

# **Fibrinogen concentration and plasma clot properties as predictors of cardiovascular disease mortality in Black South Africans**

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Dissertation accepted in fulfilment of the requirements for the degree Master of Science in Cardiovascular Dietetics at the North-West University

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## **ABSTRACT**

### **Introduction and aim:**

Cardiovascular disease (CVD) is one of the main causes of mortality, globally and in South Africa. Recent evidence obtained from prospective studies point to a potential causal role for clot structure in the development of CVD, although less is known regarding CVD mortality. The limited available studies on mortality explored different populations/ethnic groups and were subject to a wide array of inclusion criteria, whilst simultaneously being differentiated by sex, restricting firm conclusions. Prospective evidence, particularly on a population level, is still lacking. We aimed to determine the prospective association between total and  $\gamma'$  (a splice variant of) fibrinogen concentration and plasma clot properties and incident CVD mortality over 13 years in a Black South African cohort.

### **Methods:**

Data was collected from 2 010 apparently healthy Black South Africans who took part in the South African, North West Province arm of the International Prospective Urban and Rural Epidemiology Study (PURE-SA-NW). Data was collected at three time points; 2005, 2010 and 2015, of which only the baseline data is included in this mini-dissertation. Mortality status and cause of death data was obtained from Statistics South Africa and was available for all participants up until December 2018. The cause of death was documented on death certificates by a medically qualified general practitioner according to the 10<sup>th</sup> revision of the International Classification of Disease codes (ICD-10 codes). Plasma fibrinogen was measured using the Clauss method, and  $\gamma'$  fibrinogen with an enzyme-linked immunosorbent assay. Markers of plasma clot formation, structure and lysis were measured using the turbidity-based global fibrinolytic assay. Ordinary Cox models, adjusted for known CVD risk markers were used to determine the relationship of total and  $\gamma'$  fibrinogen and plasma clot properties at baseline with incident CVD mortality. CVD mortality was defined in two ways. First CVD mortality was classified when the underlying cause of death was indicated as CVD (ICD10 Codes I00 – I99), this is termed CVD<sub>Underlying</sub> (n=106 cases). A second approach was to classify CVD mortality when either underlying cause of death and/or the immediate cause of death was indicated as CVD (ICD10 Codes I00 – I99), this is termed CVD<sub>Total</sub> (n=154). Association with all-cause mortality, although not the main aim of this mini-dissertation, is also reported.

**Results:**

Originally, 2 010 participants were included in the PURE population. However, participants were removed from the dataset if they did not have complete data for the exposure variables (coagulation markers). After cleaning the dataset and excluding the participants with missing data the final sample size was n=1 645. *Certain infectious and parasitic diseases* (ICD 10 codes A00 to B99) (n=144) and *Diseases of the circulatory system* (CD 10 codes I00 to I99) (n=106) were the two main groups of underlying cause of death in this cohort. The three most prominent underlying CVD causes of death according to the ICD-10 code broad groups were hypertensive diseases (n=37), cerebrovascular diseases (n=31) and other forms of heart disease (n=29). The CVD mortality group consisted of proportionally more individuals from urban areas, had a higher percentage of smokers and alcohol consumers and lower physical activity than the survivors at baseline. They also had a higher prevalence of hypertension, and higher lipoprotein(a), glycated haemoglobin, inflammatory markers, gamma-glutamyl transferase, creatinine and glomerular filtration rate compared to the survivors. Of the coagulation markers, only total fibrinogen and clot density (maximum absorbance) had increased risk for CVD<sub>Underlying</sub> and CVD<sub>Total</sub> mortality, in a minimally adjusted model (age and gender), while significance was lost in the fully adjusted model.

**Conclusion:**

Infectious and parasitic diseases followed by diseases of the circulatory system were the main causes of death in the PURE-SA-NW cohort, while hypertensive, cerebrovascular and heart diseases were the main CVD causes of death. Total fibrinogen and clot density contributed to an increased risk of incident CVD mortality before, but not after adjustment for known CVD risk markers. These findings suggest that fibrinogen concentration and plasma clot properties did not confer additional, independent CVD mortality risk in this Black South African cohort. Although evidence from highly controlled case-control studies suggest a causal relationship for clot properties in CVD progression and mortality, the contribution of fibrinogen and plasma clot properties against the background of other CVD risk factors, such as urban living conditions, on epidemiological level, appears to be less prominent.

**Keywords:** Fibrinogen concentration, fibrin clot properties, clot formation, clot structure, clot lysis cardiovascular disease, cardiovascular disease mortality, thrombotic diseases.



## LIST OF ABBREVIATIONS AND SYMBOLS

|           |                                    |
|-----------|------------------------------------|
| $\gamma'$ | gamma prime                        |
| %         | percentage                         |
| °C        | degree Celsius                     |
| AF        | atrial fibrillation                |
| AFM       | atomic force microscopy            |
| AIDS      | acquired immunodeficiency syndrome |
| ALT       | alanine transaminase               |
| ANCOVA    | analyses of covariance             |
| ANOVA     | analyses of variance               |
| APS       | antiphospholipid syndrome          |
| AST       | aspartate transaminase             |
| au        | absorbance units                   |
| au/s      | absorbance units per second        |
| BMI       | body mass index                    |
| BP        | blood pressure                     |
| CAD       | coronary artery disease            |
| CD40L     | cluster of differentiation 40      |
| CETP      | cholesterol ester transfer protein |
| CI        | confidence interval                |

|       |                                      |
|-------|--------------------------------------|
| CLT   | clot lysis time                      |
| CRP   | C-reactive protein                   |
| CVD   | cardiovascular disease               |
| DBP   | diastolic blood pressure             |
| DMT2  | diabetes mellitus type 2             |
| DVT   | deep vein thrombosis                 |
| ECG   | electrocardiogram                    |
| ESRD  | end-stage renal disease              |
| F     | factor                               |
| FPA   | fibrinopeptide A                     |
| FPB   | fibrinopeptide B                     |
| g     | gram                                 |
| g/d   | gram per day                         |
| g/L   | gram per litre                       |
| GGT   | gamma-glutamyl transferase           |
| GLM   | generalised linear models            |
| GP    | platelet glycoprotein                |
| HbA1c | glycated haemoglobin                 |
| HCM   | hypertrophic cardiomyopathy          |
| HDL-C | high-density lipoprotein cholesterol |

|                   |                                                               |
|-------------------|---------------------------------------------------------------|
| HIV               | human immunodeficiency virus                                  |
| ICAM-1            | intercellular adhesion molecule-1                             |
| ICD-10            | International Classification of Diseases 10th Revision        |
| IL-6              | interleukin-6                                                 |
| LPL               | lipoprotein lipase                                            |
| Lp(a)             | lipoprotein (a)                                               |
| ISAK              | International Society for the Advancement of Kinanthropometry |
| IU/L              | international units per litre                                 |
| kg/m <sup>2</sup> | kilogram per square metre                                     |
| Ks                | clot permeability coefficient                                 |
| LDL-C             | low-density lipoprotein cholesterol                           |
| mg                | milligram                                                     |
| mg/L              | milligram per litre                                           |
| MI                | myocardial infarction                                         |
| mL/min            | millimetre per minute                                         |
| mmHg              | millimetre of mercury                                         |
| mmol/L            | millimole per litre                                           |
| MSc               | Master of Science                                             |
| NCD               | non-communicable diseases                                     |
| NETs              | neutrophil extracellular traps                                |

|            |                                                                          |
|------------|--------------------------------------------------------------------------|
| ng/mL      | nanogram per millilitre                                                  |
| nm         | nanometre                                                                |
| NWU        | North-West University                                                    |
| OR         | odds ratio                                                               |
| p          | p-value                                                                  |
| PAD        | peripheral artery disease                                                |
| PAI-1      | plasminogen activator inhibitor-1 activity                               |
| PE         | pulmonary embolism                                                       |
| PF4        | platelet factor 4                                                        |
| pg/mL      | picogram per millilitre                                                  |
| PT         | prothrombin                                                              |
| PURE       | Prospective Urban and Rural Epidemiology study                           |
| PURE SA-NW | Prospective Urban and Rural Epidemiology study, South Africa, North West |
| RA         | rheumatoid arthritis                                                     |
| RR         | relative risk                                                            |
| SAA        | serum amyloid A                                                          |
| SBP        | systolic blood pressure                                                  |
| SD         | standard deviation                                                       |
| SEM        | scanning electron microscopy                                             |
| SPSS       | Statistical Package for the Social Sciences                              |

|               |                                                       |
|---------------|-------------------------------------------------------|
| STEMI ST      | segment elevation myocardial infarction               |
| suPAR         | soluble urokinase-type plasminogen activator receptor |
| TAFI          | thrombin activatable fibrinolysis inhibitor           |
| TC            | total cholesterol                                     |
| TEG           | thromboelastography                                   |
| TEM           | transmission electron microscopy                      |
| TF            | tissue factor                                         |
| TG            | triglyceride                                          |
| tHcy          | total homocysteine                                    |
| TIA           | transient ischaemic attack                            |
| TNF- $\alpha$ | tumour necrosis factor-alpha                          |
| tPA           | tissue plasminogen activator                          |
| U/mL          | units per millilitre                                  |
| UN            | United Nations                                        |
| VCAM-1        | vascular cell adhesion protein-1                      |
| VKAs          | vitamin K antagonists                                 |
| VT            | venous thrombosis                                     |
| VTE           | venous thromboembolism                                |
| VWF           | Von Willebrand factor                                 |
| WC            | waist circumference                                   |

WHO

World Health Organisation

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## CHAPTER 1: INTRODUCTION

### 1.1 Background

Cardiovascular disease (CVD) is one of the leading causes of mortality worldwide, causing 17 million deaths per year and accounting for 31% of all deaths (WHO, 2018). Identifying individuals in danger of developing CVD has mainly been reliant on certain traditional risk factors or prediction models such as the Framingham or Reynolds risk scores (Ridker *et al.*, 2007). However, research suggests that these traditional risk factors and models do not sufficiently detect individuals at risk of CVD. For example, up to 20% of coronary disease patients have no traditional risk factors and 40% have only one (Hozawa *et al.*, 2007). Therefore, additional novel biomarkers are required to further improve the ability to correctly identify individuals at risk of CVD.

Various novel biomarkers for CVD risk prediction have since been researched and include markers of inflammation, coagulation, plaque instability, platelet activation and neurohormonal activation (Lowe & Rumley, 2014; Schaub *et al.*, 2012; Yun *et al.*, 2016; Kritharides, 2009; Roig-Minguell, 2004). The coagulation marker that is the focus of this study is fibrinogen concentration and its functional consequences measured as fibrin clot properties.

Fibrinogen is a protein found in human blood plasma (1.5–4.0 g/L) and is synthesised in the liver (Huseby & Bang, 1971). It is regulated in response to inflammatory changes supporting its multifaceted functions in haemostasis and thrombosis (Pieters & Wolberg, 2019). Fibrinogen has been acknowledged for CVD risk prediction as a novel biomarker and performs a crucial role in blood coagulation as the key structural element of a thrombus (Danesh *et al.*, 2005; Kaptoge *et al.*, 2012; Koenig, 2003; Krobot *et al.*, 1992; Mora *et al.*, 2006; Pieters *et al.*, 2021; Wang *et al.*, 2017). The possible causal role of fibrinogen concentration in CVD can be attributed to numerous pathophysiologic mechanisms such as increased platelet aggregation, altered/pro-thrombotic fibrin clot structure, increased blood viscosity, inflammation and endothelial activation (De Moerloose *et al.*, 2010; Ernst & Resch, 1993; Luyendyk *et al.*, 2019; Pulanić & Rudan, 2005; Torpy *et al.*, 2009; Vilar *et al.*, 2020). Despite these potential pathophysiological mechanisms, the causal role of fibrinogen concentration in CVD development and progression (also mortality) remains to be proven.

Although there is ample evidence for the relationship between fibrinogen and CVD risk and progression, less is known regarding its role in CVD mortality. The majority of previous research available, regarding fibrinogen and CVD mortality focused only on fibrinogen concentration (not

functionality) and investigated participants with pre-existing CVDs (coronary heart disease, myocardial infarction, ischaemic stroke, peripheral artery disease and angina pectoris) or other pre-existing medical conditions (e.g., end-stage renal disease, cancer and diabetes) (Banerjee *et al.*, 1992; Benderly *et al.*, 1996; Coppola *et al.*, 2005; Doweik *et al.*, 2003; Liu *et al.*, 2020; Rahmani *et al.*, 2019; Rosengren & Wilhelmsen, 1996; Thompson *et al.*, 1995; Toss *et al.*, 1997; Yang *et al.*, 2017; Zoccali *et al.*, 2003). Each of these studies explored different populations/ethnic groups and was subject to a wide array of inclusion criteria whilst simultaneously being differentiated by sex. However, the general conclusions of these studies were that increased/elevated fibrinogen concentration contributes significantly to mortality and poor event outcomes in all populations, despite differences in population, inclusion criteria and sex (Banerjee *et al.*, 1992; Benderly *et al.*, 1996; Coppola *et al.*, 2005; Doweik *et al.*, 2003; 2002; Liu *et al.*, 2020; Rahmani *et al.*, 2019; Rosengren & Wilhelmsen, 1996; Thompson *et al.*, 1995; Toss *et al.*, 1997; Yang *et al.*, 2017; Zoccali *et al.*, 2003).

One of the pathways through which fibrinogen is thought to contribute to CVD is through altered plasma clot properties. The main consensus is that patients with CVD present with fibrin clots that form faster, are less porous, have more dense structures, have thinner and highly branched fibres, increased fibre and clot stiffness, and greater resistance to fibrinolysis (Collet *et al.*, 2006; Fatah *et al.*, 1992; Sjøland *et al.*, 2007; Undas, 2017). The aforementioned clot structure variations have been observed in both arterial and venous thrombotic diseases (Bridge *et al.*, 2014; Undas & Ariëns, 2011b). Furthermore, recent evidence—albeit limited—obtained from prospective studies also points to a potential causal role for this type of clot structure in the development and progression of CVD, including evidence for CVD mortality (Celinska-Löwenhoff *et al.*, 2018; Cieslik *et al.*, 2018; Mrozinska *et al.*, 2019). However, most of these studies investigated this relationship in specific patient samples and focused more on disease risk and event recurrence rather than mortality, limiting the generalisability of the findings. Prospective evidence for a relationship between clot properties and CVD mortality, particularly on a population level is, therefore, still lacking.

Thus, to better understand the contribution of plasma clot properties and its main precursor, fibrinogen, to CVD severity (measured as mortality), we researched the association between fibrinogen concentration and fibrin clot properties, measured at baseline in 2 010 apparently healthy black South Africans, with incident CVD mortality over a 13-year follow-up period.

## **1.2 Aim and objectives**

This mini-dissertation is affiliated with the South African arm of the PURE study conducted in the North West province (PURE-SA-NW) and falls within the aim of the approved larger study (NWU-00034-17-A1) titled: *Characterisation of the haemostatic profile of a Black South African population* in which the CVD-related effects of urbanisation in South Africa are investigated.

The study aims to determine whether total and  $\gamma'$  (a splice variant of) fibrinogen concentration and plasma clot properties is associated with incident CVD mortality over 13 years, in Black South Africans who partook in the PURE-SA-NW study. These relationships will be investigated prospectively.

### **1.2.1 Objectives**

The objectives of the study were:

- To characterise total and  $\gamma'$  fibrinogen concentration and plasma clot properties (clot formation, clot structure and clot lysis) of 2 010 apparently healthy South Africans older than 30 years, participating in the PURE-SA-NW study.
- To obtain CVD mortality data of the cohort over a 13-year period using International Classification of Diseases' 10th revision (ICD-10 codes).
- To determine the prospective association of total and  $\gamma'$  fibrinogen concentration and plasma clot properties with incident CVD mortality over 13 years.

Table 1.1: Research team

| Team member             | Qualification | Role in study                                                                                                                                                                                                           |
|-------------------------|---------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Ms Hendrina Jankowitz   | BSc Dietetics | MSc student: Critical evaluation of literature, protocol drafting and editing, obtaining mortality data, statistical analysis, interpretation of the results and writing the mini-dissertation.                         |
| Prof. Marlien Pieters   | PhD Dietetics | Supervisor of MSc student: Study conceptualisation, larger study principal investigator, guidance regarding the writing of the protocol and mini-dissertation, statistical analysis, and interpretation of the results. |
| Dr Zelda de Lange-Loots | PhD Nutrition | Co-supervisor of MSc student: Guidance regarding the writing of the protocol and mini-dissertation, statistical analysis, and interpretation of the results.                                                            |

All members of the research team are affiliated with the NWU.

### 1.3 Structure of mini-dissertation

This mini-dissertation is written in chapter format and consists of Chapters 1–5. All five chapters were edited by a qualified language editor. The technical requirements and editing were done in line with the North West University (NWU) guidelines, with referencing complying with the NWU Harvard style, as prescribed by the university. The following paragraphs provide a summary of each chapter.

In the current chapter, Chapter 1, the reader is presented with background information on the topic, the aim and objectives of the study are provided, and the research team is introduced.

Chapter 2 is a literature review that provides the background information necessary for the interpretation of the study results. The first section of Chapter 2 discusses the global burden of CVD, CVD classification and the normal physiological mechanisms of haemostasis. Succeeding that section follows the coagulation cascade, intrinsic/extrinsic pathways, and the cell-based coagulation model. The last section is divided into two subsections and examines the cross-

sectional and prospective evidence available, linking fibrinogen concentration and fibrin clot properties to CVD outcomes/events and mortality.

Chapter 3 describes the methodology of the study. In this chapter, the aims and objectives of the study are clarified. Furthermore, it includes the study design, study population, participant recruitment, inclusion/exclusion criteria and ethical approval. This chapter also contains the anthropometric measurements and biochemical analysis and includes the equipment used and standard operating procedures followed. Lastly, this chapter contains the procedure used to obtain the mortality data as well as a detailed statistical plan for data interpretation.

The results are represented in Chapter 4. In this chapter, the data is examined, processed, and represented in results tables. Various tables are included in this chapter such as the demographic, lifestyle, and biochemical variance in the population as well as all-cause and CVD mortality numbers and causes, according to the ICD-10 codes on the participants' death certificates. Although all-cause mortality is not part of the main aim of this mini-dissertation, data on all-cause mortality may be useful for the interpretation of CVD mortality and hence, were also considered. Additionally, this chapter provides evidence regarding the prospective association of total fibrinogen concentration and plasma clot properties with all-cause and CVD mortality.

Chapter 5 contains the discussion. In this chapter, the findings of the study are considered, interpreted, and discussed, and conclusions that are relevant to the study outcomes are drawn. This chapter also presents the strengths and limitations of the study and provides recommendations for future research. This mini-dissertation is concluded with a reference list and sources that are cited throughout all five chapters as well as annexures providing supporting documentation.

## CHAPTER 2: LITERATURE REVIEW

### 2.1 Introduction

According to the World Health Organization (WHO) and the Global Burden of Disease Study, cardiovascular disease (CVD) accounted for 31% of all deaths globally in 2017 (WHO, 2018). Furthermore, in 2021, the American Heart Association identified that CVD claims more lives each year than any cancer or chronic lung disease combined (Virani *et al.*, 2021). Recognising individuals susceptible to developing CVD has mainly been reliant on certain traditional risk factors or prediction models such as the Framingham or Reynolds risk scores (Cook *et al.*, 2012; Lloyd-Jones *et al.*, 2019). However, researchers suggest that these traditional risk factors and prediction models do not sufficiently detect individuals at risk of CVD. For example, up to 20% of coronary disease patients have no traditional risk factors and 40% have only one (Hozawa *et al.*, 2007). Therefore, additional novel biomarkers are required to improve the ability to correctly identify individuals at risk of CVD events and mortality.

Various novel biomarkers for CVD risk prediction have since been researched and include markers for inflammation, coagulation, plaque instability, platelet activation and neurohormonal activation (Lowe & Rumley, 2014; Schaub *et al.*, 2012; Yun *et al.*, 2016; Kritharides, 2009; Roig-Minguell, 2004). The coagulation marker that is the focus of this mini-dissertation is fibrinogen concentration and its functional consequences, measured as fibrin clot properties.

Fibrinogen is a protein found in human blood plasma that is produced in the liver (Huseby & Bang, 1971) and has been acknowledged for CVD risk prediction as a novel biomarker (Danesh *et al.*, 2005; Kaptoge *et al.*, 2012; Koenig, 2003; Krobot *et al.*, 1992; Mora *et al.*, 2006; Pieters *et al.*, 2021; Wang *et al.*, 2017). The possible causal role of fibrinogen concentration in CVD can be accredited to numerous pathophysiologic mechanisms such as increased platelet aggregation, altered/pro-thrombotic fibrin clot structure, increased blood viscosity, inflammation and endothelial activation (De Moerloose *et al.*, 2010; Ernst & Resch, 1993; Luyendyk *et al.*, 2019; Pulanić & Rudan, 2005; Torpy *et al.*, 2009; Vilar *et al.*, 2020).

Many cohort studies have indicated that there is an association between fibrinogen concentration and fibrin clot properties regarding CVD. The main consensus is that patients with CVD present with fibrin clots that form faster, are less porous, have more dense structures, have thinner and highly branched fibres, increased fibre and clot stiffness and greater resistance to fibrinolysis (Collet *et al.*, 2006; Fatah *et al.*, 1992; Sjøland *et al.*, 2007, 2021; Undas, 2017). Furthermore,

recent evidence obtained from prospective studies also points to a potential causal role for this type of clot structure in the development and progression of CVD, including evidence for CVD mortality (Celinska-Löwenhoff *et al.*, 2018; Cieslik *et al.*, 2018; Mrozinska *et al.*, 2019). Many of these studies, however, investigated this relationship in specific patient samples (case-control studies) and focused more on disease risk and event recurrence rather than mortality, limiting the generalisability of the findings. Therefore, prospective evidence, particularly on a population level, is still lacking. The majority of research available on mortality investigated participants with pre-existing CVDs (coronary heart disease, myocardial infarction, ischaemic stroke, peripheral artery disease and angina pectoris) or other pre-existing medical conditions (end-stage renal disease, cancer and diabetes) (Banerjee *et al.*, 1992; Benderly *et al.*, 1996; Coppola *et al.*, 2005; Doweik *et al.*, 2003; Lisman *et al.*, 2002; Liu *et al.*, 2020; Rahmani *et al.*, 2019; Rosengren & Wilhelmsen, 1996; Thompson *et al.*, 1995; Toss *et al.*, 1997; Yang *et al.*, 2017; Zoccali *et al.*, 2003). Individually, these studies examined various populations or ethnic groups, was subject to a broad range of inclusion criteria and was also separated by sex. Despite the variations in the samples the overall conclusions of the studies' authors are that elevated/increased fibrinogen concentration significantly contributes to mortality and poor event outcomes in all populations (Banerjee *et al.*, 1992; Benderly *et al.*, 1996; Coppola *et al.*, 2005; Doweik *et al.*, 2003; Lisman *et al.*, 2002; Liu *et al.*, 2020; Rahmani *et al.*, 2019; Rosengren & Wilhelmsen, 1996; Thompson *et al.*, 1995; Toss *et al.*, 1997; Yang *et al.*, 2017; Zoccali *et al.*, 2003).

