 0.086^{c} 0.657^{d}
Q2 (1.12 < 1.35)	4.53 (4.42-4.64)		8.66 (8.30-9.03)		0.598 (0.575-0.620)		62.0 (60.6-63.3)	
Q3 (1.35 < 1.61)	4.57 (4.46-4.67)		8.51 (8.14-8.88)		0.576 (0.554-0.598)		62.2 (61.0-63.4)	
Q4 (≥1.61)	4.54 (4.44-4.65)		8.72 (8.34-9.10)		0.581 (0.557-0.605)		62.6 (61.5-63.6)	
	(r = -0.029; p = 0.396)		(r = 0.033; p = 0.328)		(r = -0.031; p = 0.353)		(r = 0.055; p = 0.104)	
C22:6n3 DHA (%)								
Q1 (<3.19)	4.65 (4.54-4.77)	0.322	7.80 (7.46-8.14)*†	<0.0001 0.076^{a}	0.533 (0.512-0.554)*†	<0.0001 <0.0001^{a}	58.1 (56.9-59.4)*†	<0.0001 <0.0001^{a} <0.0001^{b} 0.001^{c}
Q2 (3.19 < 3.86)	4.57 (4.46-4.68)		8.33 (7.98-8.69)$^{\bullet}$		0.554 (0.533-0.575)$^{\bullet\#}$		62.3 (61.0-63.7)*	
Q3 (3.86 < 4.54)	4.53 (4.42-4.63)		8.82 (8.45-9.19)*		0.617 (0.594-0.640)$^{*\bullet}$		62.9 (61.8-64.0)†	
Q4 (≥4.54)	4.53 (4.42-4.64)		9.32 (8.92-9.72)$^{\dagger\bullet}$		0.637 (0.613-0.662)$^{\dagger\#}$		64.7 (63.5-65.8)*	
	(r = -0.047; p = 0.169)		(r = 0.184; p < 0.0001)		(r = 0.228; p < 0.0001)		(r = 0.277; p < 0.0001)	

Table 4.6. ***(continued)***

Fatty acid quartiles	Lag time (min)	ANOVA p-value	Slope (au/s)	ANOVA p-value	Maximum absorbance (∆nm)	ANOVA p-value	Clot lysis time (min)	ANOVA p-value
Total n-6 (%)								
Q1 (<33.2)	4.58 (4.47-4.68)	0.250	8.09 (7.73-8.45)*	0.0327 0.301^{a}	0.531 (0.509-0.553)$^{*\dagger\bullet}$	<0.0001 0.014^{a}	59.8 (58.4-61.1)$^{*\dagger\bullet}$	0.0004 0.003^{a} <0.001^{b} 0.267^{c}
Q2 (33.2 < 36.4)	4.56 (4.45-4.68)		8.82 (8.41-9.22)*		0.602 (0.579-0.626)*		62.3 (61.1-63.6)*	
Q3 (36.4 < 38.6)	4.65 (4.54-4.76)		8.64 (8.26-9.03)		0.609 (0.584-0.633)†		62.6 (61.3-63.8)†	
Q4 (≥38.6)	4.49 (4.39-4.59)		8.72 (8.37-9.06)		0.600 (0.578-0.621)$^{\bullet}$		63.4 (62.2-64.6)$^{\bullet}$	
	(r = -0.028; p = 0.404)		(r = 0.051; p = 0.132)		(r = 0.127; p < 0.0001)		(r = 0.127; p < 0.0001)	
Total long chain n-6 (%)								
Q1 (<17.5)	4.63 (4.52-4.73)	0.112	8.14 (7.75-8.52)*†	<0.0001 0.003^{a}	0.540 (0.517-0.562)*†	<0.0001 0.023^{a}	59.6 (58.3-60.9)*†	<0.0001 <0.0001^{a} <0.0001^{b} 0.003^{c}
Q2 (17.5 < 19.2)	4.65 (4.54-4.76)		8.04 (7.71-8.37)$^{\bullet\#}$		0.572 (0.551-0.593)$^{\bullet}$		60.6 (59.4-61.9)$^{\bullet\#}$	
Q3 (19.2 < 20.9)	4.51 (4.40-4.62)		8.94 (8.55-9.34)$^{*\bullet}$		0.607 (0.582-0.631)*		63.7 (62.5-65.0)$^{*\bullet}$	
Q4 (≥20.9)	4.49 (4.39-4.60)		9.14 (8.77-9.51)$^{\dagger\#}$		0.622 (0.599-0.646)$^{\dagger\bullet}$		64.1 (62.9-65.2)$^{\dagger\#}$	
	(r = -0.075; p = 0.027)		(r = 0.169; p < 0.0001)		(r = 0.193; p < 0.0001)		(r = 0.200; p < 0.0001)	
C18:2n6 Linoleic acid (%)								
Q1 (<14.2)	4.51 (4.39-4.62)	0.094	8.71 (8.31-9.11)	0.407 0.194^{a}	0.574 (0.550-0.598)	0.459 0.268^{a}	61.6 (60.4-62.8)	0.902 0.904^{a} 0.134^{b} 0.790^{c} 0.116^{d}
Q2 (14.2 < 16.4)	4.68 (4.56-4.79)		8.73 (8.35-9.11)		0.600 (0.576-0.624)		62.2 (60.9-63.4)	
Q3 (16.4 < 18.6)	4.51 (4.41-4.61)		8.49 (8.13-8.86)		0.582 (0.559-0.605)		62.1 (60.8-63.5)	
Q4 (≥18.6)	4.59 (4.49-4.69)		8.33 (7.98-8.69)		0.586 (0.564-0.607)		62.2 (61.0-63.4)	
	(r = 0.024; p = 0.472)		(r = -0.073; p = 0.030)		(r = -0.003; p = 0.939)		(r = -0.015; p = 0.667)	

Table 4.6. ***(continued)***

Fatty acid quartiles	Lag time (min)	ANOVA p-value	Slope (au/s)	ANOVA p-value	Maximum absorbance (∆nm)	ANOVA p-value	Clot lysis time (min)	ANOVA p-value
C20:3n6 Eicosatrienoic acid (%)								
Q1 (<2.46)	4.66 (4.55-4.76)		8.30 (7.94-8.66)		0.567 (0.544-0.589)		60.5 (59.2-61.8)*	<0.0001
Q2 (2.46 < 2.81)	4.59 (4.48-4.69)	0.206	8.59 (8.21-8.96)	0.430	0.591 (0.567-0.615)	0.172	60.8 (59.5-62.0)†	0.0001^{a}
Q3 (2.81 < 3.26)	4.51 (4.41-4.62)		8.69 (8.30-9.08)	0.223^{a}	0.581 (0.559-0.603)	0.257^{a}	62.6 (61.3-63.8)	0.0003^{b}
Q4 (≥3.26)	4.52 (4.41-4.63)		8.69 (8.31-9.08)		0.603 (0.578-0.627)		64.3 (63.2-65.4)*†	0.281^{c}
	(r = -0.064; p = 0.058)		(r = 0.035; p = 0.298)		(r = 0.058; p = 0.088)		(r = 0.148; p < 0.0001)	<0.0001^{d}
C20:4n6 Arachidonic acid (%)								
Q1 (<13.0)	4.64 (4.54-4.75)		7.98 (7.59-8.36)*†		0.533 (0.511-0.555)*†		60.0 (58.7-61.4)*†	<0.0001
Q2 (13.0 < 14.5)	4.59 (4.48-4.70)	0.342	8.16 (7.82-8.51)$^{\bullet\#}$	<0.0001	0.578 (0.555-0.602)*	<0.0001	60.9 (59.7-62.1)$^{\bullet\#}$	0.0002^{a}
Q3 (14.5 < 16.1)	4.53 (4.42-4.64)		8.91 (8.54-9.28)$^{*\bullet}$	0.004^{a}	0.604 (0.582-0.627)†	0.008^{a}	63.3 (62.0-64.6)$^{*\bullet}$	<0.0001^{b}
Q4 (≥16.1)	4.52 (4.40-4.63)		9.22 (8.84-9.59)$^{\dagger\#}$		0.626 (0.602-0.649)*		63.9 (62.8-65.0)$^{\dagger\#}$	0.084^{c}
	(r = -0.061; p = 0.074)		(r = 0.192; p < 0.0001)		(r = 0.210; p < 0.0001)		(r = 0.183; p < 0.0001)	
C22:5n6 Osbond acid (%)								
Q1 (<0.52)	4.64 (4.54-4.75)		8.70 (8.31-9.08)		0.590 (0.566-0.614)		61.9 (60.6-63.2)	0.892
Q2 (0.52 < 0.65)	4.53 (4.43-4.64)	0.456	8.80 (8.45-9.16)	0.230	0.606 (0.584-0.627)	0.151	61.8 (60.6-63.0)	0.673^{a}
Q3 (0.65 < 0.87)	4.56 (4.45-4.67)		8.29 (7.93-8.65)	0.479^{a}	0.575 (0.551-0.598)	0.328^{a}	62.4 (61.2-63.7)	0.382^{b}
Q4 (≥0.87)	4.54 (4.43-4.65)		8.48 (8.08-8.88)		0.571 (0.547-0.595)		61.9 (60.6-63.2)	0.288^{c}
	(r = -0.027; p = 0.426)		(r = -0.055; p = 0.103)		(r = -0.048; p = 0.153)		(r = 0.022; p = 0.518)	0.579^{d}