This literature review addresses various concepts relating to fibrinogen and CVD such as the global burden of CVD and associated risks, CVD classification, physiology and mechanisms of haemostasis, fibrin clot properties as well as the association between fibrin clot properties and CVD and related mortality.

## **2.2 The global burden of cardiovascular disease and associated risks**

In 2017, the WHO indicated that approximately 17,8 million people die of CVD annually, representing 32% of all global deaths (WHO, 2018). Heart attacks and strokes (thrombotic disorders) contribute to 85% of global CVD deaths. The majority (75%) of CVD deaths take place in low- and middle-income countries like South Africa (insert ref).

According to Statistics South Africa and the Heart and Stroke Foundation of South Africa, CVD is responsible for almost one in six deaths (17.3%), accounting for 215 deaths per day (Byrne *et al.*, 2016) (WHO, 2018) (StatsSA, 2015). CVD mortality is increasing significantly in South Africa and is currently one of the top three causes of death in the country (Pillay-van Wyk *et al.*, 2016;

Sivadasanpillai *et al.*, 2015), second only to human immunodeficiency virus/acquired immunodeficiency syndrome (WHO, 2014; WHO, 2018). Furthermore, it is expected that by 2030, the number of premature deaths in people of working age (35–64 years-old) due to CVD will increase by 41% (Pillay-van Wyk *et al.*, 2016).

Many factors contribute to the risk of developing CVD. A number of these factors that are widely acknowledged as established CVD risk factors have been combined into risk scores, which are used to predict an individual's risk of developing CVD. Two examples of these scoring systems / prediction models that are frequently used are the Framingham risk score and the Reynolds risk score (Ridker *et al.*, 2007). The Framingham risk score has been used effectively to estimate an individual's probability of having a CVD event, based on six widely acknowledged risk factors, namely age, sex, total cholesterol, high-density lipoprotein (HDL) cholesterol, smoking status and systolic blood pressure (Cook *et al.*, 2012). The Framingham risk score determines the percentage risk of an individual experiencing a CVD event within 10 years (Hosein *et al.*, 2020). An individual's score is calculated and estimated as having <10% (low), 10–20% (intermediate) and >20% (high risk) for CVD events (Hosein *et al.*, 2020). The Reynolds risk score also includes age, sex, total cholesterol, high-density lipoprotein cholesterol, smoking status / tobacco use, systolic blood pressure and additionally, considers C-reactive protein (CRP) and family history of CVD events before the age of 60 (Klisić, 2018). However, the data suggest that the use of these traditional risk factors alone does not adequately identify an individual's risk for CVD development, progression, and mortality. For example, up to 20% of coronary disease patients have no traditional risk factors and 40% have only one. In addition, a European study conducted on the accuracy of the Framingham equation with 15 406 men and women between the ages 45-64 years concluded that individual's risk is significantly underpredicted in certain socioeconomic groups and different ethnicities (Brindle *et al.*, 2005, Cook *et al.*, 2012, Smith *et al.*, 2003). This study proposed that additional markers could be included to the equation to increase the accuracy of the equation (Brindle *et al.*, 2005, Cook *et al.*, 2012, Smith *et al.*, 2003). This highlights the need for the identification of additional novel biomarkers to further improve the ability to accurately identify individuals at risk for CVD (Hozawa *et al.*, 2007). Biomarkers that have since been identified to influence and contribute to CVD include, but are not limited to, inflammatory markers such as interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF- $\alpha$ ), vascular cell adhesion protein-1 (VCAM-1) and CRP (Kritharides, 2009); coagulation markers such as fibrinogen, fibrin clot properties and plasminogen activator inhibitor-1 (PAI-1) (Lowe & Rumley, 2014); markers of plaque instability, namely myeloperoxidase, myeloid-related protein and pregnancy-associated plasma protein-A (Schaub *et al.*, 2012); markers of platelet activation such as P-selectin, cluster

of differentiation 40 protein (CD40L), platelet factor-4 (PF4) and platelet glycoprotein (GP) IIb/IIIa (Yun *et al.*, 2016) and also; markers of neurohormonal activation such as renin-angiotensin-aldosterone system, vasopressin and endothelin (Roig-Minguell, 2004).

The biomarker that is the focus of this mini-dissertation is fibrinogen concentration and its functional consequences, measured as fibrin/plasma clot properties. Fibrinogen has been significantly acknowledged for CVD risk prediction as a novel biomarker and performs a crucial role in blood coagulation as the key structural element of a thrombus (Danesh *et al.*, 2005; Kaptoge *et al.*, 2012; Koenig, 2003; Krobot *et al.*, 1992; Mora *et al.*, 2006; Pieters *et al.*, 2021; Wang *et al.*, 2017). The general findings obtained from literature indicated that fibrinogen concentration can significantly contribute to CVD events and outcomes (including mortality) regardless of different study populations being studied (Benderly *et al.*, 1996; Kaptoge *et al.*, 2012; Toss *et al.*, 1997; Wang *et al.*, 2017a; Woodward *et al.*, 2009; Yang *et al.*, 2017).

In addition to the existing literature, fibrinogen has also been associated with established cardiovascular risk factors, implying that the elevation of fibrinogen may be a physiological pathway by which these risk factors exert their influence (Krobot *et al.*, 1992; Vilar *et al.*, 2020). There are numerous mechanisms by which fibrinogen may increase cardiovascular risk (Vilar *et al.*, 2020). Firstly, fibrinogen binds specifically to activated platelets through the GP IIb/IIIa receptor, contributing to platelet aggregation and the promotion of thrombogenesis (Torpy *et al.*, 2009; Vilar *et al.*, 2020). Secondly, elevated levels of fibrinogen stimulate prothrombotic fibrin clot formation, which promotes atherosclerosis (Torpy *et al.*, 2009; Vilar *et al.*, 2020). Thirdly, fibrinogen is a significant contributor to elevated plasma viscosity, which contributes to thrombus size, structure and stability (Byrnes & Wolberg, 2017; Vilar *et al.*, 2020). Finally, fibrinogen is an acute-phase protein that is elevated in inflammatory states (Torpy *et al.*, 2009).

In addition, fibrinogen can undergo a series of molecular changes, such as alternative variant splicing and post-translational modifications which can influence fibrinogen plasma concentrations and biochemical properties (altered clot properties), and therefore may affect the risk of CVD (Surma & Banach, 2021). One of the most commonly occurring variants, is fibrinogen gamma prime (fibrinogen  $\gamma'$ ), of which the concentration typically ranges from 8–15% of total fibrinogen concentration (Baker & Ariëns, 2018; Surma & Banach, 2021; Wolberg, 2016). Fibrinogen  $\gamma'$  is an extended  $\gamma$  chain produced as a result of alternative mRNA processing in intron 9. An alternative polyadenylation signal in intron 9 leads to the translation of a 20-amino acid extension which replaces the 4 amino acids of exon 10 (Farrell, 2014; Lovely *et al.*, 2011; Surma

& Banach, 2021; Uitte de Willige *et al.*, 2009). This structure causes fibrinogen  $\gamma'$  to create clots that are denser, with less branching and a firm, closely connected network (Ariëns, 2013; Farrell, 2014; Lovely *et al.*, 2011). Furthermore, these clots have been known to be heterogenous in structure and have an increased resistance to lysis (Appiah *et al.*, 2015; Duval & Ariëns, 2017; Uitte de Willige *et al.*, 2009). The current data available proposes a positive association between increased fibrinogen  $\gamma'$  levels and arterial thrombosis, while decreased fibrinogen  $\gamma'$  levels have been associated with venous thrombosis (Ariëns, 2013; Cheung *et al.*, 2008; Duval & Ariëns, 2017; Lovely *et al.*, 2002; Macrae *et al.*, 2016; Mannila *et al.*, 2007; Uitte de Willige *et al.*, 2005).

Before the relationship between clot properties, as the functional consequence of fibrinogen and CVD is discussed in more detail, a classification of the different types of CVD is provided below.

### **2.3 CVD classification**

CVD is an umbrella term that can be used to define different types of diseases that affect the heart and blood vessels (Mc Namara *et al.*, 2019) such as diseased vessels, structural problems, infection and blood clots (Mc Namara *et al.*, 2019). Thus, CVD can be categorised according to mechanisms of function, area of affliction and cause of disease. For this study, CVDs have been divided into thrombotic and non-thrombotic diseases, as illustrated in Figure 2.1. Non-thrombotic diseases include myocarditis, hypertrophic cardiomyopathy and arrhythmias (Mc Namara *et al.*, 2019). However, the focus of this study is on thrombotic CVD since fibrinogen, being a major coagulation protein, is mainly associated with thrombotic disorders.

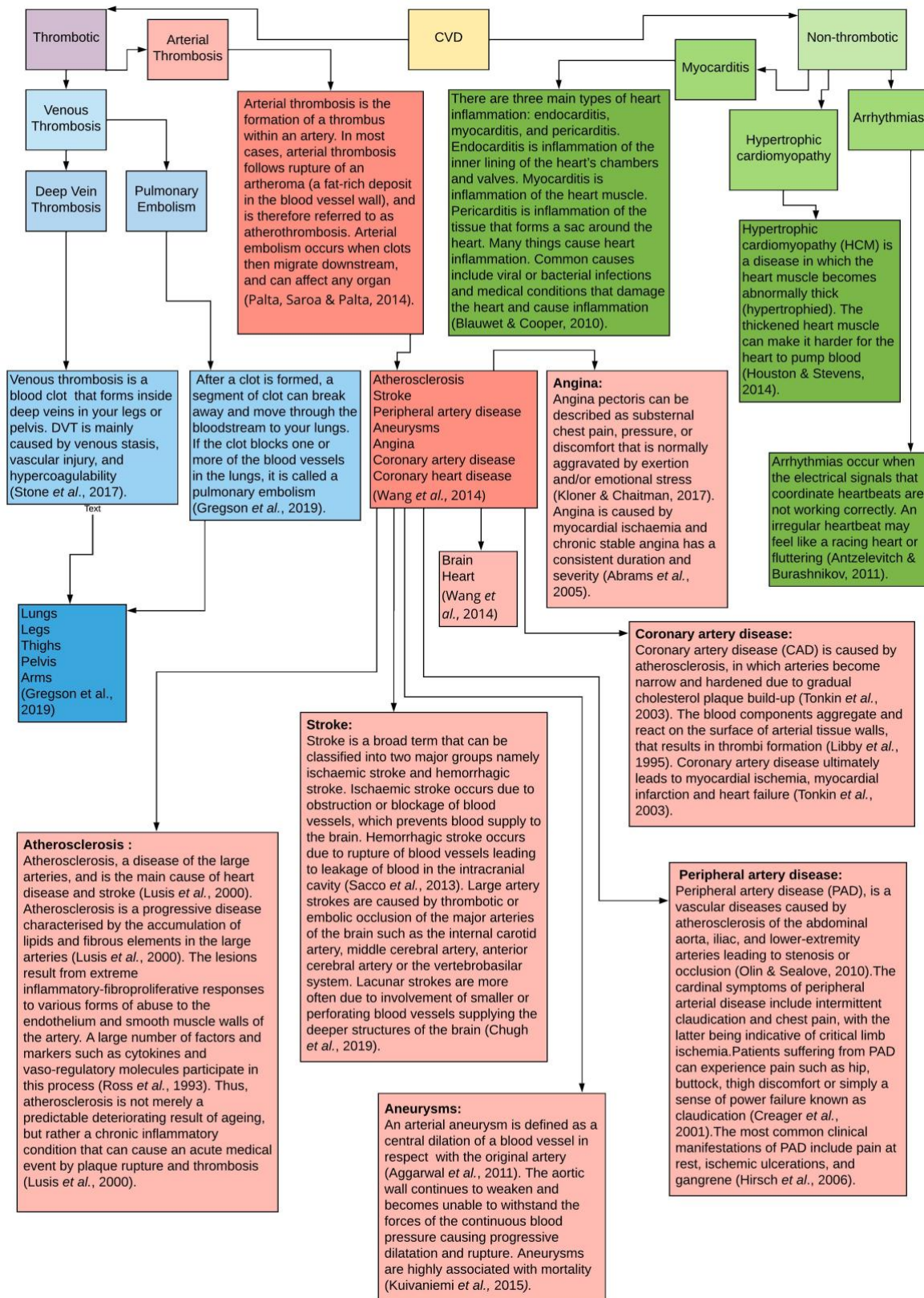


Figure 2.1 Cardiovascular disease classification

Thrombosis can be defined as the pathological formation of a blood clot and can occur within the arterial or venous systems. Thrombus formation contributes to various CVD conditions including MI, stroke, pulmonary embolism (PE) and limb ischaemia (Oklu, 2017).

Although thrombosis can occur in both the arterial and the venous systems, the aetiology of the disease differs, depending on the location. Arterial thrombosis mostly occurs after the erosion or rupture of atherosclerotic plaque (Previtali *et al.*, 2011). Cardiac ischaemia, stroke and MI are the most harmful clinical manifestations of atherothrombosis (Prandoni *et al.*, 2003; Previtali *et al.*, 2011; Yusuf *et al.*, 2004).

In contrast, venous thrombosis is mostly caused by three elements supporting thrombus formation in the veins: venous stasis, vascular injury and hypercoagulability (Stone *et al.*, 2017). Venous thrombosis (VT) can be classified into two main clinical events: deep VT (DVT) in the limbs and PE, which results from emboli breaking off from the clot formed in the limb, travelling to and occluding smaller arteries in the lungs (Previtali *et al.*, 2011).

Thrombotic events and diseases are normally attributed to an imbalance in naturally occurring blood-clotting proteins or clotting factors, which can lead to abnormalities in haemostasis (Stone *et al.*, 2017). In the next section the physiology and mechanisms will be reviewed.

## **2.4 Physiology and mechanisms of haemostasis**

### **2.4.1 The coagulation cascade**

Haemostasis is the natural protective physiological response to vascular damage that occurs when blood components are exposed to the sub-endothelial layers of the vessel wall (Mackman *et al.*, 2007). The response process that occurs when the body experiences blood loss due to injury involves four major physiological steps (Niewiarowski, 1968; Smith *et al.*, 2015):

- 1) Constriction of the blood vessel.
- 2) Formation of a temporary platelet plug.
- 3) Activation of the coagulation cascade.
- 4) Formation of the fibrin clot.

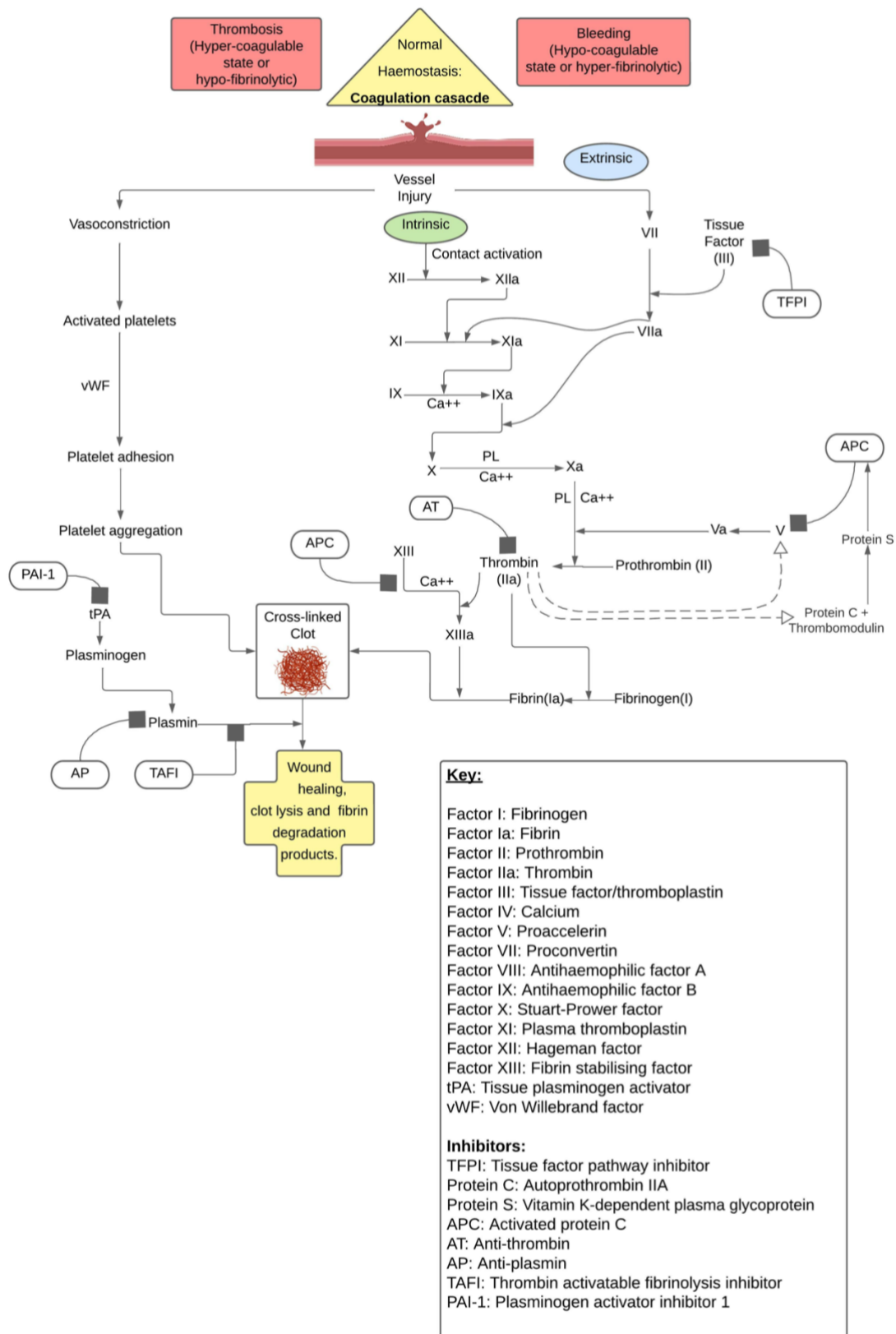


Figure 2.2 Normal physiologic mechanism of haemostasis as adapted from Adams and Bird (2009).

As presented in Figure 2.2, vasoconstriction will occur as a result of blood vessel injury and passing platelets will adhere to and collect at the injury site (Mason & Saba, 1978; Periyah *et al.*, 2017). The activation of and connection between the passing platelets and the injury site is dependent on the Von Willebrand factor (VWF) (Holthenrich & Gerke, 2018). This factor is activated by the platelet aggregation response (Periyah *et al.*, 2017). The activation of platelets results in the exposure of anionic phospholipids that serve as sites for the collection of blood coagulation enzyme complexes (DeSancho & Rand, 2003). The above-mentioned mechanism leads to the formation of a primary platelet plug (Periyah *et al.*, 2017). These coagulation complexes can form from two major pathways, namely the extrinsic and intrinsic pathways and form part of the coagulation cascade (Palta *et al.*, 2014). The most conventional perspective regarding the regulation of blood coagulation is that the initiation phase is provoked by the extrinsic pathway, while the intrinsic pathway triggers amplification (Mackman *et al.*, 2007). The extrinsic pathway comprises transmembrane receptor tissue factor (TF) and plasma factor VII/VIIa (FVII/FVIIa) while the intrinsic pathway consists of plasma FXI, FIX and FVIII (Mackman *et al.*, 2007).

#### **2.4.2 The extrinsic and intrinsic pathways**

In the extrinsic pathway, exposure of sub-endothelial TF complexed with activated FVII catalyses the production of FXa (activated FX) (Grover & Mackman, 2019).

The intrinsic pathway is a waterfall-based model (Grover & Mackman, 2019). This pathway involves the chronological activation of FXII and FXI (Grover & Mackman, 2019). FXII is converted to FXIIa through auto-activation generated by coming into contact with charged injury surfaces. FXIIa is of crucial importance for normal fibrin formation (Renné *et al.*, 2012). FXIIa together with activated FVII and TF convert FIX to FIXa (activated factor). This also catalyses the generation of FXa causing FXa in complex with its cofactor, FVa, to form the prothrombinase complex that catalyses the cleavage of prothrombin (FII) to thrombin (FIIa) (Mackman *et al.*, 2007).

However, there are limitations to the coagulation cascade model and certain clinical trials drew attention to this (Monroe & Hoffman, 2006). While these pathways play an important role in coagulation, they cannot operate as independent pathways *in vivo*, as suggested by the cascade model (Monroe & Hoffman, 2006).

In 2001, an alternative model was proposed in which coagulation is regulated by the properties of cell surfaces (Monroe & Hoffman, 2006). This model accentuates the importance of coagulation

proteins and certain cellular receptors (Hoffman & Monroe, 2001) and suggests that coagulation transpires not simply as a "cascade" but in three overlapping stages, namely initiation, amplification and propagation supported by cell surfaces (Hoffman & Monroe, 2001).

### **2.4.3 The cell-based coagulation model**

Figure 2.3 illustrates the different phases of the cell-based model. The cell-based model incorporates the role of cells in coagulation and provides an integrated understanding of haemostasis (Hoffman & Monroe, 2001). The cell-based model suggests that coagulation occurs in overlapping phases and requires the participation of two different cell types, namely TF-bearing cells and platelets (Ho & Pavey, 2017). The cell-based model illustrates that TF is not fundamentally only expressed in endothelial cells, but also surrounds the membranes of cells near the blood vessels, for instance, the smooth muscle cells and fibroblasts (Ferreira *et al.*, 2010).

The initiation phase of the model occurs when cells that express TF on their surface area are exposed to blood components at the site of injury (Ferreira *et al.*, 2010). Once TF binds to FVII in the blood, it activates the FVIIa/TF complex which controls the activation of small amounts of FIX and FX (Hoffman & Monroe, 2001; Pérez-Gómez & Bover, 2007). FXa is linked to FVa and forms a complex called prothrombinase on the cell surface (Ferreira *et al.*, 2010). Furthermore, a small amount of thrombin (FIIa) is constantly produced and activated by FXa/FVa outside the vascular space; thus, it can be assumed that some activity in the TF pathway continues in the extravascular space (Ferreira *et al.*, 2010; Hoffman & Monroe, 2001).

During the amplification phase, platelets and cofactors are stimulated to start large-scale thrombin production (Hoffman & Monroe, 2001). The thrombin generated, along with calcium, activates factors XI, IX, VIII and V in a positive feedback process (Pérez-Gómez & Bover, 2007; Smith, 2009; Swanepoel *et al.*, 2015). Additionally, the modest amounts of thrombin (FIIa) supplied from the extracellular space react with platelets and form the FVIII/VWF complex (Pérez-Gómez & Bover, 2007). The chemotactic mechanisms of these factors simultaneously arriving at the surface of platelets activate the propagating phase (Swanepoel *et al.*, 2015).

The propagation phase can be identified by the large volume of thrombin that is generated on the platelet surface (Monroe. *et al.*, 2002; Vine, 2009). Firstly, FIX binds to FVIIIa on the platelet surface, forming the tenase complex (Pérez-Gómez & Bover, 2007). A large amount of FXa is then produced directly on the platelet surface by the FIXa/FVIIIa complex (Hoffman, 2003). FXa

and FVa bind, resulting in the formation of the prothrombinase complex, which converts large amounts of prothrombin into thrombin (Hoffman, 2003; Pérez-Gómez & Bover, 2007). This large amount of thrombin is responsible for the cleavage of fibrinogen into fibrin monomers, which polymerise to eventually form a cross-linked fibrin mesh.

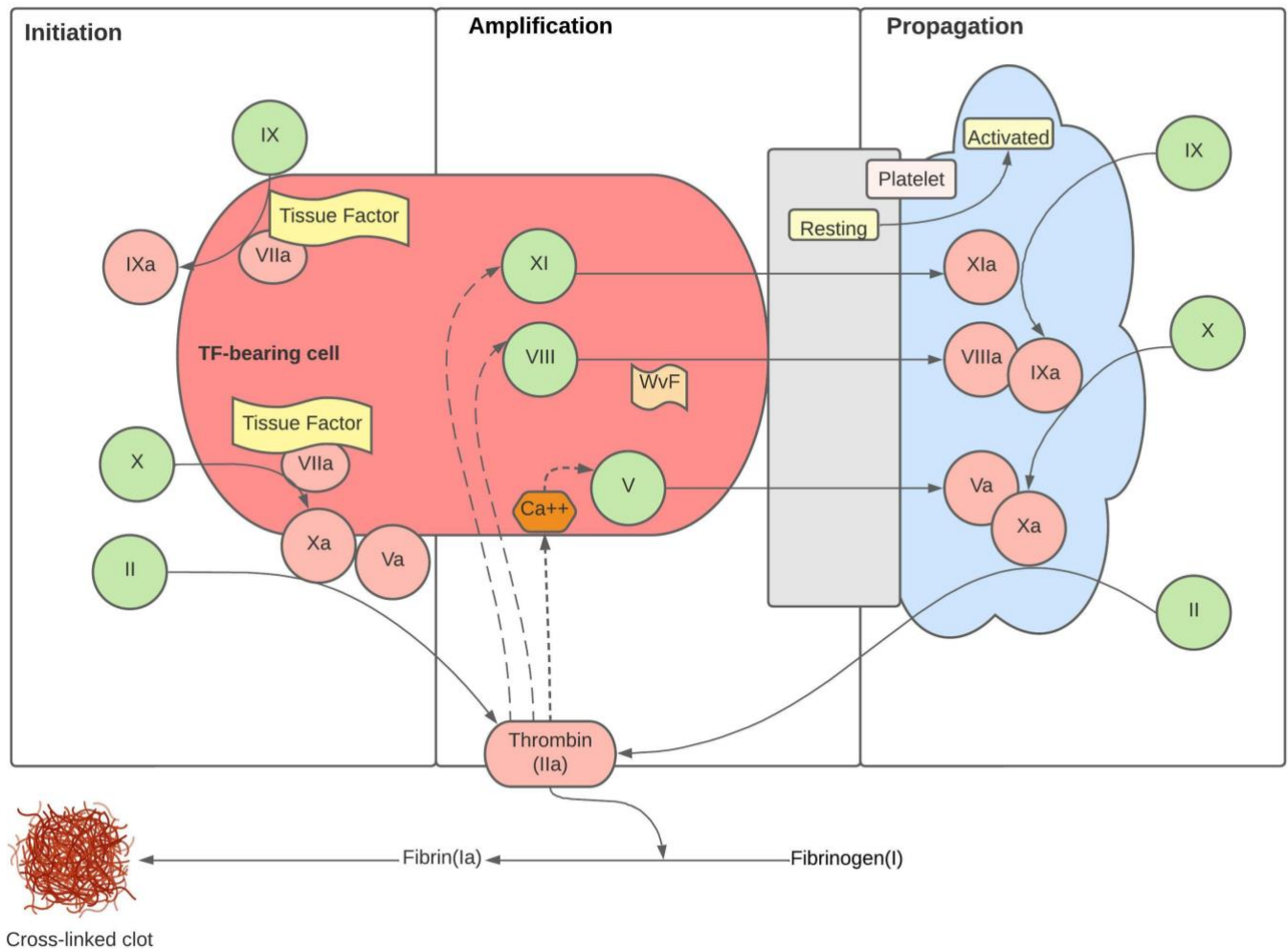


Figure 2.3 Phases of the cell-based model as adapted from Swanepoel *et al.* (2015).

Fibrinogen is a fibrous globulin-type protein found in generous amounts (1.5–4.0 g/L) in human blood plasma and is synthesised in the liver (Huseby & Bang, 1971; Weisel & Litvinov, 2017; Vilar *et al.*, 2020). It is regulated in response to inflammatory changes supporting its multifaceted functions in haemostasis and thrombosis (Pieters & Wolberg, 2019). Fibrinogen is a hexamer that comprises two sets each of A $\alpha$ -, B $\beta$ - and  $\gamma$ -chains. Each molecule contains two outer D regions connected to a central E region by a coiled-coil segment (Mosesson, 2005). The central E region

houses the N-termini of the A $\alpha$ , B $\beta$  and  $\gamma$  chains, while the C termini of the B $\beta$  and  $\gamma$  chains are situated in the D region. The C terminal of the A $\alpha$  chain folds back on the A $\alpha$  chain itself and terminates in the  $\alpha$ C domain. Figure 2.4 illustrates the composition of the fibrinogen molecule.

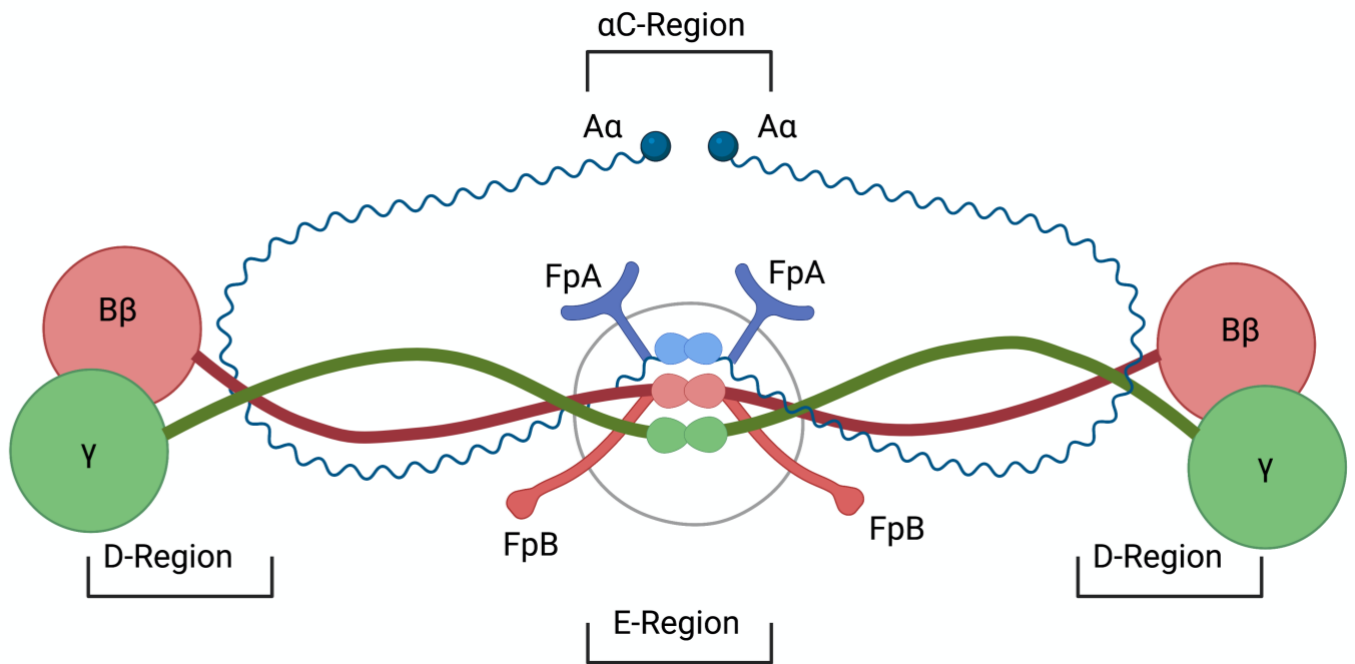


Figure 2.4 Fibrinogen molecule as adapted from Köhler *et al.* (2015). Figure created with BioRender.

Figure 2.5 illustrates the final step in coagulation where fibrinogen is polymerised to form a fibrin meshwork. Fibrinogen is activated by thrombin, which cleaves small peptides, called fibrinopeptides A and B, from the centre of the fibrinogen molecule (E-domain), converting them into fibrin monomers (Weisel, 2005). The cleavage of the fibrinopeptides permits “knob-hole” interactions amongst fibrin monomers (Chernysh *et al.*, 2012). The removal of the fibrinopeptides reveals regions on the E-domain that are called knob-A and knob-B. These knobs interact with holes a and b on the D-domain of adjacent fibrin monomers, resulting in the formation of double-stranded, half-staggered oligomers (Chamarthy, 2014). These oligomers elongate to form protofibrils with the addition of more monomers. Protofibrils then aggregate laterally to form fibrin fibres which finally, become cross-linked and insoluble by the action of FXIIIa (Chamarthy, 2014; Janmey *et al.*, 2009). FXIII crosslinks glutamine and lysine residues within and between the protofibrils (Chernysh *et al.*, 2012; Janmey *et al.*, 2009). This action causes the formation of a stabilised, insoluble clot with increased resistance to mechanical, chemical and proteolytic reactions (Chernysh *et al.*, 2012; Swieringa *et al.*, 2018). Clot properties differ significantly, depending on the circumstances during the polymerisation of fibrin (Weisel, 2007).

When the vessel injury starts to heal, the platelets contract, which internally causes the clot to shrink (Palta *et al.*, 2014). Furthermore, plasminogen then becomes activated by tissue plasminogen activator (tPA) to form plasmin, which is responsible for the lysis of the clot. As a result, this restores the flow of blood in the damaged blood vessels (Palta *et al.*, 2014).

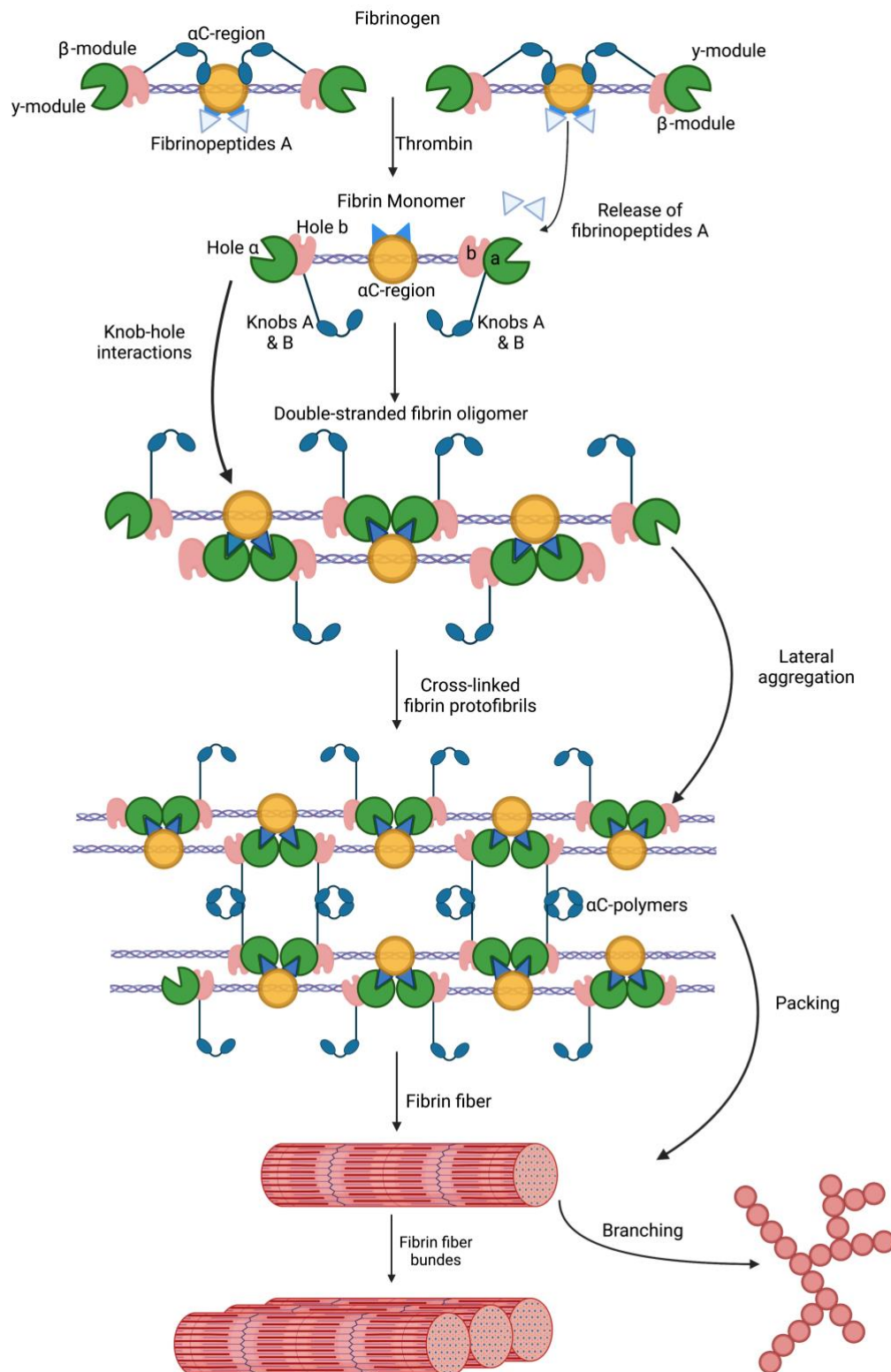


Figure 2.5 Fibrin polymerisation. The conversion of fibrinogen into fibrin monomers and protofibrils. Image compiled from Sjøland *et al.*, (2007); Weisel (2005); Weisel and Litvinov (2013). Figure created with BioRender.

#### **2.4.4 Fibrin clot properties**

Fibrin clot formation is the last step in the multifactor coagulation cascade; a mechanically stable clot is necessary to prevent blood loss and promote wound healing (Weisel & Litvinov, 2017). However, fibrin clot properties can be altered by several lifestyle and genetic factors that affect molecular interactions in the process of fibrin clot formation and in turn, affect the fibrin clot structure (Bridge *et al.*, 2014). Certain alterations can result in the clot becoming a significant contributor to the development and progression of thrombotic diseases (La Corte *et al.*, 2011b) while others can result in unwanted bleeding.

The prothrombotic fibrin clot phenotype is characterised by a combination of properties including stiff fibrin clots consisting of dense fibre networks displaying low porosity, with resistance to plasmin-mediated lysis (Collet *et al.*, 1996; Glover *et al.*, 1975; Lominadze *et al.*, 2010; Ryan *et al.*, 1999). Increasing evidence indicates that these prothrombotic clot properties are strongly associated with CVD (La Corte *et al.*, 2011a) with more recent prospective evidence pointing to a causal role (Celinska-Löwenhoff *et al.*, 2018; Cieslik *et al.*, 2018; Mrozinska *et al.*, 2019). This is discussed in the next section.

#### **2.5 Fibrin clot properties and CVD**

The probability of a thrombotic event occurring is controlled by the balance between two main processes which include prothrombotic drivers and the efficiency of the fibrinolytic system (Barrabés & Galian, 2010; Farag *et al.*, 2018; Lucking *et al.*, 2013). Cumulative research has demonstrated that reduced or compromised fibrinolytic capacity promotes thrombosis risk in patients with CVD (Farag *et al.*, 2018; Leander *et al.*, 2012; Okafor & Gorog, 2015). Similarly, studies have revealed that there is strong evidence for a consistent association between the altered, prothrombotic fibrin clot phenotype (described above) and atherothrombotic CVD disorders (Bridge *et al.*, 2014; La Corte *et al.*, 2011b; Sjøland *et al.*, 2007; Undas & Ariëns, 2011a). Studies on plasma or fibrin clot properties can provide information regarding clot formation, fibre and whole clot density, fibre branching, fibre thickness, clot porosity, the mechanical properties of fibres and whole clots as well as fibrinolysis (Bridge *et al.*, 2014).

The main consensus is that patients with CVD present with fibrin clots that form faster; have a less porous, more dense structure; have thinner and highly branched fibres, increased fibre and clot stiffness and greater resistance to fibrinolysis (Collet *et al.*, 2006; Fatah *et al.*, 1992; Sjøland *et al.*, 2007; Undas, 2017). The aforementioned clot structure variations have been seen in both

arterial and venous thrombotic diseases (Bridge *et al.*, 2014; Undas & Ariëns, 2011b). The next section provides a summary of the cross-sectional association between fibrin clot properties and CVD while Section 2.5.2 presents prospective evidence pointing to a potential causal role for prothrombotic fibrin clot properties in predicting CVD progression and severity, including mortality.

## **2.5.1 Cross-sectional evidence**

### **2.5.1.1 Coronary artery disease: myocardial infarction**

Coronary artery disease (CAD) refers to the existence of plaque in coronary arteries, supported by catheterisation or computed tomography angiography (Montalescot *et al.*, 2013). Altered clot properties such as longer clot lysis times were identified in patients with CAD in comparison with patients with no history of CAD (Collet *et al.*, 2006; Guimarães *et al.*, 2009; Kouvelos *et al.*, 2011; Meade *et al.*, 1993; Siniarski *et al.*, 2020; Undas *et al.*, 2010). When comparing stable CAD (the presence of atherosclerosis that is asymptomatic) with advanced CAD (obstructive clots with symptoms such as angina or past events), clots from patients with advanced CAD were documented to be less permeable, had longer clot lysis times and were denser than clots from stable CAD (Collet *et al.*, 2006; Siniarski *et al.*, 2020; Undas *et al.*, 2010; Undas *et al.*, 2009b). Additionally, studies have indicated that age and sex also correlate with unfavourable clot properties in patients with advanced CAD, such as patients older than 60 years of age who have decreased clot permeability and longer clot lysis times, when compared to younger controls. Furthermore, men with CAD have generally been found to have a predisposition to form denser and more compact clots than women (Siniarski *et al.*, 2020; Stępień *et al.*, 2009; Undas *et al.*, 2007). Minor clot abnormalities were also identified in close relatives of patients suffering from MI; however, these abnormalities were less severe (Mills *et al.*, 2002; Undas & Ariëns, 2011b). This suggests a genetic underpinning.

### **2.5.1.2 Ischaemic Stroke**

Ischaemic stroke, irrespective of the phase or origin, has been associated with abnormal fibrin properties (Undas *et al.*, 2009a; Undas *et al.*, 2010). In patients who have experienced cerebrovascular ischaemic events and cryptogenic stroke, it was reported that the clots presented with a less porous network, longer clot lysis time and were more compact overall, in comparison with healthy controls (Pretorius *et al.*, 2011b; Skov *et al.*, 2014). Moreover, poor clot permeability and reduced fibrinolysis have also been observed in patients suffering from acute phase (sudden onset) ischaemic stroke, suggesting that hypo-fibrinolysis is a distinctive feature of ischaemic

stroke and can be used to improve diagnosis and treatment (Rooth *et al.*, 2011; Undas & Ariëns, 2011a). Regarding age and sex, 75% of all strokes occur in individuals over 65 years old; compared to men, women tend to have a higher stroke risk, are more likely to experience repeated strokes and are more likely to have more critical strokes. Evidence regarding the clot properties of stroke patients relating to age and sex is, however, limited (Mozaffarian *et al.*, 2015).

#### **2.5.1.3 Peripheral arterial disease (PAD)**

PAD patients have been identified as having less permeable, rigid clots that are composed of thick fibres that are more resistant to lysis in comparison with healthy controls (Bhasin *et al.*, 1991; Okraska-Bylica *et al.*, 2012; Undas & Ariëns, 2011b). Characteristics such as dense fibrin clots and reduced susceptibility to lysis have also been documented in long-term follow-up participants in the context of PAD progression (Undas *et al.*, 2011). Additionally, close relatives of patients with PAD have shown similar unfavourable clot properties, indicating that fibrin characteristics in PAD may again, provide evidence of genetic regulation (Bhasin *et al.*, 2008; Parry *et al.*, 2004; Valentine *et al.*, 2000).

#### **2.5.1.4 Venous thromboembolic disorders**

In 30–50% of patients with venous thromboembolic (VTE) disorders, which includes DVT and PE, routine screening fails to identify predisposing thrombotic factors (Stone *et al.*, 2017; Undas & Ariëns, 2011b; Whiteman & Hassouna, 2000). Increasing evidence, mostly collected from case-control studies now proposes that a prothrombotic clot phenotype can be used to identify patients predisposed to VTE (Bridge *et al.*, 2014; Cieslik *et al.*, 2018; Undas & Ariëns, 2011b). Patients with VTE demonstrate increased fibrin generation and produce clots that are less permeable, denser and have increased lysis time compared to controls. Furthermore, the following studies demonstrated that relatives of VTE patients also formed clots with lower clot permeability, lower compaction, higher maximum clot absorbency and prolonged clot lysis time compared to asymptomatic controls with no history of thrombotic events (Curnow *et al.*, 2007; Meltzer *et al.*, 2010; Mrozińska *et al.*, 2018; Undas *et al.*, 2009).

Factors associated with the development of PE from DVT remain poorly understood (Martinez *et al.*, 2014). To understand the clot properties of DVT and PE patients better, Martinez *et al.* (2014) conducted a study with isolated acute DVT and PE patients. All patients were categorised into one of two groups, isolated acute DVT or PE. Patients diagnosed with both DVT and PE were classified as PE. They found that patients with DVT had less permeable, more compact clots that lysed less efficiently than plasma clots obtained from patients with PE (Martinez *et al.*, 2014;

Undas *et al.*, 2009c). In addition to the altered clot structure, Martinez *et al.* (2014) also reported altered functional and viscoelastic clot properties (documented by a decrease in lag time and an increase in the rate of viscoelastic property formation). The key differences between patients with acute DVT and PE is that *in vitro* clots from PE patients exhibit faster lysis times—possibly due to lower fibre density—as well as the earlier establishment of viscoelastic properties (Martinez *et al.*, 2014). The above-mentioned differences in clot properties between DVT and PE can potentially aid in predicting the risk of embolisation in VTE (Martinez *et al.*, 2014).