Table 4.6. ***(continued)***

Fatty acid quartiles	Lag time (min)	ANOVA p-value	Slope (au/s)	ANOVA p-value	Maximum absorbance (∆nm)	ANOVA p-value	Clot lysis time (min)	ANOVA p-value
Total n-6/n-3 (%)								
Q1 (<5.28)	4.58 (4.48-4.68)	0.561	8.89 (8.48-9.29)*	0.0003 0.157[a]	0.592 (0.567-0.617)*	0.0001 0.044[a]	63.6 (62.4-64.8)*	0.0001 0.001[a] 0.045[b] 0.002[c]
Q2 (5.28 < 6.18)	4.50 (4.40-4.61)		9.00 (8.64-9.37)†		0.622 (0.599-0.646)$^{\dagger\bullet}$		62.3 (61.0-63.5)†	
Q3 (6.18 < 7.16)	4.59 (4.47-4.70)		8.42 (8.05-8.79)		0.580 (0.558-0.601)†		62.6 (61.4-63.8)$^{\bullet}$	
Q4 (≥7.16)	4.61 (4.50-4.71)		7.95 (7.61-8.30)*†		0.547 (0.526-0.569)$^{*\bullet}$		59.6 (58.2-61.0)$^{*\dagger\bullet}$	
	(r = 0.026; p = 0.437)		(r = -0.114; p = 0.001)		(r = -0.095; p = 0.005)		(r = -0.168; p < 0.0001)	
Total long chain n-6/n-3 (%)								
Q1 (<2.30)	4.50 (4.28-4.71)	0.923	8.67 (7.67-9.67)	0.575 0.724[a]	0.557 (0.510-0.605)	0.041 0.519[a]	63.7 (60.1-67.4)	0.017 0.027[a] 0.025[b] 0.002[c]
Q2 (2.30 < 3.36)	4.57 (4.49-4.64)		8.70 (8.42-8.98)		0.603 (0.586-0.621)*†		62.8 (62.0-63.7)*	
Q3 (3.36 < 3.87)	4.57 (4.46-4.68)		8.41 (8.05-8.78)		0.573 (0.550-0.596)*		61.7 (60.5-62.9)	
Q4 (≥3.87)	4.59 (4.47-4.71)		8.45 (8.08-8.82)		0.570 (0.547-0.592)†		60.5 (59.1-61.9)*	
	(r = -0.005; p = 0.883)		(r = -0.037; p = 0.274)		(r = -0.042; p = 0.212)		(r = -0.109; p = 0.001)	

ALA: alpha-linolenic acid; DHA: docosahexaenoic acid; DPA: docosapentaenoic acid; EPA: eicosapentaenoic acid; MUFAs: monounsaturated fatty acids; PUFAs: polyunsaturated fatty acids; SFAs: saturated fatty acids

Data given as mean (95% confidence interval)

$^{*\dagger\bullet\#}$values with the same symbol differed significantly from each other

[a] p-values adjusted for fibrinogen; [b] p-values adjusted for fibrinogen and plasminogen activator inhibitor type-1 activity (PAI-1 activity); [c] p-values adjusted for body mass index (BMI); [d] p-values adjusted for PAI-1 activity; (Pearson correlation coefficient; correlation p-value)

The results presented in this chapter are discussed in detail in Chapter 5.

CHAPTER 5 – DISCUSSION AND CONCLUSION

5.1. Introduction

Cardiovascular disease (CVD) is a major cause of death both globally (WHO, 2017; Barquera *et al.*, 2015) and in South Africa (Stats SA, 2017). Although CVD was less prevalent among black South Africans in the past (Walker *et al.*, 2004), it is now on the rise in this population owing to changing lifestyles and dietary patterns caused by increasing urbanisation and westernisation (Vorster *et al.*, 2011; Nojilana *et al.*, 2016). Consequently, traditionally low blood levels of atherogenic lipids are now also increasing among black South Africans (Oosthuizen *et al.*, 2002; Peer *et al.*, 2014). It is well established that elevated triglyceride and low-density lipoprotein cholesterol (LDL-C) levels and low high-density lipoprotein cholesterol (HDL-C) levels are associated with increased CVD risk (Lusis, 2000; Harchaoui *et al.*, 2009; Boullart *et al.*, 2012; Catapano *et al.*, 2016; Nordestgaard, 2016). Although research is only starting to focus on individual fatty acids, certain plasma fatty acids have also been associated with increased or decreased CVD risk (Khaw *et al.*, 2012; Mozaffarian *et al.*, 2013). Black South Africans are furthermore known to have increased haemostatic risk factors such as high fibrinogen levels (Pieters *et al.*, 2011) which is also associated with increased CVD risk (Bridge *et al.*, 2014).

Haemostasis and blood lipids are interlinked on various levels, with the inflammatory pathway being one of these. For instance, the haemostatic factors fibrinogen, plasminogen and plasminogen activator inhibitor type-1 (PAI-1) all increase during inflammation (Binder *et al.*, 2002; de Moerloose *et al.*, 2010; Sugimoto *et al.*, 2017) and oxidised LDL-C has been shown to increase inflammation, contributing to the development of atherosclerosis (Lusis, 2000). Furthermore, as detailed in Chapter 2, evidence exists for direct interactions between haemostatic factors (such as fibrinogen) and blood lipids, with emerging evidence also linking plasma fatty acids to fibrin clot properties.

Thus far, there is only a handful of studies which investigated the relationship between blood lipids, fibrinogen and clot properties and nearly no studies focussing on plasma fatty acids, fibrinogen γ' and clot properties. With CVD prevalence increasing among black South Africans and with the concomitant increase in atherogenic blood lipids in the presence of existing high levels of fibrinogen, black South Africans are an ideal population in which to investigate these associations. We therefore aimed to determine the relationship of fibrin clot properties with different blood lipid fractions and plasma phospholipid fatty acids (as detailed in the objectives below) in 1 281 black South Africans who participated in the Prospective Urban and Rural Epidemiological Study in South Africa (PURE-SA) in 2010.

The objectives were:

1. To determine the relationship of total and γ' fibrinogen (a common splice variant of fibrinogen) concentration with blood lipids namely total cholesterol, LDL-C, HDL-C, non-HDL-C, triglyceride concentration and the lipid ratio LDL-C/HDL-C. Fibrinogen being the structural protein responsible for fibrin clot formation.

2. To determine the relationship of total and γ' fibrinogen concentration with composite (saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids (including n-3 and n-6 polyunsaturated fatty acids)) and individual plasma phospholipid fatty acids.

3. To determine the relationship of plasma clot properties (namely clot formation, clot structure, and clot lysis) with above-mentioned blood lipids and plasma phospholipid fatty acids.

4. To determine the interaction of total and fibrinogen γ' with above-mentioned blood lipids and plasma phospholipid fatty acids in determining clot properties.

The results of this study, provided in Chapter 4, are discussed next, starting with the relationship of total and γ' fibrinogen and plasma clot properties with blood lipids, followed by the relationship with plasma phospholipid fatty acids. The possible limitations of this study are then provided, followed by the conclusion and suggestions for future studies.

5.2. The relationship of total and γ' fibrinogen and plasma clot properties with blood lipids

We found no association between any of the blood lipids and γ' fibrinogen in our study, and to our knowledge no studies investigating associations between blood lipids and γ' fibrinogen are available in the literature. We however found a strong positive relationship between LDL-C (and other LDL-C driven lipid fractions) and total fibrinogen concentration, as well as an association with denser clots with a higher rate of lateral aggregation and lower lysis rate. The association between LDL-C and these clot properties remained after adjustment for fibrinogen, suggesting that its relationship with clot properties is not solely through a higher fibrinogen concentration but that other mechanisms might also be at play, such as a direct interaction with the clot formation process or binding to fibrin fibres.