#### **2.5.1.5 Diabetes mellitus type 2**

Diabetes mellitus type 2 (DMT2) is a metabolic disorder associated with increased cardiovascular risk (Alzahrani & Ajjan, 2010). Hyperglycaemia (>10 mmol/l) in DMT2 relates to negative structural changes in the fibrin network, compared to healthy controls (Dunn *et al.*, 2005). These changes are generally considered to be the result of an increased fibrinogen concentration observed in DMT2 as well as the post-translational modification process to the fibrinogen molecule, namely glycation, which results from the increased blood glucose levels (Bryk *et al.*, 2021; Dunn *et al.*, 2005). Unfavourable clot characteristics reported in DMT2 include the formation of denser fibrin clots that are resistant to fibrinolysis (Bryk *et al.*, 2021; Dunn *et al.*, 2005; Kearney *et al.*, 2017; Konieczynska *et al.*, 2014; Neergaard-Petersen *et al.*, 2014; Pretorius *et al.*, 2015; Pretorius *et al.*, 2011a; Rollini *et al.*, 2013; Soma & Pretorius, 2015; Soma *et al.*, 2016). In addition, decreased binding of tPA, increased production of the fibrinolytic inhibitor (TAFI) and increased  $\alpha$ 2-antiplasmin (another fibrinolytic inhibitor) cross-linking contributed to the resistance of fibrin clots to lysis in DMT2 (Dunn *et al.*, 2006).

Sex-specific associations regarding clot formation have indicated that women with DMT2 have reduced fibrinolysis and denser compacted clots than men. However cardiometabolic risk factors such as vascular abnormalities, impaired renal function and smoking were correlated with prothrombotic clot structure, regardless of sex (Alzahrani *et al.*, 2012).

#### **2.5.1.6 Other diseases**

Patients suffering from end-stage renal disease (ESRD) are at high risk of developing CVD and experience chronic inflammation as part of the disease condition (Sjøland *et al.*, 2007; Yao *et al.*, 2004). Abnormal fibrin clot properties such as reduced permeability and resistance to fibrinolysis have been identified in patients suffering from both ESDR and in patients who receive chronic haemodialysis (Sjøland *et al.*, 2007; Undas *et al.*, 2008). Plasma markers of inflammation such

as CRP and IL-6 correlated notably with most of the fibrin clot structure properties (Sjøland *et al.*, 2007; Undas & Ariëns, 2011a).

Another inflammatory disorder connected to CVD is rheumatoid arthritis (RA)(Del Rincón *et al.*, 2001; Kwaśny-Krochin *et al.*, 2010; Undas *et al.*, 2009). Clot analysis from RA patients revealed denser fibrin networks that are more resistant to lysis (Bezuidenhout *et al.*, 2020; Kwaśny-Krochin *et al.*, 2010). Patients with RA also have significantly elevated levels of serum amyloid A (SAA), CRP and intercellular adhesion molecule-1 (ICAM-1) which contribute to coagulation initiation, rate of fibrin cross-linking and time to reach final thrombus size (Bezuidenhout *et al.*, 2020; Page *et al.*, 2019; Peshkova *et al.*, 2020).

### **2.5.2 Prospective studies**

While ample evidence demonstrating a cross-sectional association between CVD and the prothrombotic clot phenotype exists (as detailed above), recent evidence obtained from prospective studies also points to a potential causal role for this type of clot structure in the development and progression of CVD as well as CVD mortality (Celinska-Löwenhoff *et al.*, 2018; Cieslik *et al.*, 2018; Mrozinska *et al.*, 2019).

Celinska-Löwenhoff *et al.* (2018) investigated whether abnormal plasma clot properties are predictive of recurrent thromboembolism in antiphospholipid syndrome (APS). They found that the primary outcome (symptomatic recurrent VTE, ischaemic stroke and/or myocardial infarction) was observed in 33 (26.2%) of APS patients and in only 16 (15.2%) of the controls. Reduced clot permeability and prolonged clot lysis time predicted recurrent thromboembolic events in both APS patients and controls. The study concluded that the formation of denser fibrin networks could be a new risk factor for frequent thromboembolism in patients suffering from APS (Celinska-Löwenhoff *et al.*, 2018).

Additionally, Cieslik *et al.* (2018) investigated whether altered plasma clot properties increased the risk of recurrent DVT. This was the first study that indicated that pro-thrombotic fibrin clot phenotypes, including poor clot permeability and fibrin clots' resistance to lysis, could be a novel risk factor for DVT recurrence in 320 patients (aged 18–70 years) after anticoagulation withdrawal in a follow-up of more than three years (Cieslik *et al.*, 2018). The study concluded that a prothrombotic fibrin clot phenotype, including lower clot permeability and prolonged clot lysis time, are characteristics of patients with recurrent DVT.

Janion-Sadowska *et al.* (2019) conducted a predictive study that explored whether clot permeability can predict clinically significant outcomes (primary outcomes: transient ischaemic attack (TIA) or ischaemic stroke and secondary outcomes: nonmajor bleedings, minor bleedings, and all-cause mortality) in patients with arterial fibrillation (AF) who had been treated with rivaroxaban (Janion-Sadowska *et al.*, 2019). The study enrolled 232 of 300 screened consecutive patients with AF on rivaroxaban (20 mg/d [76.3%] or 15 mg/d [23.7%]) for at least three months. The study concluded that weaker clot networks can predispose patients to minor bleeds while reduced clot permeability in AF patients can predict thromboembolic and clinically significant bleeding events during rivaroxaban therapy. Thus, denser clot structure characterised by lower clot permeability values may be valuable in improving the evaluation of thromboembolic and bleeding dangers in patients with AF who are taking rivaroxaban.

Similarly, Mrozinska *et al.* (2019) investigated whether abnormal clot properties could predict cancer diagnoses following unprovoked VTE. In that study, 369 patients aged <70 years without malignancy were recruited during routine screening; the study determined plasma clot permeability and clot lysis time along with several prothrombotic markers two to eight months after VTE. The study concluded that patients with the prothrombotic clot phenotype (low clot permeability and increased clot lysis time) had the highest risk of a cancer diagnosis within the first three years of follow-up (Mrozinska *et al.*, 2019).

Drabik *et al.* conducted a study in 2020, using clot lysis time to predict stroke during anticoagulant therapy in patients with atrial fibrillation (AF). In this study, fibrin clots of 236 patients with AF, receiving vitamin K antagonists (VKAs) were analysed, using *ex vivo* plasma clot lysis time, a measure of global fibrinolysis, along with VWF, PAI-1 and other fibrinolytic modulators. The primary outcome of this study was ischaemic cerebrovascular events, and the secondary endpoints were death or major bleeding. The study concluded that clot lysis time was not associated with death or major and minor bleeding events but that impaired fibrinolysis did predict thromboembolic events in AF patients receiving VKA (Drabik *et al.*, 2020).

Zabczyk *et al.* (2020) investigated the role of prothrombotic fibrin clot properties in acute PE, in patients with high mortality risk. This study enrolled 126 normotensive acute PE patients (aged  $58 \pm 14$  years) and 25 age- and sex-matched healthy controls. The study concluded that longer clot lysis time was associated with an eightfold increased risk of PE-related death. Furthermore, prothrombotic fibrin clot properties were associated with a higher early mortality risk in acute PE

patients. The study suggests that these biomarkers can play a prognostic role in clinical settings (Ząbczyk *et al.*, 2020).

Furthermore, a recent long-term follow-up study by Bryk *et al.* (2021) investigated the role of plasma clot properties in incident cardiovascular mortality, in 133 DMT2 patients aged 43–83 years. Plasma fibrin clot turbidity, permeation, compaction, and the efficiency of clot lysis along with thrombin generation and fibrinolytic proteins were evaluated at the time of enrolment. The study was conducted over a median follow-up period of 72 months and cardiovascular mortality (n=16) was recorded. This study is the first to confirm that the formation of denser fibrin clots resistant to fibrinolysis could be a risk factor for long-term cardiovascular mortality in DMT2 (Bryk *et al.*, 2021).

A study by Gołąb *et al.* (2022) investigated whether impaired fibrinolysis observed in CVD is associated with long-term mortality following coronary artery bypass grafting (Gołąb *et al.*, 2022). The study population included 292 patients who had experienced planned coronary artery bypass grafting (Gołąb *et al.*, 2022). In this study, the research team measured fibrinolysis extent preoperatively as plasma clot lysis time and all-cause mortality were documented during an average follow-up period of 13.8 years (Gołąb *et al.*, 2022). The results indicated that long-term CVD mortality and all-cause mortality were associated with clot lysis time, LDL-C and CRP (to varying degrees) following a coronary artery bypass grafting; thus, indicating that hypo-fibrinolysis could be a risk factor for long-term CVD mortality and all-cause mortality in patients, following coronary artery bypass grafting surgery (Gołąb *et al.*, 2022). However, the study had various limitations such as its small sample size, death recorded without evaluating other clinical outcomes and the exclusion of confounding factors such as medication or newly diagnosed diseases (Gołąb *et al.*, 2022).

Lastly, in an *in vivo* mouse study, the causal/predictive relationship between hyperfibrinogenaemia, thrombosis and resistance to thrombolysis was studied (Machlus *et al.* 2011). The study aimed to establish whether elevated fibrinogen directly contributes to thrombosis and to recognise the mechanisms involved. The study used *in vivo* animal models to evaluate fibrinogen's effects on thrombus formation and stability. Furthermore, cell and TF-based *ex vivo* and *in vitro* techniques were used to recognise biochemical and biomechanical mechanisms by which fibrinogen controls fibrin formation, structure, and function. The study concluded that hyperfibrinogenaemia independently stimulates thrombus formation and stability via increased fibrin network density and resistance to lysis. These results establish hyperfibrinogenaemia in the

aetiology of both arterial and VT/VTE. The results from this study point to fibrin as a potential therapeutic target in the management of thrombotic pathologies (Machlus *et al.*, 2011).

## **2.6 Conclusion**

In conclusion, CVD accounted for 31% of all deaths worldwide in 2017 and is a global health concern (Mensah *et al.*, 2019). Identifying individuals susceptible to developing CVD remains difficult and traditional prediction models (Framingham/Reynolds risk scores) fail to predict 20–40% of patients at risk of CVD (Hozawa *et al.*, 2007). Thus, additional novel biomarkers such as fibrinogen concentration and functionality—measured as fibrin/plasma clot properties—have been considered to further improve the ability to identify at-risk individuals.

Although increased fibrinogen concentration and prothrombotic fibrin clot properties have been firmly established as CVD risk markers, the precise nature of this association and the causal role of fibrin(ogen) in CVD remains unclear. Most studies thus far have investigated this association cross-sectionally by comparing the clot properties of healthy individuals to that of participants with existing CVD. To date, only a limited number of studies have investigated the prospective causal role of fibrin clot properties in incident CVD and/or CVD-related mortality, with most of these investigating specific patient samples, thereby limiting the generalisability of the findings, especially at population level. Therefore, this topic remains highly relevant, especially in developing countries such as South Africa where CVD is on the rise.

Thus, to better understand the contribution of plasma clot properties and their main precursor, fibrinogen, to CVD development, progression, and disease severity (measured as mortality), our team researched the association between total and  $\gamma'$  fibrinogen concentration and plasma clot properties, measured in 2 010 apparently healthy Black South Africans, with incident CVD mortality over a 13-year follow-up period.

## **CHAPTER 3: METHODS**

### **3.1 Introduction**

Cardiovascular disease (CVD) is one of the main causes of mortality worldwide. CVD mortality is escalating in South Africa and is presently one of the country's top three causes of death. Many novel biomarkers concerning CVD have been researched and include fibrinogen concentration and fibrin/plasma clot properties. Recent evidence obtained from prospective studies points to a potential causal role for clot structure in the development and progression of CVD, although less is known regarding its role in CVD mortality. The limited available studies on mortality explored different populations/ethnic groups and were subject to a wide array of inclusion criteria whilst simultaneously being differentiated by sex, thereby hindering their ability to draw firm conclusions. Prospective evidence is still lacking, particularly on a population level. With that in mind, the researchers involved in this dissertation aimed to determine the prospective association of total and  $\gamma'$  fibrinogen concentration and plasma clot properties with incident CVD mortality, over 13 years, in a Black South African cohort.

The objectives of this study were:

- To characterise total and  $\gamma'$  fibrinogen concentration and plasma clot properties (clot formation, clot structure and clot lysis) of 2 010 apparently healthy South Africans older than 30 years.
- To obtain the CVD mortality data of the cohort over a 13-year period, using International Classification of Diseases' 10th revision (ICD-10 codes).
- To determine the prospective association of total and  $\gamma'$  fibrinogen concentration and plasma clot properties with incident CVD mortality over 13 years.

### **3.2 Ethics approval**

Ethics approval for the current study (NWU-00034-17-A1-03) was obtained from the Health Research Ethics Committee of the North-West University (NWU-HREC) (Annexure A). This study is affiliated with the PURE-SA study conducted in the North West Province, South Africa (ethics numbers: 04M10 and NWU-00016-10-A1). The study was conducted in accordance with the Declaration of Helsinki Ethical Principles and Good Clinical Practices (World Medical Association, 2004; World Medical Association, 2013).

### **3.3 Study design**

The international PURE study is a large-scale, prospective, epidemiological study aimed at investigating the relationships between lifestyle behaviours, risk factors and the incidence of non-communicable diseases in rural and urban communities across 27 low-, middle- and high-income countries, including South Africa (Teo *et al.*, 2010). The Western Cape and North West provinces are currently the only two study sites in South Africa. For this MSc mini-dissertation, the data collected in the North West province (PURE SA-NW) were used as fibrinogen and plasma clot property data is only available for the North West province site. Baseline data were collected in 2005 and follow-up data in 2010 and 2015. However, only the baseline data were included in this mini-dissertation as the relatively high attrition rate of the PURE study resulted in the sample size becoming too small to include the data collected in 2010 and 2015. Mortality data are available for all 2 010 participants up to December 2018.

#### **3.3.1 Study population**

The cohort consisted of Setswana-speaking individuals residing in Ikageng, Zonderwater, Extensions 7 and 11 (urban sites) and Ganyesa and Tlaskgameng (rural sites). Upon approval by all the stakeholders, potential participants were approached. If they gave verbal consent, a trained fieldworker would then describe the study to prospective participants in their home languages (English, Afrikaans, or Setswana).

#### **3.3.2 Recruitment**

Permission to access and recruit community members was agreed to on a judicial and goodwill basis by the South African Department of Health, Potchefstroom and Ganyesa city mayors, tribal chiefs, community leaders and household heads, respectively (Annexure B).

Fieldworkers conducted the recruitment, using a door-to-door approach, starting at randomly selected addresses in each community and speaking to each potential participant separately. The fieldworkers had no conflicts of interest and were trained before the study had commenced. They were selected from the communities and were able to communicate in the home languages of the study participants. Participants were regularly reminded that they could withdraw from the study at any stage.

Based on a power calculation published by Vorster (2002) for the Transition and Health during Urbanisation of South Africans (THUSA) study, a group of 2 000 or more participants was required

for the PURE study (Vorster, 2002). Therefore, a total of 6 000 households were contacted during recruitment. The chosen communities and households included were deemed to be stable regarding their movements in and out of the area.

### **3.3.3 Inclusion and exclusion criteria**

Eligible individuals who wanted to participate in the PURE study had to meet the following criteria: be apparently healthy, older than 30 years and not using any acute or chronic medication (apart from blood pressure medication). Individuals also had to be inclined to take part in follow-up data collection for the following ten years. Lastly, individuals were eligible to participate if they were voluntarily enthusiastic and willing to be involved in the study. Participants were included if they were able to understand the information given to them before voluntarily signing a written informed consent form. Consequently, individuals who were inebriated or not capable of being fully informed at the time of consent were not permitted to take part. The heads of households were approached before conducting the interviews and they had to approve the involvement of any household members. If participation was denied, a non-complier questionnaire would be completed and the household as a unit was omitted from the study.

Once the individual had been informed, participants had the opportunity to sign the informed consent form. After completion of informed consent documentation, the participants were asked to complete validated interviewer-based questionnaires regarding their physical and psychological health, lifestyle, socioeconomic status, and background.

The participants were well informed about the data collection dates and travel arrangements and were given all the necessary information relating to their participation in the study. Participants had the freedom to decide whether to show up and participate in the data collection. If the participant did not report for data collection, no further participation was required. Initially, 3 750 participants completed the informed consent forms and questionnaires; 2 010 individuals decided to participate further and took part in the baseline data collection. The researchers were available for questioning during data collection.

### **3.4 Data collection methods**

All of the analyses, measurements, and sample collection described in the following sections were carried out by appropriately trained personnel, including a registered nurse. In case of an emergency, medical assistance was available at all times. The procedures described above were

carried out in accordance with the revised version (2000) of the 1975 Helsinki Declaration (World Medical Association, 2004; World Medical Association, 2013).

### **3.4.1 Questionnaires (socio-demographic information)**

All questionnaires were specifically adapted and validated for use in the PURE population. Socio-demographic information was collected by contracting each household's head to complete the Family Census, the Household Questionnaire, and the Adult Questionnaire (each participant). The Family Census Questionnaire was used to record each living household member's age, relation, sex, level of education, medical problems/medication use, and smoking status, as well as the cause of death of any deceased household members. The Household Questionnaire was used to collect data on household income, amenities, water access, and sanitation. All participants completed the Adult Questionnaire, which gathered information on general demographics, tobacco and alcohol consumption habits, contraception use (women only), medication use, occupation and level of education. Urban-rural locality was reported depending on the area in which the participant resided as indicated in Section 3.3.1. Furthermore, participants were asked to complete a physical activity index questionnaire (adapted BAECKE questionnaire) and an international physical activity questionnaire to collect information about their physical activity habits (Beacke *et al.*, 1982).

### **3.4.2 Anthropometrical measurements**

Body composition is related to haemostasis and mortality and thus, was identified as a possible confounder or mediator when investigating whether fibrinogen concentration and plasma clot properties can be used to predict incident CVD mortality. All anthropometric measurements were made following the guidelines adopted at the Arlie Conference (anthropometric standardisation) (Lohman *et al.*, 1991). All the measurements were performed by qualified fieldworkers. Measurements included height to the nearest 0.1 cm (Invicta Stadiometer, IP 1465, Invicta, London, UK in 2005 and 2010; Stadiometer, Leicester height measure, Seca, Birmingham, UK in 2015); weight to the nearest 0.01 kg (Electronic scale, Precision Health Scale, A&D Company, Tokyo, Japan); and hip and waist circumferences (Holtain unstretchable metal tape in 2005 and 2010, Steel tape, Lufkin, Cooper Tools, Apex NC, USA in 2015). For all anthropometrical measurements, participants had to remove their shoes and wear minimal clothing to ensure accuracy. Privacy was ensured by performing measurements in closed separate rooms. The body mass index (BMI) formula measured in  $\text{kg/m}^2$  was used to quantify a participant's weight in

relation to their height. The formula is calculated by dividing weight (kg) by height (cm squared). The formula is expressed as:

$$BMI = \frac{Weight (kg)}{Height (m)^2}$$

### 3.4.3 Sample collection and processing

A registered nurse collected fasting blood samples (90 ml) from the antecubital vein of each participant's right arm, using sterile winged infusion sets and syringes with disposable needles. Blood collection took place from 7:00 am to 11:00 am. Upon collection, samples were taken to the on-site laboratory for centrifugation and storage. Samples were centrifuged at 2000 x g for 15 minutes within 30 minutes after collection and stored (initially at –20°C at the rural sites until being moved) at –80°C until analysis.

### 3.4.4 Plasma glucose, glycated haemoglobin, and insulin.

Haemoglobin A1c (HbA1c) was measured using the D-10 Haemoglobin Testing System (Bio-Rad, California, USA). Fasting glucose was measured with a hexokinase method using the Synchron® System(s) (Beckman Coulter Co., Fullerton, CA, USA) and reagents. An electrochemiluminescence immunoassay was used to measure serum insulin concentration on a Cobas Integra 400 (Roche Clinical System, Roche Diagnostics, Indianapolis, IN, USA).

### 3.4.5 Plasma clot properties

Blood intended for the analysis of haemostatic variables was collected in 3.8% sodium citrate tubes. Markers of plasma clot formation, structure and lysis were measured, using the global fibrinolytic assay. Tissue factor (TF) was added to plasma to form plasma clots and lysed with tissue plasminogen activator (tPA) (Lisman *et al.*, 2005). Tissue factor and tPA concentrations were modified slightly to obtain comparable clot lysis times (CLTs) of about 60 minutes in control plasma. The final concentrations in the plasma clots were 125 x diluted TF (Dade Innovin, Siemens Healthcare Diagnostics Inc., Marburg, Germany), 17 mmol/L CaCl<sub>2</sub>, 100 ng/mL tPA (Actilyse, Boehringer Ingelheim, Ingelheim, Germany) and 10 mmol/L phospholipid vesicles, (Rossix, Mölndal, Sweden) which provided a lipid surface on which the reactions took place. Turbidity curves were obtained with a Multiskan Ascent spectrophotometer at an absorbance of 405 nm and were analysed using *Origin*® software, version 8.5 (Origin lab®, 2010).

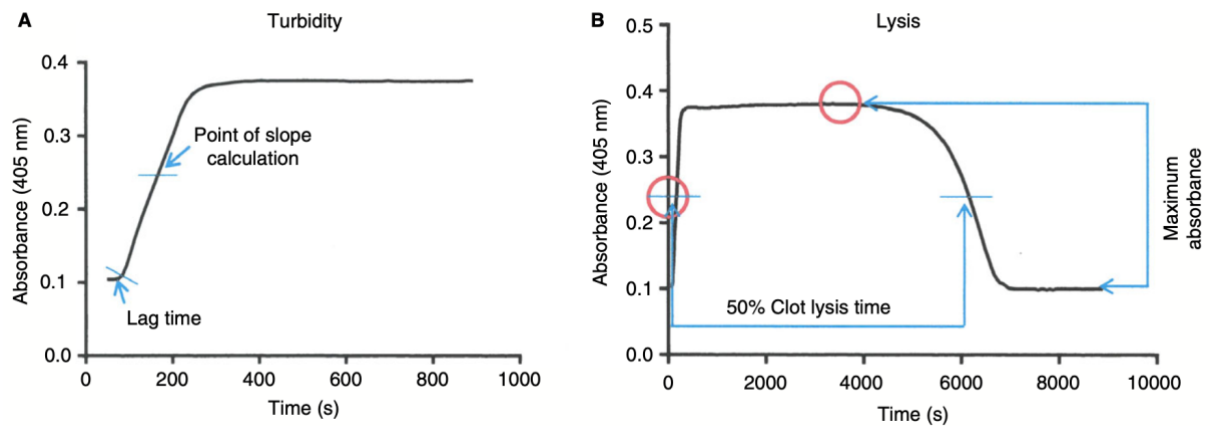


Figure 3.1 Variables calculated from turbidity curves. Image obtained from Pieters *et al.* (2018).

In Figure 3.1, the different parts of the turbidity curves are divided for clotting and lysis to reduce confusion and increase simplicity of interpretation. Observe the difference in the x-axis scale in curves A and B. Lag time (minutes) is a representation of the time required for the activation of the coagulation cascade and for fibrin protofibrils to reach lengths that are sufficient for lateral aggregation. It is calculated as the time from the onset of the measurement to the time when absorbance increased by 0.015 units. The rate of lateral aggregation is represented by the slope of the ascending turbidity curve on the side of clot formation. Maximum absorbance is an indicator of clot density and is calculated as the increase in absorbance from baseline to maximum absorbance at plateau. Clot lysis time is calculated as the time from the midpoint between baseline to maximum turbidity, to the midpoint in the transition from maximum turbidity to the final baseline turbidity (Pieters *et al.*, 2018).