Studies have found lipids incorporated into clots, with Silvain *et al.* (2011) finding cholesterol crystals present in *in vivo* thrombi from CVD patients, and Kunz *et al.* (1992) finding thrombi produced from the plasma of CVD patients to contain a higher content of cholesterol,

phospholipids and triglycerides. These patients had higher fibrinogen levels but normal total cholesterol levels. Skrzydlewski *et al.* (1976) in fact found LDL-C to directly bind to the fibrin clot by means of ion and hydrogen bonds using plasma from healthy participants, more so in the presence of normal to high levels of fibrinogen, and that increasing the concentration of LDL-C lead to firmer clots. Kunz *et al.* (1992) reported that clots from CVD patients had a shorter clotting time and were firmer compared to the healthy control participants in the study and suggested that the firmer clots could be partly due to fibrinogen and lipoproteins interacting with activated platelets. Investigations by Al-Awadhi & Dunn (2000) did not find LDL-C to markedly affect the rate of clot formation when adding lipids at various concentrations prior to clot formation, but found that the addition of higher concentrations (≥2.59 mmol/L) of LDL-C after clot formation inhibited lysis. However, this study prepared clots in a purified system, which excludes additional blood components that could mediate the effect of these lipids on clot formation. Meltzer *et al.* (2009) found total cholesterol levels to positively, but not independently, correlate with lysis times in myocardial infarction patients. Those results were likely driven by LDL-C, which was not specifically measured in that study.

Both Skrzydlewski *et al.* (1976) and Kunz *et al.* (1992) suggested that the binding of lipids to clots, thereby blocking degradation enzymes, could be a reason for the protracted lysis, with Kunz *et al.* (1992) speculating that the incorporation of lipids into clots may be due to lipoproteins binding to platelets. Curtiss & Plow (1984) found that both LDL and HDL bind to platelets with HDL having the ability to inhibit LDL binding. Another reason for the inhibited lysis observed with higher LDL-C levels could be owing to surface-bound LDL (but not HDL) binding with t-PA, which is what Simon *et al.* (1991) found, and inhibiting plasminogen activation in this way. Lipoprotein (a) (Lp(a)), which is a pro-atherogenic lipoprotein subclass with a similar chemical structure to LDL (Maranhao *et al.*, 2014), was also found to bind to t-PA in the study by Simon *et al.* (1991). In addition, Lp (a) has been shown to inhibit lysis by competing with plasminogen for binding sites on the clot due to apolipoprotein (a) (a protein that forms part of Lp (a) and that structurally differentiates Lp (a) from LDL) having a similar structure to plasminogen (Maranhao *et al.*, 2014). It was also found to associate with denser clots with thinner fibrin fibres (Undas *et al.*, 2006; Zabczyk *et al.*, 2013).

We further observed that higher HDL-C levels were associated with lower fibrinogen concentrations as well as less dense clots with a lower rate of lateral aggregation and a higher lysis rate. The association with these clot properties also remained after adjustment for fibrinogen concentration. Kunz *et al.* (1992) also found HDL-C to inversely associate with fibrinogen concentration and to correlate directly with clot formation time (in the healthy control group). Zabczyk *et al.* (2013) found higher HDL-C levels to be associated with less dense clots and faster lysis times for clots prepared from plasma of apparently healthy participants. Also, in

MI patients, Meltzer *et al.* (2009) found HDL-C levels to be inversely (but as with LDL-C also not independently) associated with lysis times. The study by Al-Awadhi & Dunn (2000) using a purified system did not find HDL-C to affect the rate of clot formation but observed that when HDL-C was added after clot formation, the highest concentration (≥2.59 mmol/L) delayed lysis, while lower concentrations enhanced lysis. The authors could not explain these unexpected results. Although Puccetti *et al.* (2001) found the reduced lysis observed with low HDL-C levels to be related to higher PAI-1 activity, we did not find this association as significance remained after adjustment for PAI-1 activity.

HDL-C also seems to bind to clots as Skrzydlewski *et al.* (1976), using chemical methods and double immunodiffusion, found HDL-C bound to clots, although less so than LDL-C. On the other hand, Talens *et al.* (2012a) found, using 2D gel electrophoresis and mass spectrometry, six proteins associated with HDL bound to clots (and specifically to fibrinogen) prepared from plasma of healthy participants, and further found more apolipoprotein A-I (associated with HDL) bound to the clot than apolipoprotein B (associated with LDL). The authors speculated that the bound apolipoprotein B is likely from Lp (a) and that the bound HDL may only be a sub fraction of HDL which could explain why HDL-C has a different effect on clot properties compared to LDL-C. More recently, Suski *et al.* (2015), using shotgun proteomics, also found HDL-related proteins (including apolipoprotein A-I) present in *ex vivo* clots formed using plasma from four acute myocardial infarction patients.

We did not observe any association between triglyceride levels with fibrinogen concentration, rate of lateral aggregation or clot density. However, higher triglyceride levels were associated with a slower lysis rate even after adjusting for PAI-1 and body mass index (BMI), suggesting that additional mechanisms may be involved. Meltzer *et al.* (2009) also found higher triglyceride levels to be independently associated with longer lysis times. Puccetti *et al.* (2001) not only found higher PAI-1 activity with higher triglyceride levels but also reduced t-PA activity (the main activator of fibrinolysis), along with inhibited lysis. Similarly, an earlier study by Yamada *et al.* (1990) also found that healthy participants with the lowest t-PA activity levels had higher concentrations of serum total cholesterol and triglycerides, and lower levels of HDL-C, compared to participants with normal serum lipids and t-PA activity levels. Mehta *et al.* (1987) found that an inhibitor of t-PA (not specified in the study) correlated positively with serum triglyceride levels, but not with serum total cholesterol levels, in patients with CVD. Mussoni *et al.* (1992) did not find fibrinolytic activity to differ between patients with hypertriglyceridemia and healthy controls. The authors suggested the concurrent higher PAI-1 and t-PA antigen levels observed in the hypertriglyceridemic patients could explain those results, although LDL-C levels did not differ significantly between the groups and were not elevated which could also have played a role.

We did not find an association between the time it took for clot formation to initiate (lag time) and any of the blood lipids. A possible reason for this may be that the turbidity-based assay we used may not be sensitive enough to detect significant differences between blood lipid quartiles in an apparently healthy population with relatively normal lipid levels.

Although we did not investigate associations of oxidised *vs.* non-oxidised lipids with clot properties, it warrants further investigation as oxidation appears to play a role in the effect of blood lipids on clot properties. Azizova *et al.* (2000), for example, found that highly oxidised LDL markedly decreased clot stability and inhibit fibrinolytic activity compared to non-oxidised or low oxidised LDL, and suggested that oxidised LDL alters clot properties by modifying fibrin molecules. Talens *et al.* (2013) found oxidised HDL to also decrease clot firmness (but not native HDL or oxidised LDL) and speculated that the mechanism involved platelets. Both studies used thromboelastography to measure clot firmness.

5.3. The relationship of total and γ' fibrinogen and plasma clot properties with plasma phospholipid fatty acids

We observed associations between total fibrinogen and clot properties and many of the composite and individual fatty acids. Several individual fatty acids had an opposite relationship with the haemostatic outcome variables than that of its composite group, highlighting the need to evaluate the effect of individual fatty acids on thrombosis and CVD risk, and not to merely make conclusions based on composite groups. As with blood lipids, we found no relationship between γ' fibrinogen concentration and any of the plasma fatty acids. It therefore does not seem as though plasma lipids and fatty acids are involved in the regulation processes of γ' fibrinogen gene expression.

When investigating composite plasma phospholipid SFA levels, we found higher levels to be associated with prothrombotic clots – clots had a higher rate of lateral aggregation, a denser structure, and took longer to lyse. These associations were largely driven by stearic acid (C18:0) which comprised a large proportion (14.9% of the total phospholipid fatty acids) of the composite SFAs. Higher fibrinogen levels could explain the association with the denser clot structure but could not fully explain the associations with faster lateral aggregation and longer lysis times. It was interesting to note that despite palmitic acid (C16:0) contributing the largest proportion (27.3% of the total phospholipid fatty acids) to the composite SFA group, it did not drive the association observed with the composite SFAs as the association was driven by stearic acid.

As with blood lipids, studies have also found fatty acids to bind (Muraoka & Okuda, 1977) or be incorporated into clots (Rabai *et al.*, 2007). Tanka-Salamon *et al.* (2016), using a turbidimetric assay and investigating the effects of fatty acid salts on clots in a purified system, found that stearate markedly decreased the rate of clot formation. Further investigations using scanning electron microscopy (SEM) found stearate to also significantly thicken fibrin fibres in a purified system. Poole (1955), who also investigated the effects of fatty acid salts by recalcifying citrate treated plasma, found that the addition of stearate to the plasma accelerated clotting. One way in which stearic acid may increase thrombosis is by activating factor XII, along with stimulating platelet aggregation, which is what Hoak (1994) found when injecting stearic acid sodium salts (unbound fatty acids) into dogs. In terms of lysis, stearic acid does not seem to slow down lysis by inhibiting plasmin as Muraoka & Okuda (1977), who investigated the effect of free fatty acids, found plasmin activity (prepared from serum) to only be slightly inhibited (15.7%) in the presence of stearic acid. Thijssen *et al.* (2005) concluded that a diet high in stearic acid involving healthy participants (with adherence confirmed by measuring serum phospholipid stearic acid levels) did not appear to be particularly thrombogenic. However, this study did not measure clot properties, but other markers of coagulation (factor VII, prothrombin fragments, and plasma fibrinogen concentration) and lysis (PAI-1 activity and t-PA/PAI-1 complexes) and did not find these to differ among participants following diets high in stearic, oleic, or linoleic acid, respectively. Because of the limited number of studies available and the differences in study designs (purified fibrinogen *vs*. plasma, the addition of fatty acids to plasma *vs.* dietary intake and the different outcome measures) used in the above-mentioned studies, it is as of yet difficult to draw firm conclusions regarding the effect of stearate on fibrin clot properties. In addition, it remains challenging to interpret results of individual fatty acids as the fatty acid composition of plasma lipids is both a reflection of fatty acid metabolism and dietary intake (FAO, 2010).

In contrast to SFAs, we found higher composite MUFAs to be associated with lower fibrinogen concentration and the formation of pro-fibrinolytic clots (clots had a lower rate of lateral aggregation, were less dense and lysed quicker). Oleic acid (C18:1n9) was likely the driving fatty acid behind these observations as it was the MUFA that contributed the most (8.65%) to the total plasma phospholipid fatty acids. These associations of the pro-fibrinolytic clot phenotype with plasma oleic acid could not be ascribed to the associated lower fibrinogen concentration alone as significance remained after adjustment for fibrinogen concentration. The study by Tanka-Salamon *et al.* (2016) using a purified system found that oleate caused clots to form slower and slightly reduced the fibrin fibre diameter, while the study by Poole (1955) found the addition of oleate to plasma accelerated clot formation. In terms of lysis, Muraoka & Okuda (1977) and Higazi *et al.* (1994) both found oleic acid to inhibit plasmin, with Muraoka & Okuda (1977) finding oleic acid to bind to the clot, which would be expected to lengthen lysis times.

However, both studies investigated the effect of free fatty acids whereas we investigated phospholipid fatty acids. A more recent study by Rabai *et al.* (2007) found that oleic acid (released from phospholipids) shortened lysis times in formed clots by accelerating the activation of plasminogen to plasmin and through increasing fibrin's affinity for t-PA but inhibited plasminogen activation in a fluid phase. The true effect of composite and individual MUFAs on clot properties, however, is not completely clear with the reason largely being the limited number of studies conducted on the topic along with the differences in study and experimental designs between the available studies. Certain characteristics of the current study population further complicate the interpretation, namely their low total fat intake (median 70.6 g/day; 24.3% of total daily energy intake), and in particular low MUFA consumption (7% of total daily energy intake).

When investigating composite PUFAs, we found higher levels of both n-6 and n-3 fatty acids to be associated with higher fibrinogen concentration and a pro-thrombotic clot profile, namely a higher rate of lateral aggregation and denser clots (which were largely explained by the concomitant increase in fibrinogen levels) and longer lysis times (which was largely explained by the concomitant increase in BMI).

While plasma phospholipid n-3 fatty acids have been strongly linked to decreased CVD risk in the past (Hallgren *et al.*, 2001; Mozaffarian *et al.*, 2013) we, however, observed that higher total n-3 fatty acids were associated with higher fibrinogen concentration and the formation of pro-thrombotic clots that had a faster rate of lateral aggregation, had a denser structure and longer lysis times. The association between n-3 fatty acids and rate of lateral aggregation was mediated in full by the associated higher fibrinogen concentration but was mediated only partly in the case of clot density, suggesting the involvement of additional mechanisms. Docosahexaenoic acid (DHA) (C22:6n3) contributed the most to total phospholipid n-3 fatty acids (3.9%) and was found to be the driving fatty acid behind these results, although DHA itself has been associated with decreased CVD risk (Simon *et al.*, 1995; Mozaffarian *et al.*, 2013). However, an important characteristic of the PURE-SA population is a traditionally low fat, high carbohydrate diet, and in particular a very low n-3 fatty acid intake (Richter *et al.*, 2014). Despite these low intakes, the plasma DHA levels were considered sufficient (median of 52-109 mg per day depending on gender and if rural or urban) (Richter *et al.*, 2014). In this population, plasma DHA correlated more strongly with dietary alpha-linolenic acid (ALA) (C18:3n3) than with dietary DHA intakes (Richter, 2014) (with the body likely having an increased ability to convert dietary ALA into DHA (FAO, 2010) in the presence of low DHA intakes) suggesting that this population might have an upregulated metabolic conversion of fatty acids. This may be ethnic specific, as fatty acid metabolism has been demonstrated to be affected by genetic variation. Polymorphisms within the fatty acid desaturase gene clusters were for example

identified in Africans and are associated with enhanced metabolism of medium to long chain PUFAs (Mathias *et al.*, 2012; Sergeant *et al.*, 2012). It may be that blood levels of DHA originating from ALA conversion has a different effect on clot properties than when originating from dietary intake of DHA, but this will need further investigation.

Another n-3 fatty acid, eicosapentaenoic acid (EPA) (C20:5n3) is also thought to protect against CVD (Mozaffarian *et al.*, 2013). In agreement with this, we found the highest blood levels of EPA to be significantly associated with cardio-protective clot properties, namely a lower rate of lateral aggregation, a less dense clot structure and accelerated lysis. These associations were largely explained by the concomitant lower fibrinogen concentrations in the highest quartile of EPA levels. However, the PURE-SA population has a generally low EPA status (mean 0.52%) with even individuals in the highest quartile having relatively low EPA levels. The traditionally low intake of fish in this population likely explains these low plasma levels. It is therefore possible that the effects of EPA would be more pronounced in populations with higher blood levels. Al-Awadhi & Dunn (2000), using a purified system, did not find EPA to affect clot formation, which agrees with our finding that fibrinogen concentration mediated the association in our study, as in purified systems, fibrinogen concentration is kept constant. It could however also suggest that other blood or plasma components may play a role in its effect on clot formation. The same study also observed increased lysis if EPA was added before, but not after clot formation, in which case EPA was found to inhibit lysis, suggesting that EPA may exert its effect on fibrin clot properties during clot formation. One of the mechanisms through which EPA may influence clot properties could be through its conversion to local-acting hormones (eicosanoids) namely the 3-series prostaglandins, thromboxane A3 and leukotriene B5 which have anti-inflammatory effects and inhibit platelet aggregation and clotting (Mori, 2017; Nagy & Tiuca, 2017).

The n-3 fatty acid ALA was the only fatty acid for which we observed an association with lag time, with slower initiation of clotting associated with higher ALA levels. We further found higher ALA levels to be associated with clots with a lower rate of lateral aggregation, a less dense structure and a faster lysis rate. The faster lysis rate was mediated through the associated lower fibrinogen concentration. Although results from animal studies cannot simply be extrapolated to humans, in agreement with our data, Holy *et al.* (2011) found that a diet high in ALA inhibited thrombus formation in mice. That study further found higher ALA tissue levels in the experimental mice, compared to control, and concluded that ALA appeared to have a direct antithrombotic effect and did not depend on being converted to long chain n-3 fatty acids, with ALA itself inhibiting tissue factor (which binds with factor VII and initiates the extrinsic pathway of haemostasis (Nemerson, 1966; Jesty & Nemerson, 1974)) and reducing platelet activation. The effect of composite n-3 fatty acids on fibrinogen concentration and clot properties depend

strongly on the relative concentrations of the constituent n-3 fatty acids as these individual fatty acids respectively demonstrate significant associations with fibrinogen and clot properties.

Upon investigating total n-6 fatty acids, we observed higher levels were associated with higher fibrinogen concentrations and clots that had a higher rate of lateral aggregation, that were denser (both largely explained by the concomitant higher fibrinogen concentration) and lysed slower (which could be explained by the associated higher BMI). The prothrombotic clot profile appeared to be driven by arachidonic acid (C20:4n6) (the second most abundant n-6 fatty acid (14.5%)) as none of the other individual n-6 PUFAs had any association with the measured clot properties. Arachidonic acid, released from cell membranes, has been shown to be pro-inflammatory and prothrombotic by activating the endothelium through its conversion to pro-inflammatory eicosanoids (prostaglandins 2-series, thromboxane A2 and leukotriene B4), thus increasing platelet adhesion and aggregation as well as clot formation (Bogatcheva *et al.*, 2005; Nagy & Tiuca, 2017).

5.4. Study limitations

This study has a few possible limitations. Firstly, it was cross-sectional in design which has the inherent limitation of only being able to measure associations, as causality cannot be inferred. Secondly, the use of a direct method to measure fibre diameter, such as scanning electron microscopy (SEM), might have been better than using maximum absorbance from turbidimetry, which is a more indirect approach. However, SEM is impractical for studies with large sample sizes as it is extremely time consuming and costly and is rarely used in studies with samples sizes larger than 20 to 30 participants. In addition, it does not allow the study of native fibrin fibres as the sample undergoes drying before visualisation on the microscope. Furthermore, we used the Friedewald Formula (Friedewald *et al*., 1972) to calculate LDL-C values in this study which may not be as accurate as other methods (Sathiyakumar *et al.*, 2018). As with all studies, there is a possibility of residual confounding, as it is not possible to control for all factors that may influence our study variables, although adjustments were made for known covariates.