### 3.4.6 Total fibrinogen concentration

Total fibrinogen concentrations were measured using the modified Clauss method on a Dade Behring BCS coagulation analyser (Multifibrin U-test Dade Behring, Deerfield, IL, USA). This analysis measures prothrombin time, where thrombin is produced rapidly, and fibrin fibre formation occurs. Functional fibrinogen was then quantified from a standard curve, using a series of diluted calibration plasma at 25%, 50% and 100%, and was expressed as g/L.

### 3.4.7 Fibrinogen $\gamma'$ concentration

An enzyme-linked immunosorbent assay (ELISA) using a Santa Cruz Biotechnology (Santa Cruz, USA) 2.G2.H9 mouse monoclonal coating antibody against the human  $\gamma'$  fibrinogen chain and an

Abcam (Cambridge, USA) goat polyclonal HRP-conjugated antibody against human fibrinogen was employed to measure fibrinogen  $\gamma'$  concentrations (De Willige *et al.* 2005). Fibrinogen  $\gamma'$  was reported as a relative concentration of total fibrinogen concentration (reported as percentage (%)  $\gamma'$  fibrinogen).

### **3.4.8 Inflammatory markers**

High sensitivity CRP and albumin were quantified in serum, using the Konelab20i™ auto-analyser (Thermo Fischer Scientific, Vantaa, Finland). The Elecsys diagnostic test was used to measure serum interleukin 6 (IL-6) through enzyme immunoassays (Elecsys 2010, Roche, Basel, Switzerland). Samples for homocysteine (Hcy) analysis was collected in EDTA tubes. The Abbott automated immunoassay analyser (AxSYM) was used to measure plasma Hcy concentrations. An indirect enzymatic method (Spectrolyze PAI-1, Trinity Biotech, Bray, Ireland) was used to quantify plasminogen activator inhibitor type-1 activity in citrated plasma (PAI-1<sub>act</sub>).

### **3.4.9 Blood lipids**

Serum lipids (triglyceride (TG), high-density lipoprotein-cholesterol (HDL-C) and total cholesterol (TC)) were quantified, using the Konelab20i™ auto-analyser (Thermo Fischer Scientific, Vantaa, Finland). Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald–Levy–Fredrickson formula (Friedewald *et al.*, 1972). Serum lipoprotein (a) (Lp(a)) concentration was measured by means of particle-enhanced immunoturbidimetry (PreciControl Lp(a) Gen.2, Roche Diagnostics, Mannheim, Germany).

### **3.4.10 Renal and hepatic function markers**

Serum, creatinine, uric acid, urea, alanine aminotransferase (ALT), aspartate aminotransferase (AST) alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) were quantified using the Konelab20i™ auto-analyser (Thermo Fischer Scientific, Vantaa, Finland). Glomerular filtration rate (GFR) was reported in mL/min/1.73m<sup>2</sup> and was calculated using the MDRD formula ( $=32788 \times \text{serum Cr}^{-1.154} \times \text{age}^{-0.203} \times 0.742$  (if female)  $\times 1.21$  (if Black)) (Levey *et al.*, 1999). Estimated glomerular filtration rate (eGFR) was also reported in mL/min/1.73m<sup>2</sup> and accounted for race, sex and age using an individual's creatinine levels (eGFR CKD-EPI(Cr) in mL/min/1.73m<sup>2</sup> if creatinine in  $\mu\text{mol/l} = 141 \times \min(\text{Scr}/\kappa, 1)^\alpha \times \max(\text{Scr}/\kappa, 1)^{-1.209} \times 0.993\text{Age} \times 1.018$  [if female]  $\times 1.159$  [if Black];  $\kappa = 61.88$  for female and 79.56 for male;  $\alpha = -0.329$  for female and  $-0.411$  for male).

### **3.4.11 Blood pressure**

Participants were resting and seated with their right arms supported at heart level for blood pressure (BP) measurements. The Omron HEM-757 (Omron Healthcare, Kyoto, Japan) was used to determine brachial systolic and diastolic BP (bSBP, bDBP) and heart rate (HR). Pulse pressure (PP) was calculated as the difference between SBP and DBP. Blood pressure was measured in duplicate and the mean was used.

### **3.4.12 Human immunodeficiency virus (HIV)**

Before and after HIV testing, qualified HIV/AIDS counsellors provided HIV guidance. Participants who indicated on their informed consent form that they wanted to receive their test results in confidence were given their results in private along with the appropriate follow-up guidance based on their results. Individuals who tested positive initially received referral letters for confirmatory testing and treatment, while those who tested negative received counselling on the importance of maintaining a negative HIV status. HIV testing was carried out in accordance with the requirements outlined in the UNAIDS/WHO Policy Statement (UNAIDS/WHO, 2004). A First Response HIV card test 1-2.0 (Transnational Technologies Inc. PMC Medical, Nani Daman, India) was used for initial HIV testing, followed by confirmatory tests with a Pareeshak card test (BHAT Bio-tech, India).

### **3.4.13 Mortality data**

First, we obtained approval from the North West Department of Health to obtain the cause of death of the PURE participants. Next, mortality status and cause of death data were obtained from the death certificates of the participants from Statistics South Africa (StatsSA). StatsSA has authority over access to the data and granted the research team access to the mortality data after each member signed a user agreement (Annexure C). Cause of death was documented by a medically qualified general practitioner and indicated on death certificates, according to the ICD-10 codes (WHO, 2015). CVD-related ICD-10 codes (I00–I99) were included to determine CVD mortality in the PURE-SA-NW study statistical analyses. In addition to CVD mortality, all-cause mortality was considered as an outcome, although this is not part of the main aim of this mini-dissertation.

### 3.5 Statistical analyses

Statistical analyses were performed with SPSS® version 27 (IBM Corp, 2015). All statistical tests were two-tailed and the type-I error rate was set to 5% ( $\alpha = 0.05$ ).

Normality was assessed by the Shapiro–Wilk test. Because most of the variables were not normally distributed, continuous data were presented as median (interquartile range, [IQR]). Non-normally distributed data were compared by using the Mann–Whitney U test or the Kruskal–Wallis analysis of variance (ANOVA) with multiple comparisons of means *post hoc* tests, depending on whether comparisons were made between two, three or more groups. For data with a normal distribution, t-tests and ANOVA with Tukey’s honest significant difference *post hoc* tests were used. Categorical data were presented as numbers (percentages and counts) and compared with Pearson’s chi-squared test.

CVD mortality was defined in two ways. First, CVD mortality was classified when the underlying cause of death was indicated as CVD (ICD-10 Codes I00–I99); this is termed CVD<sub>Underlying</sub> (n=106 cases). A second approach was to classify CVD mortality when either the underlying cause of death and/or the immediate cause of death was indicated as CVD (ICD-10 Codes I00–I99); this is termed CVD<sub>Total</sub> (n=154).

Ordinary Cox models were run to determine the contribution of the exposure variables to all-cause, CVD<sub>Underlying</sub> and CVD<sub>Total</sub> mortality, with baseline hazards defined by strata of age (below and above 55 years) and by sex. To investigate for residual confounding factors, the unadjusted estimates were compared to those derived from a multivariate, adjusted model by considering classical CVD risk factors such as ever having used alcohol, ever having smoked, education, locality of residency (urban or rural), BMI (continuous), HIV status, hypertension, CRP (continuous) and LDL-C (continuous). The hazard ratio of mortality was performed by quartiles of CLT, total fibrinogen,  $\gamma$ -fibrinogen, lag time, slope, and maximum absorbance. The linear trend of mortality over quartiles of the exposure was reported by the Wald p-value of a model having the exposure recorded as that specific midpoint of a quartile (for example, a participant in the first quartile of the exposure had the exposure recoded as the midpoint of the first quartile).

The sample size adequacy was investigated according to Schoenfeld (1983), using the *powerpi* function of the *powerSurvEpi* package of the R software version 4.0.2. The researchers confirmed that the sample size of the present study is sufficient to detect an HR above 1.3, considering a

type-I error rate of 5% ( $\alpha$ ) and a type-II error rate below 10% ( $1-\beta$  or statistical power  $> 0.9$ ). According to our power calculation, the inclusion of supplementary covariates increases the minimum HR detectable to 1.4.

Only the baseline fibrinogen and plasma clot data collected in 2005 were included in the prediction models as the relatively high attrition rate of the PURE study resulted in the sample size becoming too small to additionally include the data collected in 2010 and 2015.

The results are presented in the next chapter.

## CHAPTER 4: RESULTS

### 4.1 Introduction

The researchers involved in this mini-dissertation aimed to determine the prospective association of fibrinogen concentration and plasma clot properties with incident CVD mortality over 13 years, in a Black South African cohort. The following objectives were developed for the study. Firstly, to characterise total and  $\gamma'$  fibrinogen concentration and plasma clot properties (clot formation, clot structure and clot lysis) of apparently healthy South Africans older than 30 years, participating in the PURE-SA-NW study. Secondly, to obtain CVD mortality data of the cohort over a 13-year period, using the International Classification of Diseases' 10th revision codes (ICD-10 codes). Lastly, to determine the prospective association of total and  $\gamma'$  fibrinogen concentration and plasma clot properties with incident CVD mortality, over 13 years. Mortality data were obtained from Statistics South Africa (StatsSA) and various statistical analyses were conducted to analyse these relationships, as indicated in Chapter 3, detailing the methods employed for the study. This mini-dissertation is affiliated with the larger PURE study, using the data that had been collected in 2005 at baseline to conduct the primary statistical and descriptive analyses.

Originally, 2 010 participants were included in the PURE cohort. However, participants were removed from the dataset if they did not have complete data for the exposure variables (coagulation markers), which included total fibrinogen,  $\gamma'$  fibrinogen, lag time, slope, maximum absorbance and clot lysis time (CLT). After cleaning the dataset and excluding the participants with missing data, the final sample size was  $n=1\ 645$ .

### 4.2 Mortality trends in PURE

All-cause mortality is presented in Table 4.1 and the number of participants ( $n=499$ ) who had passed away from 2005 to 2018 is indicated. The main group classification was compiled according to the ICD-10 codes provided by StatsSA. According to the data, *Certain infectious and parasitic diseases* ( $n=144$ ) and *Diseases of the circulatory system* ( $n=106$ ) were the two main groups of causes of death in the study population. The *Diseases of the circulatory system* group reflects CVD mortality. Furthermore, it is important to note that 47 of the participants were categorised in the *Symptoms and signs not elsewhere classified (R00\_R99)* group and these participants' causes of death, therefore, remain unknown.

Table 4.1 All-cause mortality– Main groups (underlying cause of death)

| Main group                                                   | N (%)<br>n=499    |
|--------------------------------------------------------------|-------------------|
| <b>Certain infectious and parasitic diseases (A00_B99)</b>   | <b>144 (28.9)</b> |
| Neoplasms (C00_D48)                                          | 48 (9.62)         |
| Diseases of the blood and immune mechanism (D50_D89)         | 16 (3.21)         |
| Endocrine, nutritional and metabolic diseases (E00_E90)      | 22 (4.41)         |
| Mental and behavioural disorders (F00_F99)                   | 2 (0.40)          |
| Diseases of the nervous system (G00_G99)                     | 11 (2.20)         |
| <b>Diseases of the circulatory system (I00_I99)</b>          | <b>106 (21.2)</b> |
| Diseases of the respiratory system (J00_J99)                 | 60 (12.0)         |
| Diseases of the digestive system (K00_K93)                   | 16 (3.21)         |
| Diseases of the skin and subcutaneous tissue (L00_L99)       | 2 (0.40)          |
| Diseases of the musculoskeletal system, etc. (M00_M99)       | 2 (0.40)          |
| Diseases of the genitourinary system (N00_N99)               | 7 (1.40)          |
| <b>Symptoms and signs not elsewhere classified (R00_R99)</b> | <b>47 (9.42)</b>  |
| External causes of morbidity and mortality (V01_Y98)         | 16 (3.21)         |

On death certificates, two types of causes of death are coded; the first indicates the underlying cause of death, e.g., disease condition, while a second (up to a fourth) code is also provided for the immediate cause of death, e.g., the causal event. An individual can, therefore, have more than one ICD-10 code indicated on his/her death certificate. For this study, CVD mortality was investigated in two groups, namely individuals who had ICD-10 codes within the *Diseases of the circulatory system* group as the underlying cause of death (Table 4.2), termed CVD<sub>Underlying</sub> and individuals with ICD-10 codes within the *Diseases of the circulatory system* group for both underlying and immediate causes of death (Table 4.3), termed CVD<sub>Total</sub>.

Table 4.2 illustrates the participants who died of an underlying CVD cause of death (n=106) i.e., the ICD-10 codes which include any code between I00\_I99. The three most prominent underlying

CVD causes of death according to ICD-10 code broad groups were hypertensive diseases (n=37), cerebrovascular diseases (n=31) and other forms of heart disease (n=29), respectively.

Table 4.2 CVD Underlying mortality – Broad groups (underlying cause of death)

| <b>Underlying CVD broad group</b>                                                         | <b>N=106<br/>n (%)</b> |
|-------------------------------------------------------------------------------------------|------------------------|
| <b>Hypertensive diseases (I10_I15)</b>                                                    | <b>37 (34.9)</b>       |
| Ischaemic heart diseases (I20_I25)                                                        | 4 (3.78)               |
| Pulmonary heart disease and diseases of pulmonary circulation (I26_I28)                   | 2 (1.98)               |
| <b>Other forms of heart disease (I30_I52)</b>                                             | <b>29 (27.4)</b>       |
| <b>Cerebrovascular diseases (I60_I69)</b>                                                 | <b>31 (29.2)</b>       |
| Diseases of arteries, arterioles, and capillaries (I70_I79)                               | 2 (1.98)               |
| Diseases of veins, lymphatic vessels, and lymph nodes, not classified elsewhere (I80_I89) | 1 (0.94)               |

Similarly, Table 4.3 portrays the total CVD mortality (underlying and immediate) (n=154). In addition, here, the three most prominent CVD causes of death were hypertensive diseases (n=54), cerebrovascular diseases (n=41) and other forms of heart disease (n=40), respectively. Both total and underlying CVD, therefore, have the same three most prominent broad group causes of CVD death.

Table 4.3 CVD Total mortality – Broad groups (underlying and immediate cause of death)

|                                                                                           |                              |
|-------------------------------------------------------------------------------------------|------------------------------|
| <b>Total CVD broad group</b>                                                              | <b>N=154</b><br><b>n (%)</b> |
| <b>Hypertensive diseases (I10_I15)</b>                                                    | <b>54 (35.1)</b>             |
| Ischaemic heart diseases (I20_I25)                                                        | 8 (5.2)                      |
| Pulmonary heart disease and diseases of pulmonary circulation (I26_I28)                   | 5 (3.2)                      |
| <b>Other forms of heart disease (I30_I52)</b>                                             | <b>41 (26.6)</b>             |
| <b>Cerebrovascular diseases (I60_I69)</b>                                                 | <b>40 (26.0)</b>             |
| Diseases of arteries, arterioles and capillaries (I70_I79)                                | 2 (1.30)                     |
| Diseases of veins, lymphatic vessels, and lymph nodes, not classified elsewhere (I80_I89) | 3 (1.95)                     |
| Other and unspecified disorders of circulatory system (I99)                               | 1 (0.65)                     |

The descriptive data of the 1 645 participants at baseline in 2005 are presented in Table 4.4. Of these individuals, n=1 146 were survivors and n=499 had passed away (all-cause mortality). As previously mentioned, CVD mortality was divided into two main groups and included underlying CVD mortality (n=106) and total CVD mortality (n=154) (underlying and immediate).

The median age for the total sample at baseline was 48.2 years and there were more women (63%) than men (37%). Fifty-six per cent of the participants were ever tobacco users (current/previous), 43.9% were alcohol users (current/previous), 51.5% (n=841) had hypertension and 22.9% (women only) were using hormonal contraceptives. A limited number of participants in the total population were taking medication, 267 participants were using anti-hypertensive medication, 22 were using medication for diabetes and only one participant was taking lipid-lowering drugs.

### **4.3 Comparison of descriptive data at baseline between incident CVD mortality cases and survivors**

Continuous data were presented as median (interquartile range, [IQR]). The participants in the CVD<sub>(Underlying/Total)</sub> and all-cause mortality groups were older than the survivors. Proportionally, more men had passed away in all mortality groups. For both all-cause and CVD mortality (both total and underlying), proportionally more individuals were from urban areas. A higher percentage of smokers, alcohol users and lower physical activity were recorded across the board in both CVD<sub>(Underlying/Total)</sub> and all-cause mortality in comparison with survivors. WC and BMI were significantly lower in the all-cause mortality group than the survivors but did not differ between the survivors and individuals who had died of CVD. Proportionally, more individuals in all mortality groups had hypertension and/or were using antihypertensive medication, compared to survivors. Fibrinogen concentration was significantly higher in the all-cause mortality but not in the CVD mortality groups, compared to the survivors. Lag time (time to clot formation) was significantly longer in all mortality groups, maximum absorbance (clot density) was significantly higher in the CVD<sub>(Underlying/Total)</sub> groups and CLTs were shorter in all mortality groups, compared to the survivors.

The lipid profiles indicated that TC and LDL-C were significantly lower in the all-cause mortality but not in the CVD mortality groups, compared to the survivors while lipoprotein (a) (Lp(a)) was elevated in the CVD<sub>Underlying</sub> group. Only minor differences were observed between the mortality groups and survivors in terms of glucose metabolism markers. The CVD<sub>Total</sub> group had a small but statistically significant higher HbA1c than the survivors while insulin was significantly lower in the all-cause mortality group.

A significantly higher percentage of the all-cause mortality group had HIV, compared to the survivors (25.1% vs. 13.8%), while the proportion was significantly lower in the CVD<sub>Underlying</sub> group (6.6%). All inflammatory markers except albumin were significantly higher in the all-cause mortality group than in the survivors, while only interleukin-6 (IL-6), c-reactive protein (CRP) and homocysteine were higher in the CVD mortality groups. Albumin was significantly lower in all mortality groups, compared to the survivors. All liver enzymes (GGT, ALT and AST) were higher in the all-cause mortality group, compared to the survivors, while only GGT was higher in the CVD<sub>(Underlying/Total)</sub> groups. Regarding kidney function, uric acid was higher in all mortality groups and creatinine and eGFR in the CVD<sub>(Underlying/Total)</sub> groups, while urea was significantly lower in the all-cause mortality group than the survivors.

Table 4.4 Descriptive data at baseline in 2005

| Variable                                   | Total Sample<br>(n=1 645) | Survivors<br>(n=1 146) | All cause<br>(n=499) | CVD <sub>Underlying</sub><br>(n=106) | CVD <sub>Total</sub><br>(n=154) |
|--------------------------------------------|---------------------------|------------------------|----------------------|--------------------------------------|---------------------------------|
| <b>Demographic</b>                         |                           |                        |                      |                                      |                                 |
| Age (Years)                                | 48.2 (41.6; 56.0)         | 46.7 (40.9; 54.1)      | 51.6 (43.9; 59.7) *  | 57.2 (50.6; 64.5) *                  | 56.3 (50.1; 64.1) *             |
| Sex n (%)                                  |                           |                        |                      |                                      |                                 |
| Women                                      | 1 036 (63.0)              | 782 (68.2)             | 354 (50.9) *         | 59 (55.7) *                          | 83 (53.9) *                     |
| Men                                        | 609 (37.0)                | 364 (31.8)             | 245 (49.1) *         | 47 (44.3) *                          | 71 (46.1) *                     |
| Rural                                      | 883 (53.7)                | 654 (57.1)             | 229 (45.9) *         | 42 (39.6) *                          | 59 (38.3) *                     |
| Urban (n / %)                              | 762 (46.3)                | 492 (42.9)             | 270 (54.1) *         | 64 (60.4) *                          | 95 (61.7) *                     |
| <b>Lifestyle</b>                           |                           |                        |                      |                                      |                                 |
| Ever tobacco users n (%) <sup>a</sup>      | 924 (56.4)                | 601 (52.4)             | 323 (64.7) *         | 67 (63.2) *                          | 92 (59.7) *                     |
| Ever alcohol consumers (n (%) <sup>b</sup> | 722 (43.9)                | 444 (38.7)             | 278 (55.7) *         | 53.0 (50.0) *                        | 75 (48.7) *                     |
| Contraceptive use n (%) <sup>c</sup>       | 377 (22.9)                | 307 (26.8)             | 70 (14.0) *          | 13 (12.3) *                          | 19 (12.3) *                     |
| Physical activity index                    | 7.47 (5.86; 8.76)         | 7.71 (6.10; 8.86)      | 6.67 (5.52; 8.35) *  | 6.29 (5.36; 8.26) *                  | 6.29 (5.39; 8.18) *             |
| <b>Anthropometry</b>                       |                           |                        |                      |                                      |                                 |
| Waist circumference (cm)                   | 76.9 (70.2; 87.4)         | 78.2 (70.7; 88.8)      | 74.7 (69.4; 84.3) *  | 81.3 (73.4; 88.2)                    | 79.7 (72.7; 88.5)               |
| Body mass index (kg/m <sup>2</sup> )       | 22.9 (19.3; 28.5)         | 23.9 (19.8; 29.8)      | 20.9 (18.3; 25.2) *  | 23.1 (20.3; 28.0)                    | 23.0 (19.5; 27.8)               |
| <b>Blood pressure</b>                      |                           |                        |                      |                                      |                                 |
| Hypertension n (%) <sup>d</sup>            | 841 (51.5)                | 543 (47.4)             | 298 (59.7) *         | 83 (78.3) *                          | 116 (75.3) *                    |
| <b>Medication use</b>                      |                           |                        |                      |                                      |                                 |
| Hypertension drugs n (%) <sup>e</sup>      | 267 (16.2)                | 157 (13.7)             | 110 (22.0) *         | 38 (35.8) *                          | 52 (33.8) *                     |
| Lipid lowering drugs n (%) <sup>f</sup>    | 1 (0.10)                  | 1 (0.10)               | -                    | -                                    | -                               |
| Diabetes drugs n (%) <sup>g</sup>          | 22 (1.30)                 | 12 (1.00)              | 10 (2.00)            | 1 (0.90)                             | 6 (3.90) *                      |
| <b>Coagulation markers</b>                 |                           |                        |                      |                                      |                                 |
| Total fibrinogen (g/L)                     | 2.90 (2.30; 5.30)         | 2.90 (2.30; 5.00)      | 3.10 (2.30; 5.40) *  | 3.00 (2.30; 5.30)                    | 3.00 (2.30; 5.55)               |
| $\gamma'$ Fibrinogen (%)                   | 10.1 (7.13; 14.5)         | 10.2 (7.23; 14.7)      | 9.82 (6.73; 14.2)    | 8.88 (6.18; 14.7)                    | 9.22 (6.54; 14.4)               |
| Lag time (min)                             | 5.02 (3.65; 6.24)         | 4.90 (3.50; 6.12)      | 5.30 (3.89; 6.45) *  | 5.50 (3.92; 6.57) *                  | 5.54 (4.05; 6.58) *             |
| Slope (x10 <sup>-3</sup> au/s)             | 6.40 (4.00; 9.43)         | 6.50 (4.06; 9.50)      | 6.16 (3.86; 9.24)    | 6.60 (3.72; 9.17)                    | 6.14 (3.59; 9.16)               |
| Maximum absorbance ( $\Delta$ au)          | 0.44 (0.34; 0.53)         | 0.43 (0.34; 0.53)      | 0.45 (0.35; 0.54)    | 0.47 (0.38; 0.56) *                  | 0.46 (0.37; 0.56) *             |
| Clot lysis time (min)                      | 57.4 (51.1; 64.0)         | 58.0 (52.4; 64.9)      | 54.5 (47.8; 61.7) *  | 55.7 (49.0; 62.6) *                  | 55.8 (49.3; 64.4) *             |