5.5. Conclusion

Our study suggests that blood lipids and plasma phospholipid fatty acids are associated with altered fibrinogen levels and clot properties. In further support of evidence regarding blood lipids and CVD risk, we found LDL-C levels to be associated with a pro-thrombotic clot profile, while higher HDL-C concentrations appear to be protective. These relationships were only partly mediated by fibrinogen concentration suggesting potential direct interaction of the lipids with clot formation and/or properties. Plasma phospholipid fatty acids were associated with both prothrombotic and pro-fibrinolytic clot phenotypes. Individual fatty acids were often found

to have opposite effects on clot properties than its composite group, highlighting the importance of investigating the effects of individual fatty acids instead of drawing conclusions based on composite groups. Furthermore, the PURE-SA population is unique in that this population has a traditionally low fat, high carbohydrate diet possibly leading to upregulated fatty acid metabolism, which should be considered in the interpretation of the results. The drawing of firm conclusions remains hindered by the limited number of studies conducted on the topic along with the differences in study and experimental designs between the available studies and therefore warrants further investigation.

5.6. Suggestions for future studies

Suggestions for future studies include:

- As the PURE-SA study is a longitudinal study, future investigations should include a prospective investigation into the relationship between blood lipids and plasma phospholipid fatty acids and clot properties to better infer causality.
- Mechanistic studies which aim to elucidate the direct interaction of lipids and fatty acids with clot properties.
- Investigating the effect of lipid and fatty acid oxidation on clot properties.
- Investigating the effect of dietary DHA *vs.* DHA metabolically converted from dietary ALA on clot properties.
- Investigating the genetic variants related to fat metabolism in this population and how these affect clot properties.

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7. ADDENDA

Addendum A – HREC ethics approval certificate

NORTH-WEST UNIVERSITY
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NOORDWES-UNIVERSITEIT

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Institutional Research Ethics Regulatory Committee
Tel: +27 18 299 4849
Email : Ethics@nwu.ac.za

2016/09/02

ETHICS APPROVAL CERTIFICATE OF STUDY

Based on approval by **Health Research Ethics Committee (HREC),** after being reviewed at the meeting held on **08/06/2016**, the North-West University Institutional Research Ethics Regulatory Committee (NWU-IRERC) hereby **approves** your study as indicated below. This implies that the NWU-IRERC grants its permission that provided the special conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: The relationship between blood lipids, fatty acids and plasma clot properties in black South Africans.

Study Leader/Supervisor: Dr Z de Lange
Student: B Kahler

Ethics number:

N	W	U	-	0	0	0	6	3	-	1	6	-	A	1
Institution				Study Number						Year			Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: Single study
Commencement date: 2016-08-01 **Risk:** Minimal

Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation up to a maximum period of three years.

Special conditions of the approval (if applicable):

- Translation of the informed consent document to the languages applicable to the study participants should be submitted to the HREC (if applicable).
- Any research at governmental or private institutions, permission must still be obtained from relevant authorities and provided to the HREC. Ethics approval is required BEFORE approval can be obtained from these authorities.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:

- *The study leader (principle investigator) must report in the prescribed format to the NWU-IRERC via HREC:*
  - *annually (or as otherwise requested) on the monitoring of the study, and upon completion of the study*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *Annually a number of studies may be randomly selected for an external audit.*
- *The approval applies strictly to the proposal as stipulated in the application form. Would any changes to the proposal be deemed necessary during the course of the study, the study leader must apply for approval of these amendments at the HREC, prior to implementation. Would there be deviated from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility the NWU-IRERC and HREC retains the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process.*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the study are revealed or suspected,*
    - *it becomes apparent that any relevant information was withheld from the HREC or that information has been false or misrepresented,*
    - *the required amendments, annual (or otherwise stipulated) report and reporting of adverse events or incidents was not done in a timely manner and accurately,*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *HREC can be contacted for further information or any report templates via Ethics-HRECApply@nwu.ac.za or 018 299 1206.*

The IRERC would like to remain at your service as scientist and researcher, and wishes you well with your study. Please do not hesitate to contact the IRERC or HREC for any further enquiries or requests for assistance.

Yours sincerely

Prof LA Du Plessis
Digitally signed by Prof LA Du Plessis
Date: 2016.09.05 17:26:30 +02'00'

Prof Linda du Plessis
Chair NWU Institutional Research Ethics Regulatory Committee (IRERC)

Addendum B – PURE-SA 2010 informed consent form

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NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

PURE-SA Project (Prospective Urban and Rural Epidemiology)

INFORMED CONSENT FORM (including the PRIMER-study)

I, the undersigned .....(full names and surname)

read / listened to the information on the project in PART 1 and PART 2 of this document and I declare that I understand the information. I had the opportunity to discuss aspects of the project with the project leader and I declare that I participate in the project as a volunteer. I hereby give my consent to be a subject in this project. I understand that I have the right to withdraw at any time from the study.

I agree to be tested for HIV	Yes	No
I want to know my HIV-status	Yes	No
I agree to give a blood sample	Yes	No

I hereby also declare that I am aware that:

1. This blood sample will be used for the purpose of
   a. Isolating DNA to look at genetic factors that are currently associated with Type 2 Diabetes (i.e. the Calpain10, Adiponectin, Leptin and Leptin Receptor genes), or genetic factors that may be associated with Non Communicable diseases in the future. We give the assurance that all genetic tests and experiments will only focus on genotypes suspected to contribute to an increased risk of non communicable diseases of lifestyle.
   b. DNA damage due to environmental factors such as cooking methods and smoke will be tested for.
   c. Testing for liver function by determining liver enzymes such as AST, GGT,
   d. Analyses of other than genetic parameters for Diabetes Mellitus such as HbA_1C, Blood glucose and Insulin
   e. Analyses of the clotting profile and hypertension markers
   f. Analyses of bone health, iron and nutrition status

   And may be stored until such time as the above measurements/analyses will be done.
2. Body measurements such as height, weight, skin fold thicknesses, arm and leg circumferences will be taken
3. Vascular sonar will be done
4. Blood pressure to be taken
5. Pulse wave velocity measurements will be made
6. A Spirometer test to be performed to determine lung function
7. Bone density will be determined with an osteometer to detect possible osteoporosis

.....

(Signature of the subject)

Signed at **... Potchefstroom / Ganyesa ...** (delete not applicable option) on **...../...../ 2010**

Witnesses

1. ..... **2.** .....

Signed at **... Potchefstroom / Ganyesa ...** (delete not applicable option) on **...../...../ 2010**

PART 1

1. **Research Unit and Faculty:**
Africa Unit for transdisciplinary Health research (AUTHeR); Faculty of Health Sciences, North-West University

2. **Title of project/trial:**
PURE: Prospective Urban and Rural Epidemiological study

3. **Full names, surname and qualifications of project leader:**
Prof. Annamarie Kruger, Ph.D. (Nutrition)

4. Rank/position of project leader:
Research Director and project manager

5. **Aim of this project**
PURE's aim is that understanding the different lifestyle and health transitions of individuals in response to societal changes will elucidate societal and individual adaptive strategies that could diminish the adverse health effects of industrialization and urbanization on health, while retaining its benefits.

6. **Explanation of the nature of all procedures, including identification of new procedures:**
Each participant will have to fill in a number of questionnaires (Adult questionnaire, Physical activity questionnaire, Food frequency questionnaire, Health questionnaire, psychology questionnaire) with the help of field workers. A blood sample will be taken. Physical measures will be performed, including anthropometric measures (such as weight, height, and waist circumference), blood pressure, lung capacity and lung volume and bone density will be performed.

7. **Description of the nature of discomfort or hazards of probable permanent consequences for the subjects which may be associated with the project: (including possible side-effects of and interactions between drugs or radio-active isotopes which may be used.)**
It will take each participant quite a while (about 6 [six] hours) to complete all the tests and questionnaires. Discomfort may be experienced with the taking of blood samples. No measures will have permanent damage or consequences for the participants.

8. **Precautions taken to protect the subjects:**
The research nurse will be present at all times, and will be responsible for the blood sampling. She is very experienced and has performed these procedures numerous times in previous studies.

9. **Description of the benefits which may be expected from this project:**
When measures with immediate results are taken, such as blood glucose, haemoglobin, HIV status and blood pressure, the information will be communicated to the individual to seek professional help during an individual feedback session. Since this study is a longitudinal study, subjects that are high at risk will be identified over time and personal feedback and counselling will be given.
On the research site each participant will receive a cooked meal, a fruit and fruit juice. Also an amount of R50 will be paid to each participant to cover transport costs and enable each person to go for follow-up at a clinic of choice.

10. **Alternative procedures which may be beneficial to the subjects:**
There will be tested for HIV/AIDS, therefore pre-test counselling will be given. If the subject wants to know his/her status and he/her tests positive, post counselling will also be given.

PART 2

To the subject signing the consent:

You are invited to participate in a research project. It is important that you read/listen to and understand the following general principles, which apply to all participants in our research project:

1. **Participation in this project is voluntary.**

2. **It is possible that you personally will not derive any benefit from participation in this project, although the health knowledge obtained from the results may be beneficial to you and other people.**

3. **You will be free to withdraw from the project at any stage without having to explain the reasons for your withdrawal. However, we would like to request that you would rather not withdraw without a thorough consideration of your decision, since it may have an effect on the statistical reliability of the results of the project.**

4. **The nature of the project, possible risk factors, factors which may cause discomfort, the expected benefits to the subjects and the known and the most probable permanent consequences which may follow from your participation in this project, are discussed in Part 1 of this document.**

5. **We encourage you to ask questions at any stage about the project and procedures to the project leader or the personnel, who will readily give more information. They will discuss all procedures with you.**

6. **The University staff will use standardised procedures and take all possible precaution to protect the subject from risks.**

7. **All information will be kept CONFIDENTIAL and no personal information will be published without my consent.**

Prof ANNAMARIE KRUGER

Contact details: 082 771 5778 / 018 299 2095(Office)

Addendum C – Quantitative food frequency questionnaire

PURE/South Africa 2010
Quantitative Food Frequency Questionnaire

☐☐☐☐ *Subject Code*

Name of fieldworker: _____

Subject ID

☐☐ *Centre #* ☐☐☐ *Community #* ☐☐☐☐ *Household #* ☐☐ *Subject #*

Subject Initials

☐☐☐ *F M L*

Today's date: 2010 *year* ☐☐ *month* ☐☐ *day* **Day of the week:** _____

1. Name of the PARTICIPANT: _____

2. Not applicable in South Africa

3. National identity # or equivalent _____ **N/A** ☐

4. DOB: ☐☐☐☐ ☐☐ ☐☐ **AND Age** ☐☐ **years**

5. Sex: ☐ Female ☐ Male

Please think carefully about the food and drink you have consumed during the ***PAST MONTH*** (four weeks). We have divided the foods into different groups for example all the porridges and cereals together. I will go through a list of food groups and drinks with you and I would like you to tell me:

- Which foods you eat in each of the different food groups
- How the food is prepared
- How much of the food you eat at a time
- How many times a day you eat it and if you do not eat it everyday, how many times a week or a month you eat it.

To help you to describe the amount of a food you eat, I will show you pictures of different amounts of the food as well as other food models, containers, etc.

There are no right or wrong answers.

Everything you tell me is confidential. Only your subject number appears on the form.

Is there anything you want to ask now?

Are you willing to go on with the questions?

Before we start I would like to find out what type of margarine, oil and milk you **USUALLY** use in your home.

1. What type of **MARGARINE** do you USUALLY use in your home? Give brand name if possible
   - ☐ Tub/Soft margarine (brand name) _____
   - ☐ Brick/Hard margarine (brand name) _____
   - ☐ Do not use margarine in home

2. What type of **OIL** do you USUALLY use in the preparation of food in your home?
   - ☐ Sunflower oil (give brand name)_____
   - ☐ Canola oil (give brand name) _____
   - ☐ Olive oil (give brand name) _____
   - ☐ Other (give brand name) _____
   - ☐ Oil previously used _____
   - ☐ Do not use OIL ever in the home

3. What type of **MILK** do you USUALLY use in your home? Mark only ONE
   - ☐ Full cream milk / Fresh cow's milk/ Box milk full cream
   - ☐ Low fat milk / 2% milk / Box low fat or 2% milk
   - ☐ Fat free milk / Skim milk / Box fat free or skim milk
   - ☐ Powder milk (eg Elite; give brand name) _____
   - ☐ Do not use milk

4. What type of **CREAMER** do you USUALLY use in your home?
   - ☐ Cremora, Ellis Brown, Coffee Mate, Tea Mate etc
   - ☐ Cremora Lite
   - ☐ Do not use creamer

2

QUANTIFIED FOOD FREQUENCY QUESTIONNAIRE 2010

INSTRUCTIONS: Circle the subject's answer. Fill in the amount and times eaten in the appropriate columns.

I shall now ask you about the type and the amount of food you have been eating in the **LAST MONTH**. Please tell if you eat the food, how much you eat and how often you eat it. We shall start with maize meal porridge.

PORRIDGE AND BREAKFAST CEREALS								
FOOD	DESCRIPTION	AMOUNT	TIMES EATEN 2 columns to be completed				CODE	AMOUNT / WEEK
			Per day	Per week	Per month	Seldom /Never		
Maize-meal porridge	Stiff (pap)						4401	
Maize-meal porridge	Soft (slappap)						4400	
Maize-meal porridge	Crumbly (phutu)						4402	
Ting	Maize meal						4429	
	Mabella						4427	
	Other							
Mabella	Stiff						3437	
	Soft							
Morvite	Soft						4404	
Oats							3239	
Tastee wheat	Soft						3240	
Other cooked porridge	Type							
Breakfast cereals	All bran flakes						3242	
	Corn flakes plain						3243	
	Weetbix						3244	
	Rice crispies plain						3252	
	Other							

3

Do you pour milk on your porridge or cereal? Yes 1 No 2

If yes, what type of milk (whole fresh, sour, 1%, fat free, milk blend, etc) _____

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN 2 columns to be completed				CODE	AMOUNT / WEEK
			Per day	Per week	Per month	Seldom /Never		
If yes, how much milk	Whole milk/full cream milk/ fresh cow's milk						2718	
	Maas/sour milk						2787	
	Low fat / 2% milk						2772	
	Fat free / skim milk						2775	
	Other							

Do you put sugar on your porridge or cereal? Yes 1 No 2

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN 2 columns to be completed				CODE	AMOUNT / WEEK
			Per day	Per week	Per month	Seldom /Never		
If yes, how much sugar WHITE or BROWN	Cooked porridge						3989	
	Cereal						3989	
	Other porridge / cereal						3989	
	Other							

4

OTHER STARCH								
FOOD	**DESCRIPTION**	**AMOUNT**	**TIMES EATEN** 2 columns to be completed				**CODE**	**AMOUNT / WEEK**
			Per day	**Per week**	**Per month**	**Seldom /Never**		
Samp	Bought Self ground						3250	
Samp and beans	Give ratio of samp:beans						3402 (1:1)	
Samp and peanuts	Give ratio of samp:peanuts						3250 (samp) 3458	
Rice	White						3247	
	Brown						3315	
	Maize Rice						3250	
	Any fat added?							
Pasta	Macaroni, plain						3262	
	Spaghetti, plain						3262	
	Spaghetti, canned in tomato sauce						3258	
	Macaroni & cheese Milk: Fat:							
	Other specify							
Pizza	Home made: Specify topping						3353 (base+ch)	
	Bought: Specify topping						3353 (base+ch)	

5

You are being very helpful. Can I now ask you about meat?

CHICKEN, MEAT, FISH

How many times do you eat meat (beef, mutton, pork, chicken, fish) per week? _____

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN 2 columns to be completed				CODE	AMOUNT / WEEK
			Per day	Per week	Per month	Seldom /Never		
Chicken	Meat & skin, boiled						2926	
	Meat & skin, roasted/grilled/fried in chicken fat						2925	
	Meat ONLY, boiled (no skin)						1559	
	Meat ONLY, roasted (no skin)						1545	
	White meat only, cooked (NO Skin)						2964	
	Kentucky / Chicken Licken (Fried in batter/crumbs)						3018	
	Nando's						2925	
	Other							
Do you eat chicken skin?		Always 1		Sometimes 2		Never 3		
Chicken stew	With potato and onion WITH skin						4413	
	With tomato and onion WITH skin						2985	
	With vegetables WITH skin						3005	
	With tomato and onion NO skin						4379	
	With vegetables NO skin						4378	
Chicken BONE stew	With potato and onion and tomato						4414	
	Other							
Chicken feet	Nothing added						2997	
	Stew with potato, onion and tomato						4415	
Chicken head							2999	
Chicken offal	Stew with tomato and onion and sunflower oil						4416	
	Liver, cooked						2970	
	Other							

6

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN 2 columns to be completed				CODE	AMOUNT / WEEK
			Per day	Per week	Per month	Seldom /Never		
RED MEAT	**How do you like your meat?** With fat **OR** Fat trimmed							
Red meat BEEF	BRISKET, boiled/fried without added fat						4363	
	CHUCK, boiled/fried without added fat						2945	
	BRISKET, fried in added fat Type of fat:						4363	
	CHUCK, fried in added fat Type of fat:						2945	
	Beef, stewed with cabbage						3006	
	Beef, stewed with potato and onion and tomato						4417	
	Beef, stewed with vegetables						3020	
	Mince , nothing added						2921	
	Mince, tomato & onion added						2987	
	Beef **BONE** stew with potato and onion and oil						4419	
	Other							
MUTTON	Meat, with fat, cooked						2947	
	Mutton, no fat, cooked						3036	
	Mutton, chop, grilled						2927	
	Mutton, stewed with vegetables						2916	
	Other							

BRISKET: more fat and cheaper
CHUCK: less fat than the brisket, but more expensive

7

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN 2 columns to be completed				CODE	AMOUNT / WEEK
			Per day	Per week	Per month	Seldom /Never		
Beef/mutton Offal	Offal, cooked						3003	
	Stewed with vegetables							
	Liver, beef, fried/cooked						2920	
	Liver, sheep, fried/cooked						2955	
	Kidney, beef, cooked						2923	
	Kidney, sheep, cooked						2956	
	Brain, sheep, cooked						2952	
	Lung, beef, cooked						3019	
	Lung, sheep, cooked						4337	
	"Gemaldes" (lung & fat)						4409	
	Heart, beef, cooked						2968	
	Heart, sheep, cooked						2969	
	Other							
Goat meat	Grilled/roasted/cooked						4281	
	Stewed with vegetables							
	Other							
Venison/ Wild buck							2913	
Horse/Donkey							4407	
Rabbit							4327	
Other type of meat	Specify							

What type of vegetables is usually put into meat stews?