|                                                |                   |                   |                     |                     |                     |
|------------------------------------------------|-------------------|-------------------|---------------------|---------------------|---------------------|
| <b>Lipids</b>                                  |                   |                   |                     |                     |                     |
| Total cholesterol (mmol/l)                     | 4.80 (3.99; 5.90) | 4.85 (4.05; 5.95) | 4.74 (3.87; 5.74) * | 4.87 (4.05; 6.12)   | 4.95 (4.11; 6.12)   |
| HDL-C (mmol/l)                                 | 1.41 (1.06; 1.89) | 1.41 (1.08; 1.85) | 1.42 (1.03; 1.98)   | 1.51 (1.12; 1.98)   | 1.46 (1.12; 1.93)   |
| LDL-C (Friedewald) (mmol/l)                    | 2.76 (2.05; 3.63) | 2.80 (2.10; 3.68) | 2.68 (1.94; 3.51) * | 2.82 (2.10; 3.75)   | 2.85 (2.11; 3.75)   |
| Triglycerides (mmol/l)                         | 1.06 (0.80; 1.52) | 1.06 (0.80; 1.51) | 1.07 (0.81; 1.55)   | 1.04 (0.81; 1.60)   | 1.06 (0.81; 1.69)   |
| Lipoprotein (a) (nmol/l)                       | 66.4 (30.2; 126)  | 67.9 (30.0; 125)  | 64.0 (30.3; 133)    | 81.7 (35.2; 144) *  | 72.6 (33.3; 136)    |
| <b>Glucose metabolism</b>                      |                   |                   |                     |                     |                     |
| Fasting glucose (mmol/l)                       | 4.80 (4.30; 5.30) | 4.80 (4.30; 5.20) | 4.80 (4.20; 5.30)   | 4.75 (4.10; 5.40)   | 4.90 (4.20; 5.50)   |
| Haemoglobin A1c (%)                            | 5.50 (5.30; 5.80) | 5.50 (5.30; 5.80) | 5.60 (5.20; 5.90)   | 5.60 (5.30; 5.90)   | 5.70 (5.40; 6.00) * |
| Insulin ( $\mu$ U/ml)                          | 4.00 (2.20; 7.60) | 4.30 (2.40; 7.70) | 3.40 (1.70; 6.80) * | 3.70 (2.10; 6.48)   | 3.75 (2.00; 6.90)   |
| HIV infected n (%)                             | 762 (17.2)        | 158 (13.8)        | 125 (25.1) *        | 7 (6.60) *          | 18 (11.7)           |
| <b>Inflammatory markers</b>                    |                   |                   |                     |                     |                     |
| PAI-1 <sub>act</sub> (IU/ml) <sup>h</sup>      | 4.14 (1.29; 7.80) | 4.35 (1.47; 7.66) | 3.64 (0.62; 7.90) * | 4.33 (1.08; 8.73)   | 4.30 (1.12; 8.83)   |
| Interleukin 6 (pg/ml)                          | 2.83 (0.75; 5.65) | 2.43 (0.75; 4.85) | 3.92 (1.95; 8.12) * | 3.39 (1.90; 6.53) * | 3.20 (1.91; 5.91) * |
| C-reactive protein (mg/l)                      | 3.25 (0.99; 9.30) | 3.14 (0.89; 8.60) | 3.71 (1.25; 12.5) * | 3.68 (1.61; 9.44)   | 3.92 (1.61; 11.3) * |
| Homocysteine ( $\mu$ mol/l)                    | 9.22 (7.45; 12.1) | 8.96 (7.30; 11.6) | 9.97 (8.06; 13.1) * | 10.9 (8.57; 14.1) * | 10.7 (8.49; 13.4) * |
| Albumin (g/l)                                  | 43.3 (39.5; 57.8) | 43.7 (40.1; 58.7) | 42.6 (37.7; 55.2) * | 42.6 (35.1; 54.0) * | 42.4 (37.1; 54.0) * |
| <b>Liver enzymes</b>                           |                   |                   |                     |                     |                     |
| GGT (U/l) <sup>i</sup>                         | 45.9 (29.5; 87.6) | 41.7 (28.0; 72.0) | 62.0 (34.1; 131) *  | 50.0 (29.0; 113) *  | 50.9 (30.3; 115) *  |
| Alanine transaminase (U/l)                     | 17.2 (12.0; 25.3) | 17.0 (12.0; 24.0) | 18.0 (12.0; 29.2) * | 15.3 (11.8; 23.0)   | 16.8 (12.0; 27.0)   |
| Aspartate transaminase (U/l)                   | 26.0 (19.0; 39.0) | 25.2 (18.8; 37.0) | 29.0 (20.0; 49.0) * | 24.7 (18.0; 34.8)   | 25.0 (18.3; 36.6)   |
| Alkaline phosphatase (U/l)                     | 122 (96.5; 156)   | 119 (94.5; 151)   | 128 (103; 170) *    | 121 (97.6; 152)     | 125 (101; 159)      |
| <b>Kidney function</b>                         |                   |                   |                     |                     |                     |
| Urea (nmol/l)                                  | 3.87 (3.00; 5.20) | 3.95 (3.00; 5.33) | 3.61 (3.00; 4.88) * | 3.93 (3.00; 5.50)   | 4.16 (3.00; 5.50)   |
| Creatinine ( $\mu$ mol/l)                      | 67.4 (57.7; 83.0) | 67.4 (58.4; 81.8) | 67.4 (56.4; 86.3)   | 76.3 (64.5; 262) *  | 72.4 (61.1; 104) *  |
| Uric Acid (mmol/l)                             | 0.37 (0.28; 0.53) | 0.36 (0.28; 0.52) | 0.40 (0.29; 0.59) * | 0.45 (0.33; 0.68) * | 0.47 (0.33; 0.64) * |
| eGFR (mL/min/1.73m <sup>2</sup> ) <sup>j</sup> | 118 (100; 129)    | 118 (100; 129)    | 118 (100; 130)      | 106 (86.4; 119) *   | 111 (92.0; 121) *   |

Footnote: current and previous smokers<sup>a</sup>, current and previous alcohol consumers<sup>b</sup>, hormonal contraceptive use (no vs. yes)—women only<sup>c</sup>; two groups: hypertension (hypertensive)<sup>d</sup>, two groups: hypertension drugs (BP drug use)<sup>e</sup>; two groups: cholesterol drugs (CHOL drug use)<sup>f</sup>; two groups: diabetes drugs (DM drug use)<sup>g</sup>, plasminogen activator inhibitor-1 activity (PAI-1<sub>act</sub>) <sup>h</sup>; gamma-glutamyl transferase (GGT)<sup>i</sup>; estimated glomerular filtration rate (eGFR)<sup>j</sup>; \* A p-value  $\leq$  0.05 indicates significant difference from the survivors. Continuous data were presented as median (interquartile range, [IQR]).

#### 4.4 HR of mortality and mortality trend

Table 4.5 indicates the hazard ratio (HR) of mortality and mortality trend analysis for all-cause mortality. CLT, slope (rate of lateral aggregation) and maximum absorbance (clot density) had HR's  $\geq 1$ , increasing from Q<sub>2</sub> to Q<sub>4</sub> in the first model, adjusted for age and gender only. This indicates that longer CLTs, increased rate of clot formation and higher clot density were associated with increased risk for all-cause mortality. However, upon supplementary adjustment for the use of alcohol, smoking, education, locality (urban or rural), BMI, HIV status, hypertension, CRP, HbA1c and LDL-C (Model<sub>2</sub>), the associations were no longer significant. Total fibrinogen,  $\gamma'$  fibrinogen and lag time were not associated with all-cause mortality.

Tables 4.6 and 4.7 provide the hazard ratio (HR) of mortality and mortality trend analysis for CVD<sub>Underlying</sub> and CVD<sub>Total</sub> mortality. In Model<sub>1</sub>, only total fibrinogen and clot density (maximum absorbance) had an increased risk for CVD<sub>Underlying</sub> and CVD<sub>Total</sub> mortality, respectively, adjusted for age and gender. The HR for Q<sub>4</sub> of fibrinogen compared to Q<sub>1</sub> was 1.69 and for maximum absorbance, it was 1.63. None of the coagulation markers were related to CVD mortality risk in the fully adjusted models.

The covariates, which consistently contributed significantly to all-cause mortality, in the respective models (Model<sub>2</sub>) of each of the exposure variables (CLT, total fibrinogen,  $\gamma'$  fibrinogen and lag time, slope and maximum absorbance) were BMI (HR ranging from 0.97 to 0.98), rural locality (HR: 0.75–0.79), HIV positive status (HR: 2.94–3.08) and hypertension (HR: 0.64–0.67). This indicates that lower BMI, rural locality, and hypertension at baseline were associated with lower risk for incident all-cause mortality, while a positive HIV status was a strong predictor of all-cause mortality with an average three-fold increased risk. None of the covariates that had been adjusted for contributed significantly to CVD<sub>Underlying</sub> mortality. Lastly, the most significant contributors to CVD<sub>Total</sub> mortality (consistently so in the respective models of all exposures) were locality and HIV status with rural location (HR: 0.62–0.67) and a positive HIV status (HR: 0.55–0.61), reducing incident CVD<sub>Total</sub> mortality risk.

Table 4.5 Hazard ratio of mortality and mortality trend analysis for quartiles of CLT, total fibrinogen,  $\gamma'$ -fibrinogen, lag time, slope, and maximum absorbance. Analysis based on all-cause mortality (n = 499).

|                                | Deaths | P-Years | HR <sub>1</sub> (95% CI) | HR <sub>2</sub> (95% CI) |
|--------------------------------|--------|---------|--------------------------|--------------------------|
| Clot lysis time (min)          |        |         |                          |                          |
| Q <sub>1</sub> ( $\leq 51.1$ ) | 96.0   | 4 521   | 1.00 (ref.)              | 1.00 (ref.)              |
| Q <sub>2</sub> (51.2–57.3)     | 112    | 4 521   | 1.27 (0.96; 1.67)        | 1.10 (0.81; 1.49)        |
| Q <sub>3</sub> (57.4–63.9)     | 110    | 4 532   | 1.34 (1.01; 1.77)        | 1.12 (0.82; 1.55)        |
| Q <sub>4</sub> ( $\geq 64.0$ ) | 181    | 4 521   | 1.66 (1.26; 2.18)        | 1.48 (1.07; 2.06)        |
| P-trend                        |        |         | 0.0003                   | 0.09                     |
| Total fibrinogen (g/L)         |        |         |                          |                          |
| Q <sub>1</sub> ( $\leq 2.30$ ) | 130    | 4 334   | 1.00 (ref.)              | 1.00 (ref.)              |
| Q <sub>2</sub> (2.31–2.90)     | 145    | 4 664   | 1.10 (0.87; 1.40)        | 1.00 (0.77; 1.31)        |
| Q <sub>3</sub> (2.91–5.30)     | 97     | 4 147   | 0.90 (0.69; 1.18)        | 0.79 (0.58; 1.08)        |
| Q <sub>4</sub> ( $\geq 5.31$ ) | 127    | 4 950   | 1.05 (0.81; 1.36)        | 0.91 (0.68; 1.23)        |
| P-trend                        |        |         | 0.90                     | 0.55                     |
| $\gamma'$ -fibrinogen (%)      |        |         |                          |                          |
| Q <sub>1</sub> ( $\leq 7.12$ ) | 119    | 4 499   | 1.00 (ref.)              | 1.00 (ref.)              |
| Q <sub>2</sub> (7.12–10.1)     | 123    | 4 521   | 0.98 (0.76; 1.26)        | 0.88 (0.67; 1.15)        |
| Q <sub>3</sub> (10.2–14.5)     | 122    | 4 532   | 0.91 (0.70; 1.17)        | 0.98 (0.74; 1.28)        |
| Q <sub>4</sub> ( $\geq 14.6$ ) | 135    | 4 521   | 0.85 (0.67; 1.09)        | 0.96 (0.74; 1.26)        |
| P-trend                        |        |         | 0.21                     | 0.18                     |
| Lag time (min)                 |        |         |                          |                          |
| Q <sub>1</sub> ( $\leq 3.64$ ) | 148    | 4 521   | 1.00 (ref.)              | 1.00 (ref.)              |
| Q <sub>2</sub> (3.65–5.02)     | 127    | 4 477   | 0.86 (0.68; 1.10)        | 0.79 (0.61; 1.02)        |
| Q <sub>3</sub> (5.03–6.23)     | 122    | 4 576   | 0.86 (0.67; 1.09)        | 0.85 (0.65; 1.11)        |
| Q <sub>4</sub> ( $\geq 6.24$ ) | 102    | 4 521   | 0.83 (0.65; 1.08)        | 0.86 (0.64; 1.15)        |
| P-trend                        |        |         | 0.18                     | 0.43                     |
| Slope ( $\times 10^{-3}$ au/s) |        |         |                          |                          |
| Q <sub>1</sub> ( $\leq 3.99$ ) | 132    | 4 521   | 1.00 (ref.)              | 1.00 (ref.)              |
| Q <sub>2</sub> (4.00–6.40)     | 127    | 4 521   | 0.94 (0.74; 1.21)        | 0.92 (0.70; 1.21)        |
| Q <sub>3</sub> (6.41–9.42)     | 121    | 4 532   | 1.05 (0.81; 1.35)        | 0.91 (0.68; 1.21)        |
| Q <sub>4</sub> ( $\geq 9.43$ ) | 119    | 4 521   | 1.35 (1.04; 1.75)        | 1.07 (0.79; 1.46)        |
| P-trend                        |        |         | 0.0067                   | 0.80                     |
| Max Absorbance ( $\Delta$ au)  |        |         |                          |                          |
| Q <sub>1</sub> ( $\leq 0.34$ ) | 116    | 4 499   | 1.00 (ref.)              | 1.00 (ref.)              |
| Q <sub>2</sub> (0.35–0.43)     | 122    | 4 543   | 1.01 (0.78; 1.30)        | 0.87 (0.66; 1.14)        |
| Q <sub>3</sub> (0.44–0.52)     | 128    | 4 521   | 1.17 (0.91; 1.50)        | 1.05 (0.79; 1.39)        |
| Q <sub>4</sub> ( $\geq 0.53$ ) | 133    | 4 521   | 1.46 (1.13; 1.88)        | 1.13 (0.84; 1.52)        |
| P trend                        |        |         | 0.0009                   | 0.08                     |

Note. Model<sub>1</sub> is a simple Cox model stratified by age (below or above 55 years) and sex; Model<sub>2</sub> is Model<sub>1</sub> additionally adjusted for ever use of alcohol, ever smoking, education, locality (urban or rural), BMI (continuous), HIV status, hypertension, CRP (continuous), HbA1c (continuous) and LDL-C (continuous). CI: confidence interval, HR: hazard Ratio, Q: quartile, P-Years: person years, P-trend: test for linearity trend among quartiles of exposures.

Table 4.6 Hazard ratio of mortality and mortality trend analysis for quartiles of CLT, total fibrinogen,  $\gamma'$ -fibrinogen, lag time, slope, and maximum absorbance. Analysis based on CVD<sub>Underlying</sub> deaths (n = 106)

|                                | Deaths | P-Years | HR <sub>1</sub> (95% CI) | HR <sub>2</sub> (95% CI) |
|--------------------------------|--------|---------|--------------------------|--------------------------|
| Clot lysis time (min)          |        |         |                          |                          |
| Q <sub>1</sub> ( $\leq 51.1$ ) | 24     | 4 521   | 1.00 (ref.)              | 1.00 (ref.)              |
| Q <sub>2</sub> (51.2–57.3)     | 21     | 4 521   | 1.02 (0.56; 1.84)        | 0.91 (0.47; 1.76)        |
| Q <sub>3</sub> (57.4–63.9)     | 25     | 4 532   | 1.45 (0.81; 2.60)        | 1.38 (0.72; 2.65)        |
| Q <sub>4</sub> ( $\geq 64.0$ ) | 36     | 4 521   | 1.55 (0.86; 2.79)        | 1.26 (0.61; 2.58)        |
| P-trend                        |        |         | 0.12                     | 0.50                     |
| Total fibrinogen (g/L)         |        |         |                          |                          |
| Q <sub>1</sub> ( $\leq 2.30$ ) | 26     | 4 334   | 1.00 (ref.)              | 1.00 (ref.)              |
| Q <sub>2</sub> (2.31–2.90)     | 27     | 4 664   | 1.16 (0.67; 2.00)        | 0.96 (0.52; 1.77)        |
| Q <sub>3</sub> (2.91–5.30)     | 23     | 4 147   | 1.25 (0.69; 2.26)        | 1.19 (0.62; 2.28)        |
| Q <sub>4</sub> ( $\geq 5.31$ ) | 30     | 4 950   | 1.69 (0.97; 2.92)        | 1.42 (0.75; 2.68)        |
| P-trend                        |        |         | 0.05                     | 0.18                     |
| $\gamma'$ -fibrinogen (%)      |        |         |                          |                          |
| Q <sub>1</sub> ( $\leq 7.12$ ) | 27     | 4 499   | 1.00 (ref.)              | 1.00 (ref.)              |
| Q <sub>2</sub> (7.12–10.1)     | 18     | 4 521   | 0.66 (0.36; 1.20)        | 0.66 (0.35; 1.24)        |
| Q <sub>3</sub> (10.2–14.5)     | 30     | 4 532   | 0.91 (0.53; 1.55)        | 0.86 (0.48; 1.52)        |
| Q <sub>4</sub> ( $\geq 14.6$ ) | 31     | 4 521   | 0.75 (0.44; 1.26)        | 0.81 (0.47; 1.41)        |
| P-trend                        |        |         | 0.49                     | 0.80                     |
| Lag time (min)                 |        |         |                          |                          |
| Q <sub>1</sub> ( $\leq 3.64$ ) | 33     | 4 521   | 1.00 (ref.)              | 1.00 (ref.)              |
| Q <sub>2</sub> (3.65–5.02)     | 29     | 4 477   | 0.94 (0.56; 1.57)        | 0.97 (0.57; 1.66)        |
| Q <sub>3</sub> (5.03–6.23)     | 21     | 4 576   | 0.70 (0.41; 1.22)        | 0.77 (0.42; 1.40)        |
| Q <sub>4</sub> ( $\geq 6.24$ ) | 23     | 4 521   | 0.96 (0.56; 1.66)        | 1.00 (0.53; 1.88)        |
| P-trend                        |        |         | 0.77                     | 0.91                     |
| Slope ( $\times 10^{-3}$ au/s) |        |         |                          |                          |
| Q <sub>1</sub> ( $\leq 3.99$ ) | 33     | 4 521   | 1.00 (ref.)              | 1.00 (ref.)              |
| Q <sub>2</sub> (4.00–6.40)     | 30     | 4 521   | 0.87 (0.52; 1.45)        | 0.72 (0.41; 1.26)        |
| Q <sub>3</sub> (6.41–9.42)     | 23     | 4 532   | 0.90 (0.52; 1.55)        | 0.74 (0.40; 1.37)        |
| Q <sub>4</sub> ( $\geq 9.43$ ) | 20     | 4 521   | 1.22 (0.69; 2.18)        | 1.05 (0.54; 2.06)        |
| P-trend                        |        |         | 0.40                     | 0.58                     |
| Max absorbance ( $\Delta$ au)  |        |         |                          |                          |
| Q <sub>1</sub> ( $\leq 0.34$ ) | 24     | 4 499   | 1.00 (ref.)              | 1.00 (ref.)              |
| Q <sub>2</sub> (0.35–0.43)     | 32     | 4 543   | 1.24 (0.73; 2.11)        | 1.08 (0.62; 1.89)        |
| Q <sub>3</sub> (0.44–0.52)     | 23     | 4 521   | 0.99 (0.55; 1.77)        | 0.81 (0.44; 1.49)        |
| Q <sub>4</sub> ( $\geq 0.53$ ) | 27     | 4 521   | 1.63 (0.93; 2.86)        | 1.13 (0.60; 2.12)        |
| P trend                        |        |         | 0.09                     | 0.68                     |

Note. Model<sub>1</sub> is a simple Cox model stratified by age (below or above 55 Years) and sex; Model<sub>2</sub> is Model<sub>1</sub> additionally adjusted for ever use of alcohol, ever smoking, education, locality (urban or rural), BMI (continuous), HIV status, hypertension, CRP (continuous), HbA1c (continuous) and LDL-C (continuous). HR: hazard ratio, P-Years: person years, P-trend: test for linearity trend among quartiles of exposures.