8

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN 2 columns to be completed				CODE	AMOUNT / WEEK
			Per day	Per week	Per month	Seldom / Never		
Wors / Sausage							2931	
Bacon							2906	
Patties	Beef, fried						2984	
	Chicken, fried						3011	
Cold meats AND Processed meats	Polony						2919	
	Ham						2967	
	Vienna						2936	
	Frankfurter, beef & pork						2937	
	Frankfurter/Sausage, chicken						3012	
	Russian/Salami						2948	
	Other							
Canned meat	Bully beef, plain						2940	
	Bully beef with potato & onion & oil						2994	
	Other							
Meat pie BOUGHT Or HOMEMADE	Beef						2939	
	Steak and kidney						2957	
	Sausage roll						2939	
	Cornish						2953	
	Chicken						2954	
	Other							
Hamburger	Bought						4418	
	Other							
Biltong	Beef (with fat OR without fat)						3021	
Dried wors Dried sausage	Beef						2949	

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN 2 columns to be completed				CODE	AMOUNT / WEEK
			Per day	Per week	Per month	Seldom / Never		
Dried beans	Baked beans in tomato sauce						3176	
	Bean salad / Sousbone						3174	
	Soup with dried beans, beef and vegetables						3145	
	Sugar beans, cooked						3205	
	Other							
Lentils	Whole, cooked						3203	
	Lentil soup with beef and vegetables						3153	
Soya products eg. Imana, Knorr, Jileleke, Toppers	Cooked						3196	
	Soup/Gravy						4431	
	Stewed with extra potato, onion and tomato						4430	
	Other							

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FOOD	DESCRIPTION	AMOUNT	TIMES EATEN 2 columns to be completed				CODE	AMOUNT / WEEK
			Per day	Per week	Per month	Seldom / Never		
Pilchards in tomato sauce or chilli or brine	Whole						3102	
	Mashed with fried onion						3102 (70%) 3730 (30%)	
	With tomato and onion						4420	
	Other							
Fish	Hake, fried with batter/crumbs						3072	
	Hake, fried in oil						3060	
	Hake, steamed						4373	
	Moddervis / Yellow fish fried in oil						3084	
	Moddervis / Yellow fish baked with onion (NO oil added)						3089	
	Other							
Other canned fish	Tuna in oil						3056	
	Other							
Fish cakes	Bought: Fried						3080	
	Home made with potato						3098	
Fish fingers	Bought						3081	
Eggs	Boiled/poached						2867	
	Scrambled (full cream milk & oil)						2890	
	Scrambled (NO milk, ONLY oil added)						2869	
	Scrambled (NO oil, ONLY full cream milk)						2872	
	Fried in oil						2869	
	Fried in brick margarine						2877	
	Other							

VEGETABLES								
FOOD	**DESCRIPTION**	**AMOUNT**	**TIMES EATEN** 2 columns to be completed				**CODE**	**AMOUNT / WEEK**
			Per day	**Per week**	**Per month**	**Seldom /Never**		
Cabbage	**How do you cook cabbage?**							
	Boiled, nothing added						3756	
	Boiled with potato and onion and **oil**						3815	
	Boiled with potato and onion and **brick margarine**						3813	
	Fried in **oil**						3812	
	Fried in **brick margarine**						3810	
	Boiled with potato, onion and tomato and oil						4421	
	Raw with nothing added						3704	
	Other							
Spinach or morogo or beetroot leaves or other green leafy	**How do you cook spinach?**							
	Boiled, nothing added						3913	
	Boiled with **oil** added							
	Boiled with **brick margarine** added						3898	
	Boiled with **tub margarine** added						3899	
	Boiled with potato, onion and tomato and oil						4422	
	Other							
Tomato and onion gravy	With oil						4423	
	Without fat						3925	
	Canned						4192	
	Thickened with packet soup powder						4432	
	Other							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN 2 columns to be completed				CODE	AMOUNT / WEEK
			Per day	Per week	Per month	Seldom /Never		
Pumpkin (yellow) Butternut Hubbard squash Table Queen Etc	Boiled, nothing added						4164	
	Boiled with sugar only (NO fat)						3728	
	Boiled with **brick margarine** & sugar						3893	
	Boiled with **tub margarine** and sugar						4433	
	Boiled with **oil** and sugar						4428	
	Other							
Carrots	Boiled, nothing added						3757	
	Boiled with **oil** added							
	Boiled with **brick margarine** added						3816	
	Boiled with **tub margarine** added						3817	
	Boiled with **sugar** only						3818	
	Boiled with **oil** and sugar							
	Boiled with **brick margarine** and sugar						3819	
	Boiled with **tub margarine** and sugar						3820	
	Boiled with potato, onion and **oil**						3824	
	Boiled with potato, onion and **brick margarine**						3822	
	Boiled with potato, onion and **tub margarine**							
	Chakalaka						4412	
	Raw, nothing added						3709	
	Other							
Mealies/ Sweet corn	On cob – fat added **Fat**						3725	
	On cob – no fat added						3725	
	Creamed sweet corn / canned						3726	
	Whole kernel/canned						3942	
	Whole kernel, frozen, boiled						4132	
	Other							13

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN 2 columns to be completed				CODE	AMOUNT / WEEK
			Per day	Per week	Per month	Seldom /Never		
Beetroot	Salad						3699	
	Boiled, nothing added						3698	
How do you cook potatoes?								
Potatoes	Boiled/baked with skin						4155	
	Boiled/baked without skin						3737	
	Boiled with **oil** added						3873	
	Boiled with **brick margarine** added						3867	
	Boiled with **tub margarine** added						3868	
	Mashed with **whole milk** and **brick margarine**						3876	
	Mashed with **whole milk** and **oil**							
	Roasted in beef fat						3878	
	Roasted in oil						3979	
	French fries (chips) / Fried potatoes						3740	
	Other							
Sweet potatoes	**How do you cook sweet potatoes?**							
	Boiled/baked with skin						3748	
	Boiled/baked without skin						3903	
	Boiled with **sugar** and **oil** added						4434	
	Boiled with **sugar** and **brick margarine** added						3749	
	Other							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN 2 columns to be completed				CODE	AMOUNT / WEEK
			Per day	Per week	Per month	Seldom /Never		
Broccoli	Boiled						3701	
	Raw						3702	
Cauliflower	Boiled						3716	
Green beans	Boiled, nothing added						3696	
	Cooked with potato, onion and **oil**						3794	
	Cooked with potato, onion and **brick margarine**						3792	
	Other							
Mixed vegetables	Canned						4264	
	Frozen, boiled						3727	
	Other							
Salad vegetables	Mixed salad: tomato, lettuce and cucumber						3921	
	Raw tomato						3750	
	Cucumber, raw						4119	
	Coleslaw (cabbage) (mayonnaise)						3705	
	Coleslaw (cabbage) (commercial)						3707	
	Potato salad (mayonnaise)						3928	
	Baked bean salad						4424	
	Other salad vegetables							
Mayonnaise / salad dressing	Mayonnaise						3488	
	Vinegar, oil						3487	
	Other: Specify							
Other vegetables, specify + preparation								

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Now we come to fruit

FRUIT

Do you like fruit? Yes **1** No **2**

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN 2 columns to be completed				CODE	AMOUNT / WEEK
			Per day	Per week	Per month	Seldom /Never		
Apples							3592	
Banana							3540	
Pears							3582	
Oranges							3560	
Naartjie							3558	
Grapes							3550	
Peaches	Fresh						3565	
	Canned						3567	
Apricots	Fresh						3534	
	Canned						3535	
Mangoes							3556	
Guavas	Fresh						3551	
	Canned						3553	
Watermelon	Fresh						3576	
Fruit salad	Fresh						3588	
	Canned						3580	
Fig (Vye)							3544	
Avocado							3656	
Wild fruit/berries	Specify type							

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FOOD	DESCRIPTION	AMOUNT	TIMES EATEN 2 columns to be completed				CODE	AMOUNT / WEEK
			Per day	Per week	Per month	Seldom /Never		
Dried fruit	Apple, dried, raw						3600	
	Peach, dried, raw						3568	
	Mixed fruit, dried, raw						3593	
	Mixed fruit, dried and cooked with sugar						3590	
	Fruit roll, dried (all types)						3655	
	Other							
Other fruit	_____ _____							

If subject eats canned fruit: Do you have custard with the canned fruit or fresh fruit salad?