Table 4.7 Hazard ratio of mortality and mortality trend analysis for quartiles of CLT, total fibrinogen,  $\gamma'$ -fibrinogen, lag time, slope, and maximum absorbance. Analysis based on CVD<sub>Total</sub> deaths (n = 154)

|                                | Deaths | P-Years | HR <sub>1</sub> (95% CI) | HR <sub>2</sub> (95% CI) |
|--------------------------------|--------|---------|--------------------------|--------------------------|
| Clot lysis time (min)          |        |         |                          |                          |
| Q <sub>1</sub> ( $\leq 51.1$ ) | 39     | 4 521   | 1.00 (ref.)              | 1.00 (ref.)              |
| Q <sub>2</sub> (51.2–57.3)     | 29     | 4 521   | 1.02 (0.56; 1.84)        | 0.94 (0.55; 1.62)        |
| Q <sub>3</sub> (57.4–63.9)     | 34     | 4 532   | 1.45 (0.81; 2.60)        | 1.42 (0.81; 2.47)        |
| Q <sub>4</sub> ( $\geq 64.0$ ) | 52     | 4 521   | 1.55 (0.86; 2.79)        | 1.43 (0.79; 2.61)        |
| P-trend                        |        |         | 0.22                     | 0.21                     |
| Total fibrinogen (g/L)         |        |         |                          |                          |
| Q <sub>1</sub> ( $\leq 2.30$ ) | 42     | 4 334   | 1.00 (ref.)              | 1.00 (ref.)              |
| Q <sub>2</sub> (2.31–2.90)     | 37     | 4 664   | 1.16 (0.67; 2.00)        | 0.80 (0.48; 1.33)        |
| Q <sub>3</sub> (2.91–5.30)     | 34     | 4 147   | 1.25 (0.69; 2.26)        | 1.12 (0.66; 1.91)        |
| Q <sub>4</sub> ( $\geq 5.31$ ) | 41     | 4 950   | 1.69 (0.97; 2.92)        | 1.19 (0.70; 2.02)        |
| P-trend                        |        |         | 0.11                     | 0.24                     |
| $\gamma'$ -fibrinogen (%)      |        |         |                          |                          |
| Q <sub>1</sub> ( $\leq 7.12$ ) | 38     | 4 499   | 1.00 (ref.)              | 1.00 (ref.)              |
| Q <sub>2</sub> (7.12–10.1)     | 32     | 4 521   | 0.66 (0.36; 1.20)        | 0.86 (0.52; 1.41)        |
| Q <sub>3</sub> (10.2–14.5)     | 42     | 4 532   | 0.91 (0.53; 1.55)        | 1.00 (0.61; 1.62)        |
| Q <sub>4</sub> ( $\geq 14.6$ ) | 42     | 4 521   | 0.75 (0.44; 1.26)        | 0.85 (0.52; 1.37)        |
| P-trend                        |        |         | 0.20                     | 0.55                     |
| Lag time (min)                 |        |         |                          |                          |
| Q <sub>1</sub> ( $\leq 3.64$ ) | 52     | 4 521   | 1.00 (ref.)              | 1.00 (ref.)              |
| Q <sub>2</sub> (3.65–5.02)     | 42     | 4 477   | 0.94 (0.56; 1.57)        | 0.96 (0.62; 1.49)        |
| Q <sub>3</sub> (5.03–6.23)     | 30     | 4 576   | 0.70 (0.41; 1.22)        | 0.69 (0.41; 1.15)        |
| Q <sub>4</sub> ( $\geq 6.24$ ) | 30     | 4 521   | 0.96 (0.56; 1.66)        | 0.88 (0.52; 1.48)        |
| P-trend                        |        |         | 0.19                     | 0.51                     |
| Slope ( $\times 10^{-3}$ au/s) |        |         |                          |                          |
| Q <sub>1</sub> ( $\leq 3.99$ ) | 49     | 4 521   | 1.00 (ref.)              | 1.00 (ref.)              |
| Q <sub>2</sub> (4.00–6.40)     | 40     | 4 521   | 0.87 (0.52; 1.45)        | 0.72 (0.45; 1.16)        |
| Q <sub>3</sub> (6.41–9.42)     | 36     | 4 532   | 0.90 (0.52; 1.55)        | 0.83 (0.50; 1.37)        |
| Q <sub>4</sub> ( $\geq 9.43$ ) | 29     | 4 521   | 1.22 (0.69; 2.18)        | 1.04 (0.59; 1.83)        |
| P-trend                        |        |         | 0.47                     | 0.58                     |
| Max absorbance ( $\Delta$ au)  |        |         |                          |                          |
| Q <sub>1</sub> ( $\leq 0.34$ ) | 35     | 4 499   | 1.00 (ref.)              | 1.00 (ref.)              |
| Q <sub>2</sub> (0.35–0.43)     | 39     | 4 543   | 1.24 (0.73; 2.11)        | 0.90 (0.55; 1.45)        |
| Q <sub>3</sub> (0.44–0.52)     | 36     | 4 521   | 0.99 (0.55; 1.77)        | 0.85 (0.51; 1.42)        |
| Q <sub>4</sub> ( $\geq 0.53$ ) | 44     | 4 521   | 1.63 (0.93; 2.86)        | 1.34 (0.80; 2.26)        |
| P trend                        |        |         | 0.04                     | 0.19                     |

Note. Model<sub>1</sub> is a simple Cox model stratified by age (below or above 55 years) and sex; Model<sub>2</sub> is Model<sub>1</sub> additionally adjusted for ever use of alcohol, ever smoking, education, locality (urban or rural), BMI (continuous), HIV status, hypertension, CRP (continuous), HbA1c (continuous) and LDL-C (continuous). HR: hazard ratio, P-Years: person years, P-trend: test for linearity trend among quartiles of exposures.

Additionally, sensitivity analyses were conducted for thrombotic CVD conditions only and for this purpose, the data were split into two new outcome groups. The first group included only thrombotic mortality ICD-10 I-codes including I64 (unspecified stroke) and excluding haemorrhagic/hypertensive CVDs (I10–15, I60, I61, I62) vs survivors. For the second group, the same procedure was followed, except that I64 was also excluded. Because I64 is unspecified stroke, it does not distinguish between haemorrhagic and occlusive stroke and its inclusion/exclusion may, therefore, influence the results. The results did, however, not differ from the CVD<sub>Total</sub> and CVD<sub>Underlying</sub> groups (as already reported) and therefore, were not reported again.

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

## CHAPTER 5: DISCUSSION

### 5.1 Introduction

As previously reported, cardiovascular disease (CVD) is one of the foremost causes of death worldwide, contributing to 17 million deaths annually (WHO, 2018). Identifying persons at risk of developing CVD has been dependent on standard, long-established risk prediction models such as the Framingham or Reynolds risk scores (Ridker *et al.*, 2007). However, these risk scores can be ineffective in predicting CVD risk for certain individuals and thus novel biomarkers are required to improve CVD risk prediction. Fibrinogen concentration and fibrin/plasma clot properties are examples of such novel markers and are the focus of this mini-dissertation. The literature has indicated that patients with CVD generally have fibrin clots that form faster and have less porous, more dense structures; have thinner and highly branched fibres, increased fibre and clot stiffness and greater resistance to fibrinolysis (Collet *et al.*, 2006; Fatah *et al.*, 1992; Sjøland *et al.*, 2007; Undas, 2017). These characteristics contribute to CVD mortality through various mechanisms, as previously indicated in Chapter 2.

With this study the researchers aimed to determine whether total and  $\gamma'$  fibrinogen concentration and plasma clot properties are associated with incident CVD mortality in a Black South African cohort over a 13-year period. The objectives of the study were:

- To characterise total and  $\gamma'$  fibrinogen concentration and plasma clot properties (clot formation, clot structure and clot lysis) of 2 010 apparently healthy South Africans older than 30 years, participating in the Prospective Urban and Rural Epidemiology study, South Africa, North West (PURE-SA-NW) study.
- To obtain CVD mortality data of the cohort over a 13-year period using International Classification of Diseases' 10th revision (ICD-10 codes).
- To determine the prospective association of total and  $\gamma'$  fibrinogen concentration and plasma clot properties with incident CVD mortality over 13 years.

Relevant conclusions regarding the results are discussed in the following chapter. Although the results on all-cause mortality are also be interpreted, the focus of this mini-dissertation is on CVD mortality and hence, received a more detailed discussion. Furthermore, the mortality trends of the PURE cohort are compared to national data and consideration for differences is reviewed. Descriptive data at baseline which are significantly associated with incident mortality are explored as well as the possible contributions of each variable to CVD mortality. Lastly, specific focus is

placed on fibrinogen (total and fibrinogen  $\gamma$ ) and clot properties (clot lysis time (CLT), lag time, slope, and maximum absorbance) as predictors of CVD mortality.

## **5.2 Analysis of mortality trends in PURE**

The report: *Mortality and causes of death in South Africa: Findings from death notification for 2018* by Statistics South Africa (StatsSA) outlines the mortality trends in South Africa spanning 21 years from 1997 to 2018. This report indicates the most frequent causes of death in 2018, according to disease conditions. The graph below indicates the top five causes of mortality documented in the report, focusing on the underlying causes of death. According to the main group division of the ICD-10 codes, TB and HIV are both diseases which can be grouped in the *certain infectious and parasitic diseases (ICD codes: A00\_B99)* category and combined, they contributed to 10.8% of the underlying causes of mortality. Similarly, cerebrovascular diseases and other forms of heart disease can be grouped in the main group division of ICD-10 codes, *diseases of the circulatory system (I00-I99)* and together, they contribute to 10.2% of the underlying cause of mortality. Diabetes mellites (DM) is the next most common cause of death and can be grouped in the *endocrine, nutritional, and metabolic diseases (ICD10-codes: E00\_E90)* main ICD-10 code group.

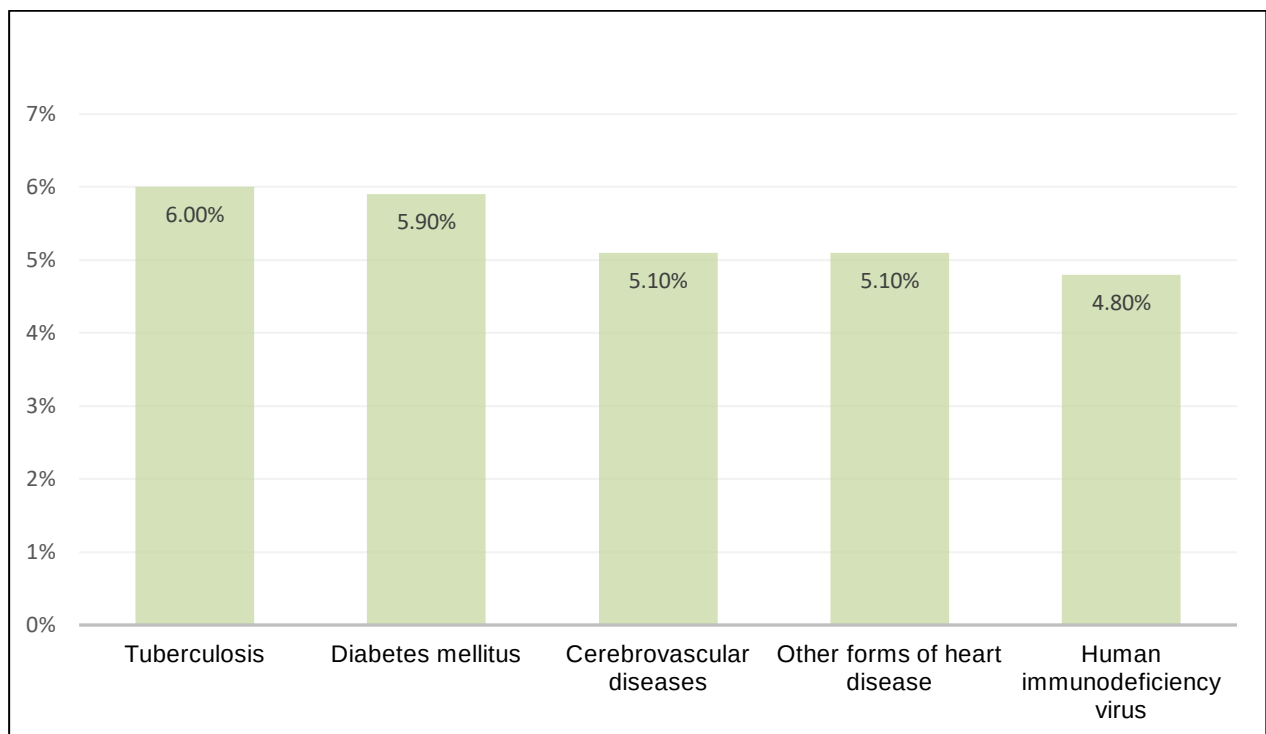


Figure 5.1 Top five leading underlying natural causes of death in 2018 (adapted from Mortality and causes of death in South Africa: Findings from death notification for 2018) (StatsSA, 2018).

The above-mentioned information includes all age groups. The median age of the mortality groups in the PURE study was 51,2 years of age, thus for a comprehensive and more accurate report the top five cause of death for the age's groups 45–64 are as follows. Ranked the highest and the third cause of death is TB (7,9%) and HIV (5,4%) respectively, both diseases which can be grouped in the *certain infectious and parasitic diseases (ICD codes: A00\_B99)* category and combined, they contributed to 13,3% of total death in the 44-64 age category. DM is the second ranked cause of death at 7,2% of deaths in this category. The fourth, fifth and sixth most common cause of death in this age category belongs to the group *diseases of the circulatory system* and include cerebrovascular diseases (5,0%), other forms of heart disease (5,0%) and hypertensive diseases (4,1%) thus contributing to a total of 14,1% of all deaths in this age category.

In the age category 65 years and above DM was the highest cause of death contributing 9,0%. The second to fifth most common cause of death were all in the category *diseases of the circulatory system* and starts with cerebrovascular diseases (8,5%), followed by hypertensive diseases (8,4%), other forms of heart disease (7,4%) and ischaemic heart diseases (4,8%).

The results found in this PURE cohort somewhat agree with the report from StatsSA (StatsSA, 2018) and various other resources (WHO, 2014; WHO, 2018) regarding the order of contribution of the different causes of death in all age groups although the size of the contribution differs. In the PURE cohort, focusing on the underlying main groups of mortality classification, *certain infectious and parasitic diseases* were also the most prominent causes of death, but with a much higher contribution at 28.8%. The second leading cause of death was *diseases of the circulatory system* at 21.2% and includes cerebrovascular and other forms of heart disease. The only prominent difference between the PURE cohort and StatSA data is that *endocrine, nutritional, and metabolic diseases*, including DM, comprised the sixth most prominent cause of death with only 4.40% of the PURE study mortality data attributed to this broad group, whereas DM is the third most prominent cause of death in all age groups South Africa at 5.90% (StatsSA, 2018). This is most likely because non-communicable diseases (NCD) such as DM were some of the exclusion criteria for the PURE cohort, thus excluding participants with prior DM diagnoses. Diseases of the respiratory system and cancer were the fourth and fifth most prominent causes of death in the PURE study. Another noteworthy observation was that 9.42% of the PURE cohort's causes of death were assigned to *symptoms and signs not elsewhere classified*. This can be a reflection of the poor quality of the collection of routine data and reflects the misclassification of mortality, especially in Black populations in SA (Anker, 1997; Kahn *et al.*, 1999).

With respect to the cardiovascular disease (CVD) broad groups, hypertensive diseases (ICD-10 codes: I10\_I15) constituted the CVD broad group (for underlying as well as total CVD) with the highest mortality rate (34.9%) in the PURE cohort (this also constituted 7.4% of all-cause mortality). This is higher than the national prevalence of 4.5% in 2018 (StatsSA, 2018). Globally, hypertensive diseases contribute to 12.8% of all deaths (WHO, 2013). A study in the United States concluded that between 2000 and 2018, hypertension-related CVD had, on average, increased by +0.5% per year (Rethy *et al.*, 2020). The relatively high incidence of hypertensive disease mortality in this study may be related to the ethnicity of the PURE population. Various studies indicated that CVD death linked to hypertension was twice as prevalent in Black populations than in their White counterparts (Carnethon *et al.*, 2017; Cooper & Rotimi, 1997; Seedat, 1983). Furthermore, in comparison with non-hypertensive individuals, hypertensive individuals have significantly higher mortality risk for all-cause and CVD mortality (Aune *et al.*, 2021; Pan *et al.*, 2020; Peer *et al.*, 2013; Wu *et al.*, 2015). Thus, concluding that the prevalence of hypertension indeed influences mortality rates (Mills *et al.*, 2020). Previous studies found that the overall

prevalence of hypertension in 17 geographically and economically varying countries was 40.8% (men, 41.4% and women, 37.7%) (Chow *et al.*, 2013; Mills *et al.*, 2020). Furthermore, low-income countries such as South Africa experienced a higher prevalence of hypertension in urban communities than rural areas (Chow *et al.*, 2013; Kandala *et al.*, 2021; Mills *et al.*, 2020). The South African National Health and Nutrition Examination Survey (SANHANES) attributed the high prevalence of hypertension in SA and other low-income countries to various reasons including treatment non-compliance, urbanisation and behavioural risk factors such as poor diet, physical inactivity and alcohol/tobacco use (Adeloye & Basquill, 2014; Kandala *et al.*, 2021).

The second highest CVD mortality broad group was cerebrovascular diseases at 29.2%. This includes non-traumatic subarachnoid, intracerebral and intracranial haemorrhage; cerebral infarction, occlusion and stenosis of precerebral and cerebral arteries not resulting in cerebral infarction, other cerebrovascular diseases, cerebrovascular disorders in diseases classified elsewhere and sequelae of cerebrovascular disease. However, the ICD-10 code I64 included in this broad group is defined as *stroke, not specified as haemorrhage or infarction*. Thus, it does not distinguish between haemorrhagic and occlusive stroke. The aetiology of these diseases differ: a haemorrhagic stroke is caused by the rupture of a blood vessel (accounts for 15–20% of acute strokes) and is often the consequence of high blood pressure, whereas occlusive stroke is caused by a thrombotic blockage or disruption of blood flow to the brain (accounts for 80–85% of all acute strokes) (Kuriakose & Xiao, 2020; Ojaghihaghighi *et al.*, 2017; Tadi & Lui, 2021). Occlusive strokes are associated with prothrombotic fibrin clot properties (increased fibrin clot density and clots that are resistant to lysis) (Pera *et al.*, 2012; Undas *et al.*, 2010), while the relationship of clot properties with haemorrhagic stroke is less clear. In participants of European descent, researchers found occlusive strokes to be ten times more likely to occur than haemorrhagic strokes (HS) but HS has a higher mortality risk (Andersen *et al.*, 2009; Bamford *et al.*, 1990; Di Carlo *et al.*, 2003; Kiyohara *et al.*, 2003; Kolominsky-Rabas *et al.*, 1998; Lauria *et al.*, 1995; Sacco *et al.*, 1982). However, in studies conducted with individuals of African descent, Black participants had relatively higher risks for HS (Lopez *et al.*, 2006; Owolabi *et al.*, 2017; Owolabi *et al.*, 2015; Thrift *et al.*, 2014). The fact that the type of stroke was not indicated on the death certificates of the PURE study participants might, therefore, have an influence on the association we found between plasma clot properties and cerebrovascular diseases (I60\_I69). Thus, to increase the validity of the results, sensitivity tests were conducted for thrombotic CVD conditions only, both including and excluding ICD-10 code I64 to determine whether or not the different types of strokes influenced the results; however, the results did not differ between the CVD<sub>Total</sub> and CVD<sub>Underlying</sub> groups and are, therefore, not discussed separately.

### **5.3 Comparison of descriptive data at baseline between incident CVD mortality cases and survivors**

#### **5.3.1 Age**

In the PURE cohort, the results indicated that CVD mortality rates (both, total and underlying) were higher in older participants. Ageing causes various functional changes in the body that can affect the likelihood of CVD mortality (Rodgers *et al.*, 2019; Yazdanyar & Newman, 2009). Physiological changes in older adults such as increased inflammation (reflected by increased levels of proinflammatory markers e.g., C-reactive protein (CRP), interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF $\alpha$ )), increased oxidative stress, increased apoptosis, and structural vascular changes (e.g., thickening of the cardiac muscle/hypertrophy) all contribute to cardiac dysfunction and deterioration (Curtis *et al.*, 2018; Meschiari *et al.*, 2017; North & Sinclair, 2012; Rodgers *et al.*, 2019; Steenman & Lande, 2017; Yazdanyar & Newman, 2009).

#### **5.3.2 Sex**

With reference to sex, the baseline results in the PURE cohort indicated that proportionally more men passed away than women. Sex differences with respect to hormones (oestrogen and testosterone) have been revealed to contribute to CVD outcomes (Pérez-López *et al.*, 2010; Phillips, 2005; Rodgers *et al.*, 2019). For example, pre-menstrual women have a generally lower prevalence of CVD in comparison to men of the same age—this is most likely due to the cardioprotective effect of oestrogen (Baker *et al.*, 2003; Iorga *et al.*, 2017; Rodgers *et al.*, 2019; Villa *et al.*, 2015; Xue *et al.*, 2009). In keeping with this, men often experience the development of CVD 10–15 years before women due to the progressive reduction of oestrogen levels following puberty (Baker *et al.*, 2003; Pérez-López *et al.*, 2010; Rodgers *et al.*, 2019). Like oestrogen, testosterone also exhibits cardioprotective properties, thus low levels of testosterone (testosterone deficiency), as is often seen in older men (due to a decrease in testosterone with age), is associated with a high incidence of CVD mortality (Araujo *et al.*, 2011; Hyde *et al.*, 2012; Jones *et al.*, 2005; Jones, 2010; Kelly & Jones, 2014; Ruige *et al.*, 2011). A potential pathway through which decreased testosterone is associated with CVD mortality is via its negative association with certain coagulation markers (plasminogen activator inhibitor-1 (PAI-1), fibrinogen and Factor VII) and thus increased thrombotic risk (Bonithon-Kopp *et al.*, 1988; Caron *et al.*, 1989; Heller *et al.*, 1982; Jeppesen *et al.*, 1996; Marques-Vidal *et al.*, 1995; Phillips *et al.*, 1994; Yang *et al.*, 1993).

### 5.3.3 Human immunodeficiency virus (HIV) status

Positive HIV status was the strongest predictor of all-cause mortality in the PURE cohort, with an average three-fold increased risk. This agrees with the finding that in the ICD-10 broad group, *certain infectious and parasitic diseases* was the leading cause of death (28.8%) in the PURE cohort. On the other hand, a positive HIV status reduced incident CVD<sub>Total</sub> mortality risk by ~ 50%. These findings should not, however, be interpreted that a positive HIV status protects against CVD mortality, but rather that individuals living with HIV more frequently pass away from causes other than CVD. For example, opportunistic diseases like tuberculosis (TB) are the most common HIV-related cause of death (Bonnet *et al.*, 2005; Chepkondol *et al.*, 2020; Mollel *et al.*, 2022; Podlekareva *et al.*, 2014; Worodria *et al.*, 2011). Furthermore, individuals living with HIV frequently experience weight loss / have poor nutritional status and research indicates that individuals living with HIV with a body mass index (BMI) <18.5 kg/m<sup>2</sup> have a significantly higher risk of all-cause (not specified) mortality (Guenter *et al.*, 1993; Khatri *et al.*, 2020; Melese *et al.*, 2017; Palenicek *et al.*, 1995; Paton *et al.*, 2006; Ssebuufu *et al.*, 2020; Süttmann *et al.*, 1995; Van der Sande *et al.*, 2004; Wheeler *et al.*, 1998). In the PURE cohort, BMI was also lower at baseline in the all-cause mortality group than in the survivors or the CVD mortality groups. Increased BMI, (discussed in Section 5.3.5) is a known risk factor for CVD, thus a lower BMI in individuals living with HIV may protect against the development of CVD.

### 5.3.4 Locality

According to the World Bank Databank and the United Nations (UN), 68% of the SA population were residing in urban locations in 2021 (Ritchie & Roser, 2018). Urban locations refer to towns, cities and suburbs, while rural areas include villages, hamlets or small towns (Pateman, 2011). In the PURE cohort, the results indicated that between 60.4% (underlying mortality) and 61.7% (total mortality) of the individuals who had died of CVD lived in urban settings. Urban locations, specifically, were also significantly associated with CVD<sub>Total</sub> mortality in the Cox regression models, with a 40% increased mortality risk. NCDs, especially CVD, are influenced by various factors which are linked not only to physiology and genetics, but also to the sociological environment and lifestyle choices (Bakilo *et al.*, 2021). In Black South Africans, urban settings have been associated with an increased prevalence of CVD due to a combination of specific risk factors related to Westernisation such as obesity, smoking, changes in dietary patterns, reduced physical activity and increased alcohol intake (Bakilo *et al.*, 2021; Bourne *et al.*, 2002; Vorster, 2002; Vorster *et al.*, 2005; Wentzel-Viljoen *et al.*, 2018; Wilna & Grobler, 2021; Wyndham, 1979).