Yes 1 No 2

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN 2 columns to be completed				CODE	AMOUNT / WEEK
			Per day	Per week	Per month	Seldom /Never		
Custard	Homemade, full cream milk or fresh cow's milk						2716	
	Homemade, lowfat milk						2779	
	Homemade, skim milk						2717	
	Commercial eg Ultramel						2716	
	Other							

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BREAD AND BREAD SPREADS

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN 2 columns to be completed				CODE	AMOUNT / WEEK
			Per day	Per week	Per month	Seldom /Never		
Bread / Bread rolls	White						3210	
	Brown						3211	
	Whole wheat						3212	
Do you spread anything on the bread?		Always 1		Sometimes 2		Never 3		
Margarine	**What brand do you have at home now?**							
	Tub, regular						3496	
	Tub, medium fat						4406	
	Tub, light/low fat						3524	
	Brick, regular						3484	
	Brick, medium fat						4405	
	Brick, lite/low fat						3528	
	Other							
Peanut butter							3485	
Jam/syrup/ honey							3985	
Marmite / Fray bentos / Oxo							4058	
Fish/meat paste							3109	
Cheese	Cheddar						2722	
	Gouda						2723	
	Other							
Sandwich spread							3522	
Achaar							3117	
Other spreads	Specify							

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FOOD	DESCRIPTION	AMOUNT	TIMES EATEN 2 columns to be completed				CODE	AMOUNT / WEEK
			Per day	Per week	Per month	Seldom /Never		
Dumpling	White flour						3210	
	Whole wheat flour						3212	
Vetkoek	White flour						3257	
	Whole wheat flour						3324	
Provita, crackers, etc	Provita						3235	
	Cream crackers						3230	
	Other savoury biscuits like Bacon kips, wheat crackers, etc						3331	

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DRINKS								
FOOD	**DESCRIPTION**	**AMOUNT**	**TIMES EATEN** 2 columns to be completed				**CODE**	**AMOUNT / WEEK**
			Per day	**Per week**	**Per month**	**Seldom / Never**		
Tea	English (normal)						4038	
	Rooibos						4054	
Coffee							4037	
White sugar	Tea						3989	
	Coffee						3989	
Brown sugar	Tea						4005	
	Coffee						4005	
Milk per cup of TEA	**Do you use milk in your TEA?** Yes / No **If YES, What type of milk do you use in TEA?**							
	Fresh / long life whole/full cream						2718	
	Fresh/long life: 2%/low fat						2772	
	Fresh/long life: fat free / skim milk						2775	
	Creamer/whitener like Ellis Brown / Cremora						2751	
	Cremora **Lite**							
	Condensed milk						2714	
	Evaporated milk						2715	
	Other							
	None							
Milk per cup of COFFEE	**Do you use milk in your COFFEE?** Yes / No **If YES, What type of milk do you use in COFFEE?**							
	Fresh/long life: whole/full						2718	
	Fresh/long life: 2%/low fat						2772	
	Fresh/long life: fat free						2775	
	Creamer/whitener like Ellis Brown						2751	
	Cremora **Lite**							
	Condensed milk						2714	
	Evaporated milk						2715	
	Other							
	None							

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FOOD	DESCRIPTION	AMOUNT	TIMES EATEN 2 columns to be completed				CODE	AMOUNT / WEEK
			Per day	Per week	Per month	Seldom / Never		
Milk as such	**What type of milk do you drink milk as such?**							
	Fresh/long life: whole / full cream milk						2718	
	Fresh/long life: 2% milk / low fat milk						2772	
	Fresh/long life: fat free / skim milk						2775	
	Condensed milk						2714	
	Sour/maas						2787	
	Other							
Milk drinks	Flavoured milk						2774	
	Milo made with full cream milk						2735	
	Milo made with skim milk						2747	
	Drinking chocolate made with water						4287	
	Other							
Yoghurt	Drinking yoghurt low fat						2756	
	Plain low fat						2734	
	Low fat sweetened with fruit						2732	
Squash	Sweet O						4027	
	Six O							
	Oros/Lecol – with sugar or other						3982	
	- artificially sweetener						3990	
	KoolAid						4027	
	Other							
Fizzy drinks Coke, fanta, etc	Sweetened						3981	
	Diet							

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FOOD	DESCRIPTION	AMOUNT	TIMES EATEN 2 columns to be completed				CODE	AMOUNT / WEEK
			Per day	Per week	Per month	Seldom / Never		
Fruit juice	Fresh/Liquifruit/Ceres						2866	
	Tropica (Dairy –fruit juice mix)						2791	
	Other							
Mageu/Motogo							4056	
Home brew							4039	
Tlokwe							4039	
Beer							4031	
Cider	Sweet						4057	
Spirits **Eg Brandy, gin, vodka, whisky, cane, etc**							4035	
Wine red							4033	
Wine White							4033	
Other specify								
WATER	Tap, borehole, dam, river, etc						4042	
	Bottled						4042	

SNACKS AND SWEETS								
FOOD	**DESCRIPTION**	**AMOUNT**	**TIMES EATEN** 2 columns to be completed				**CODE**	**AMOUNT / WEEK**
			Per day	**Per week**	**Per month**	**Seldom / Never**		
Potato crisps							3417	
Peanuts	Raw						4285	
	Roasted						3458	
Cheese curls, Niknaks, etc							3267	
Raisins							3552	
Peanuts and raisins								
Chocolates	Milk chocolate, plain						3987	
	Kit Kat etc						4024	
	Chocolate coated bars like Bar One, TV bar, etc						3997	
	Other							
Candies/Sweets	Sugus, gums, hard sweets, etc						4000	
Toffees / Fudge / caramels							3991	
Biscuits/cookies	Potch cookies						4425	
	Homemade, plain						3233	
	Commercial, plain						3216	
	Commercial, with filling						3217	
	Other							
Cakes	Butter cake, homemade with **whole milk** and **brick margarine NO icing**						3288	
	Chocolate cake, homemade with **whole milk and brick margarine NO icing**						3289	
	Icing for cake made with **brick margarine**						4014	
	Other							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN 2 columns to be completed				CODE	AMOUNT / WEEK
			Per day	Per week	Per month	Seldom / Never		
Tarts	Apple tart with a batter made with whole milk and brick margarine						3327	
	Other							
Scones	Plain made with whole milk and brick margarine						3237	
	Other							
Muffin	Bran						3407	
	Plain						3408	
	Other							
Rusks	Buttermilk, commercial						3329	
	Homemade, white						3222	
	Other							
Savouries	Sausage rolls						2939	
	Samoosas: Meat filling						3355	
	Samoosas: Vegetable filling						3414	
	Biscuits eg bacon kips						3331	
	Other							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN 2 columns to be completed				CODE	AMOUNT / WEEK
			Per day	Per week	Per month	Seldom / Never		
Jelly							3983	
	Custard added made with whole milk						2716	
	Other							
Baked pudding	Baked in a syrup						3312	
	Baked without a syrup						3429	
	Custard added made with whole milk						2716	
	Other							
Instant pudding	Made with whole milk						3266	
	Made with low fat milk						3395	
	Other							
Ice cream	Regular						3483	
	Soft serve						3518	
	Other							
Sorbet							3491	
Other specify								

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SAUCES, GRAVIES AND CONDIMENTS								
FOOD	DESCRIPTION	AMOUNT	TIMES EATEN 2 columns to be completed				CODE	AMOUNT / WEEK
			Per day	Per week	Per month	Seldom / Never		
Tomato sauce							3139	
Worcester sauce							4309	
Chutney							3168	
Pickles							3866	
White sauce	Made with whole milk and brick margarine						3142	
Packet soups	Dry powder (all types)						3158	
	Made with water (all types)						3165	
Gravy	Made from meat and thickened						3120	
Other								

WILD FRUITS, WILD BIRDS, ANIMALS OR INCECTS (hunted in rural areas or on farms)								
FOOD	DESCRIPTION	AMOUNT	TIMES EATEN 2 columns to be completed				CODE	AMOUNT / WEEK
			Per day	Per week	Per month	Seldom / Never		
MISCELLANEOUS: Please mention *ANY OTHER FOODS* used more than once/two times a week which we have NOT talked about								
INDIGENOUS/TRADITIONAL FOODS/PLANTS/ANIMALS Please tell me if you use any indigenous plants OR other indigenous foods like mopani worms, locusts ect to eat PLEASE GIVE FETAILS								