### 5.3.5 Anthropometric variables: BMI and waist circumference (WC)

The anthropometric variables assessed in the PURE sample population were BMI and WC. Both BMI and WC are generally considered to be good indicators of CVD risk (Christian *et al.*, 2009; Flint *et al.*, 2010; Ross *et al.*, 2020; Staiano *et al.*, 2012; Tran *et al.*, 2018). However, in the PURE sample, neither BMI nor WC significantly differed between survivors and the CVD (underlying and total) mortality groups. From a statement released by the American Heart Association about cardiovascular risk in ethnically diverse populations, it was concluded that certain minority groups can have higher BMI with normal levels of adiposity and lower risk for CVD, while other groups have normal BMI with high levels of adiposity and increased CVD risk (Flegal *et al.*, 2013; Rao *et al.*, 2015; Zhu *et al.*, 2005). For example, in a study conducted comparing the BMI of different ethnic groups, the BMI values predictive of having one in three CVD risk factors differed slightly in men of different ethnicities (Black men were estimated at 23.0 kg/m<sup>2</sup>, in comparison to those of White men at 23.6 kg/m<sup>2</sup>). Concurrently, in the same study, the BMI cut-off for Black women was 23.0 kg/m<sup>2</sup>, which was significantly higher than the cut-off of 21.7 kg/m<sup>2</sup> noted for White women (Rao *et al.*, 2015; Zhu *et al.*, 2005).

In contrast, BMI and WC were both significantly lower in the all-cause mortality group than in the survivors. As alluded to in Section 5.3.3, this could be due to the high contribution of infectious diseases (25.1%) to all-cause mortality and the fact that such diseases are associated with lower BMI, poor nutrient intake, protein energy malnutrition and increased mortality rates (Alebel *et al.*, 2021; Jiang *et al.*, 2019; Koethe *et al.*, 2013; Naidoo *et al.*, 2018; Sharma *et al.*, 2015; Woodd *et al.*, 2016).

### 5.3.6 Lipid profile

Regarding the lipid profile, many studies have uncovered that unfavourable lipid profiles can lead to increased progression and severe events/outcomes in CVD (Boullart *et al.*, 2012; Dayimu *et al.*, 2019; Graham *et al.*, 2012; Orozco-Beltran *et al.*, 2017; Stampfer *et al.*, 1991; Zhao *et al.*, 2021). Increased serum low-density lipoprotein cholesterol (LDL-C) and triglycerides (TG) could have an impact on the arteries' ability to constrict and decompress through the process of atherosclerosis (Karimi *et al.*, 2000; Zhao *et al.*, 2021). Atherosclerosis is a major contributor to CVD progression and mortality, of which the pathology is multifactorial (Lusis, 2000; Malekmohammad *et al.*, 2021). Two of the most important factors which contribute to atherosclerosis are cholesterol deposition (traditionally associated with an unfavourable lipid profile: ↑ LDL-C, ↓ high-density lipoprotein cholesterol (HDL-C) and ↑ TG) and chronic inflammation (Elshourbagy *et al.*, 2014;

Malekmohammad *et al.*, 2021; Ruparelia *et al.*, 2017; Yan *et al.*, 2020). There are three identifiable stages in the atherosclerosis process: the formation of fatty streaks (initiation stage), induction of atheroma (progression) and plaque formation/rupture (clinical event) (Malekmohammad *et al.*, 2021). In the early stage of atherosclerosis, fatty deposits, known as atheromatous plaques, form in the inner layers of arteries (Rafieian-Kopaei *et al.*, 2014). The formation of these plaques begins with the deposition of small cholesterol crystals in the intima and the smooth muscle beneath it, possibly caused by endothelial dysfunction. Furthermore, it involves the modification of lipids and entry into the subintima (Libby, 2000; Malekmohammad *et al.*, 2021; Rafieian-Kopaei *et al.*, 2014; Ross, 1993). LDL-C is modified by oxidation, lipolysis, proteolysis and aggregation, and the oxidised LDL-C (Ox-LDL) is used by monocyte-macrophage differentiation to synthesise foam cells (increasing inflammation) (Blankenberg *et al.*, 2003; Burke-Gaffney *et al.*, 2002; Lusis, 2000; Poznyak *et al.*, 2021; Remmerie & Scott, 2018). The plaque grows with the rapid increase of fibrous tissue (such as foam cells) in the surrounding smooth muscle, this proliferation causes bulging inside the arteries and reduces blood flow (turbulent flow of blood). Additionally, calcium is released in the plaque core, causing hardening of the arterial wall (Lusis, 2000; Malekmohammad *et al.*, 2021; Rafieian-Kopaei *et al.*, 2014; Remmerie & Scott, 2018). As the plaque continues to grow, two factors influence the vulnerability of the plaque; namely, the formation of a necrotic core and fibrous cap thinning (Libby, 2000; Linton *et al.*, 2000; Virmani *et al.*, 2002). Vulnerable plaques result in plaque rupture, exposing procoagulant and prothrombotic factors from the necrotic core of the lesion to platelets and procoagulant factors in the lumen, finally resulting in the formation of a thrombus (Linton *et al.*, 2000; Lusis, 2000; Malekmohammad *et al.*, 2021; Rafieian-Kopaei *et al.*, 2014; Stone *et al.*, 2014; Virmani *et al.*, 2002; Zhao *et al.*, 2021). In contrast, high levels of HDL-C have been shown to be cardioprotective due to their function in regulating inflammatory response and reversing cholesterol transport (Nagao *et al.*, 2018; Voight *et al.*, 2012).

Nevertheless, in the PURE cohort, none of the lipids differed significantly between the CVD mortality groups and the survivors. Several studies have suggested that this might be due to Black South Africans' favourable serum lipid profiles (Bentley & Rotimi, 2017; Sliwa *et al.*, 2012; Truswell & Mann, 1972; Vorster, 2002; Vorster *et al.*, 2005). One possible explanation for the favourable lipid profiles may be the relationship between body fatness and serum lipids. Abdominal-visceral adipose tissue (visceral fat), more so than subcutaneous adipose tissue, is associated with increased cardiometabolic risk and an unfavourable lipid profile (increased serum TG and decreased HDL-C) (Gruzdeva *et al.*, 2018; Orozco-Beltran *et al.*, 2017; Sukkriang *et al.*, 2021). Individuals of African ancestry, however, are less likely to deposit fat in these areas

(visceral and intrahepatic) and preferentially deposit in subcutaneous adipose tissue (Bentley & Rotimi, 2017; Hosain et al., 2010; Katzmarzyk et al., 2010). In addition, the INTERHEART study found that the relative risk (reported as odds ratio (OR)) of abdominal obesity to predict CVD in Africans was less than half that in individuals of European descent (Steyn *et al.*, 2005). Furthermore, the Framingham Heart Study equations (developed based on the correlation between risk factors and CVD events) found that serum lipids were not highly predictive of CVD mortality in African American individuals (Bentley & Rotimi, 2017; Grundy *et al.*, 2001) which agrees with the findings of our study. The favourable lipid profile may also be related to ethnic differences in lipid metabolism genes. A study conducted on women of different ethnicities (in South Africa) found that the protective lipid profile in Black women (compared to White women) was associated with polymorphisms of the lipoprotein lipase (LPL) and cholesteryl ester transfer protein (CETP) (lipid metabolism) genes (Ellman *et al.*, 2015).

### **5.3.7 Inflammatory markers: IL-6, CRP and homocysteine**

Concerning the inflammatory markers, the PURE CVD mortality groups (underlying and total) presented with higher levels of CRP and IL-6 than the survivors. Various sources have indicated that inflammation is a key attributor to CVD onset, progression and severity among others, through its contribution to atherosclerosis (Abrams, 2005; Aulin *et al.*, 2015; Gager *et al.*, 2020; Harris *et al.*, 1999; Kaura *et al.*, 2022; Løfblad *et al.*, 2021; Luc *et al.*, 2003; Ridker *et al.*, 2018; Strandberg & Tilvis, 2000; Volpato *et al.*, 2001; Zimmermann *et al.*, 1999). As mentioned in Section 5.3.5, atherosclerosis has a multifactorial physiology and inflammation forms an integral part of its onset and progression (Falk *et al.*, 1995; Libby *et al.*, 2018; Shah & Lecis, 2019). Inflammation contributes to atherosclerosis by promoting several mechanisms including the upregulation/amplification of cytokines, which modulate plaque formation and growth; the destabilisation of collagen, affecting the structural stability of the fibrous cap; and increased plaque calcification. These mechanisms contribute to plaque rupturing and increase the mechanical stress of thrombi leading to CVD events/mortality (Alfaddagh *et al.*, 2020; Hansson *et al.*, 2015; Kelly-Arnold *et al.*, 2013; Kojima *et al.*, 2017; Krystel-Whittemore *et al.*, 2016; Libby & Ridker, 2006; McLaren *et al.*, 2011; Proudfoot *et al.*, 2000; Ramji & Davies, 2015).

Similar to CRP and IL-6, homocysteine was significantly higher in the CVD mortality groups than the survivors in the PURE cohort. Higher levels of homocysteine have been associated with CVD mortality related to thrombotic events; however, a causal role has not yet been confirmed (Boushey *et al.*, 1995; Finch & Joseph, 2010; Nygård *et al.*, 1997; Peng *et al.*, 2015; Stampfer & Verhoef, 1997; Ueland *et al.*, 1992). Potential pathways through which homocysteine can

contribute to thrombosis include the inhibition of anticoagulant pathways, an increase in tissue factor, a decrease in nitric oxide and increased epithelial damage—all causing arteriosclerotic plaque formation (Balint *et al.*, 2020; Eberhardt *et al.*, 2000; Esse *et al.*, 2019; Harker *et al.*, 1976; Harker *et al.*, 1974; Liu *et al.*, 2012; Welch *et al.*, 1997). Furthermore, in a meta-analysis conducted by Peng *et al.* in 2015, from European countries reporting data of 23 623 participants, the study found that elevated homocysteine levels significantly increased CVD mortality risk by 68% (relative risk (RR) 1,68; 95% confidence interval (CI): 1.04–2.70) (Peng *et al.*, 2015).

### **5.3.8 Inflammatory markers: Albumin**

In contrast to the abovementioned, the albumin levels in the CVD mortality groups of the PURE cohort were significantly lower than those of the survivors. Serum albumin is a negative acute-phase protein and is, therefore, inversely related to inflammation (Ahmed *et al.*, 2012; Chien *et al.*, 2017; Ronit *et al.*, 2020). Several studies have indicated that low serum albumin is associated with CVD mortality and may even be causal (Ahmed *et al.*, 2012; Chien *et al.*, 2017; Danesh *et al.*, 1998; Gillum & Makuc, 1992; Klonoff-Cohen *et al.*, 1992; Kuller *et al.*, 1991; Manolis *et al.*, 2022; Phillips *et al.*, 1989; Shaper *et al.*, 2004). Low levels of albumin can contribute to CVD pathogenesis in various ways but two mechanisms have been directly linked to CVD development: reduced cholesterol transport (hypertriglyceridaemia) and chronic inflammation (synthesis of positive acute-phase proteins rather than albumin in the inflammatory response) (Bhatt *et al.*, 2019; Fasano *et al.*, 2005; Gillum & Makuc, 1992; Gopal *et al.*, 2010; Ridker *et al.*, 2017). Albumin also acts as an antioxidant (reducing oxidative stress) and has been demonstrated to have antithrombotic properties (Ahmed *et al.*, 2012; Høstmark, 2003; Paar *et al.*, 2017).

### **5.3.9 Liver enzymes: Gamma-glutamyl transferase (GGT)**

In the PURE sample, GGT was the most notable liver enzyme that was significantly higher in the CVD groups than the survivors. Several prospective studies have investigated the potential independent role of GGT in CVD mortality, however, results are inconsistent (Breitling *et al.*, 2011; Haring *et al.*, 2009; Kengne *et al.*, 2012; Koehler *et al.*, 2014; Ruttman *et al.*, 2005; Sung *et al.*, 2015; Wang *et al.*, 2017b; Wannamethee *et al.*, 2008). With respect to CVD mortality, GGT could be connected through various mechanisms contributing to atherosclerosis and include the formation of atherosclerotic plaques, development of the fibrous cap, plaque erosion and rupture, increased platelet aggregation, oxidation of LDL-C and increased production of inflammatory markers (Bradley, 2016; Emdin *et al.*, 2005; Jiang *et al.*, 2013; Kengne *et al.*, 2012; Koehler *et al.*

*al.*, 2014; Ndrepepa & Kastrati, 2016). Additionally, elevated GGT could be an initial marker of oxidative/cellular stress and could be linked to the pathogenesis of endothelial dysfunction (an early characteristic of the atherothrombotic process) (Bradley, 2016; Lee *et al.*, 2004; Yilmaz *et al.*, 2013).

#### **5.3.10 Kidney function: Uric acid**

In the PURE cohort, uric acid was significantly higher in all mortality groups than survivors. Various studies indicated that significantly higher serum uric acid was observed in all-cause and CVD mortality (Kleber *et al.*, 2015; Kuo *et al.*, 2012; Li *et al.*, 2016; Muiesan *et al.*, 2016; Rahimi-Sakak *et al.*, 2019). Elevated uric acid is a biomarker associated with kidney disease. Patients with normal kidney function have a lower risk of CVD mortality than patients with abnormal kidney function (Go *et al.*, 2004; Luo *et al.*, 2019; Matsushita *et al.*, 2010). Even though the exact mechanism is not well understood, elevated uric acid could contribute to atherosclerosis through the promotion of vascular smooth muscle cell proliferation (due to the release of pro-inflammatory, pro-oxidative and vasoconstrictive compounds) (Corry *et al.*, 2008; Kanellis *et al.*, 2003; Kang *et al.*, 2005; Kimura *et al.*, 2021; Muiesan *et al.*, 2016). Furthermore, Li *et al.* (2016) conducted a meta-analysis in adult participants with 29 prospective studies and found that coronary artery or heart disease (CAD or CHD) mortality increased with every 1 mg/dL increase in uric acid (RR 1.02 in males and 2.44 in females) (Li *et al.*, 2016).

#### **5.4 Relationship of total and $\gamma'$ fibrinogen and plasma clot properties at baseline with incident CVD mortality**

The main findings in the Cox regression models and the mortality trend analysis for all-cause mortality were that longer clot lysis times (CLTs) increased the rate of clot formation and higher clot density was associated with increased risk of all-cause mortality in the minimally adjusted models. The associations did not, however, remain significant after full adjustment for known CVD risk markers. In terms of CVD<sub>Underlying</sub> and CVD<sub>Total</sub> mortality, total fibrinogen concentration and clot density were associated with an increased CVD mortality risk; in the initial model, however, after full adjustment, the coagulation markers no longer contributed significantly to CVD mortality risk. These findings are discussed in the following sections.

##### **5.4.1 Total fibrinogen concentration**

To date, many studies have examined the association of fibrinogen with CVD risk prediction and mortality (Danesh *et al.*, 2005; Graversen *et al.*, 2016; Kengne *et al.*, 2013; Mora *et al.*, 2006;

Peters *et al.*, 2013; Kaptoge *et al.*, 2012; Veeranna *et al.*, 2013; Woodward *et al.*, 2009; Yang *et al.*, 2017). Numerous pathophysiologic mechanisms have been proposed to explain the potential causal role of fibrinogen in CVD onset, progression, and mortality. These include increased platelet activation, elevated blood viscosity, altered fibrin clot structure (prothrombotic), increased systemic inflammation, and enhanced endothelial activation (De Moerloose *et al.*, 2010; Ernst & Resch, 1993; Luyendyk *et al.*, 2019; Pulanić & Rudan, 2005; Torpy *et al.*, 2009). The central conclusion obtained from the literature that investigated the association between fibrinogen and CVD—despite variations in inclusion criteria, ethnic groups, sex or the additional inclusion of other disease conditions—was that fibrinogen concentration significantly contributed to CVD events/outcomes (mortality as well as occurrence/reoccurrence of non-fatal events)(Benderly *et al.*, 1996; Kaptoge *et al.*, 2012; Toss *et al.*, 1997; Wang *et al.*, 2017a; Woodward *et al.*, 2009; Yang *et al.*, 2017). A number of these studies found that elevated total fibrinogen was independently associated with CVD risk/events/mortality (Danesh *et al.*, 2005; Kannel *et al.*, 1992; Kaptoge *et al.*, 2012; Veeranna *et al.*, 2013; Woodward *et al.*, 2009; Yang *et al.*, 2017), also after adjustment for known CVD risk markers. For example, the National Health and Nutrition Examination Survey (NHANES), an ongoing study, used fibrinogen data collected between 1999 and 2002 and found that fibrinogen mediated the relationship between existing CVD at baseline and incident CVD mortality and that fibrinogen was one of only three risk markers (fibrinogen, osmolality and GGT) of the 22 biomarkers investigated which significantly contributed to incident CVD mortality after adjustment for established CVD risk markers (Pieters *et al.*, 2021). In contrast, some of these studies found that after adjusting for traditional risk markers, total fibrinogen no longer contributed to CVD risk prediction independently, questioning its causal role (Kengne *et al.*, 2013; Wattanakit *et al.*, 2005). One of these studies, the ARIC study (atherosclerosis risk in communities) conducted with 766 participants (70 women and 577 men) with previous cardiovascular events (CHD or stroke) found that the association of fibrinogen with the risk of recurrent CVD events/mortality was positive but not statistically significant (similar results to those obtained from our study) (Wattanakit *et al.*, 2005). Thus, indicating that fibrinogen may not be an independent risk factor for CVD mortality. Possible reasons for the discrepancy in the literature regarding fibrinogen's causal contribution to CVD might be that some of the risk prediction studies were conducted with participants who had no history of CVD at baseline (Danesh *et al.*, 2005; Peters *et al.*, 2013; Kaptoge *et al.*, 2012; Veeranna *et al.*, 2013; Woodward *et al.*, 2009), while others included individuals with pre-existing CVD at baseline (Pieters *et al.*, 2021; Wattanakit *et al.*, 2005; Yang *et al.*, 2017). Another factor to consider is that the outcomes of the different studies differed, potentially affecting the results. Some of the studies focused on CVD mortality (Danesh

*et al.*, 2005; Graversen *et al.*, 2016; Kengne *et al.*, 2013; Peters *et al.*, 2013; Wang *et al.*, 2017a) while others included a combination of non-fatal (first or recurrent) and fatal CVD events (Mora *et al.*, 2006; Kaptoge *et al.*, 2012; Wattanakit *et al.*, 2005; Yang *et al.*, 2017). Another important discussion point to consider is that in the PURE cohort, no differentiation was made between thrombotic and non-thrombotic CVD mortality. In the PURE study, 'CVD mortality' was used as an umbrella term and included all forms of CVD, regardless of the aetiology of the disease. Previous studies suggest that haemostatic and inflammatory markers such as fibrinogen may have a stronger association with thrombotic CVD events in comparison to non-thrombotic events (Empana *et al.*, 2008; Francis *et al.*, 1980; Lowe, 2011; Wannamethee *et al.*, 2009). As mentioned previously in Chapter 4 (Results), we attempted to do sensitivity testing for thrombotic CVD mortality only but found that the results did not differ from that of the overall CVD mortality. It should also be kept in mind that the number of thrombotic CVD mortality cases was rather limited, which has power implications, prohibiting firm conclusions to be drawn from this data.

#### **5.4.2 Fibrinogen $\gamma'$ concentration**

In the PURE cohort results, fibrinogen  $\gamma'$  did not significantly contribute to CVD mortality (total/underlying) in either of the HR models (Model<sub>1</sub>/Model<sub>2</sub>). Fibrinogen  $\gamma'$  is a fibrinogen subtype (chain variant) which is caused by alternative splicing of intron 9 during mRNA processing, resulting in a 16 amino acid extension of the fibrinogen  $\gamma$  chain (Appiah *et al.*, 2015; Ariëns, 2013; Chung & Davie, 1984; Fornace Jr *et al.*, 1984; Francis *et al.*, 1980; Wolfenstein-Todel & Mosesson, 1980). Fibrinogen  $\gamma'$  influences clot structure through interfering with fibrin polymerisation and protofibril formation, resulting in clots with thinner, tightly interknit fibres, and a heterogeneous network structure affecting clot density (Ariëns, 2013; Cooper *et al.*, 2003; Falls & Farrell, 1997). Additionally, the compromised clot structure is consequently more resistant to lysis (Ariëns, 2013; Carlisle *et al.*, 2010; Collet *et al.*, 2000; Cooper *et al.*, 2003; Gersh *et al.*, 2009; Kim *et al.*, 2014; Mutch *et al.*, 2003). As a result, several studies have investigated the cross-sectional association of fibrinogen  $\gamma'$  with CVD and found a positive link between fibrinogen  $\gamma'$  concentration and arterial thrombotic events/conditions (e.g., myocardial infarction [MI] and CAD), independent of total plasma fibrinogen levels (Cheung *et al.*, 2008; Drouet *et al.*, 1999; Lovely *et al.*, 2002; Lovely *et al.*, 2011; Mannila *et al.*, 2007). In contrast, decreased levels have been linked to venous thrombosis (e.g., pulmonary embolism [PE]) (Ariëns, 2013; Cheung *et al.*, 2008; Duval & Ariëns, 2017; Lovely *et al.*, 2002; Macrae *et al.*, 2016; Mannila *et al.*, 2007; Uitte de Willige *et al.*, 2005).

To date, only one prospective study has been conducted to investigate the prospective relationship between fibrinogen  $\gamma'$  and CVD. A study by Appiah *et al.* (2015) on plasma samples collected between 1993 and 1995 in 10 601 adults found that fibrinogen  $\gamma'$  was associated with CHD, ischaemic stroke (IS), peripheral artery disease (PAD), heart failure (HF), and CVD mortality (18-year follow-up) in unadjusted analysis but after adjustment for traditional risk markers for CVD, total fibrinogen and CRP the significance of the association was drastically weakened (Appiah *et al.*, 2015). These authors concluded that fibrinogen  $\gamma'$  does not contribute independently to CVD outcomes (all CVD mortality) (Appiah *et al.*, 2015). Similar to the study by Appiah *et al.* (2015), the CVD outcomes of this mini-dissertation did not differentiate between arterial and venous thrombosis (different pathophysiology), possibly explaining why fibrinogen  $\gamma'$  did not associate with incident CVD mortality.

#### **5.4.3 Plasma clot structure and properties**

The results obtained for clot properties in the PURE cohort indicated that of the plasma clot properties measured, only clot density contributed to an increased risk of CVD mortality (in the unadjusted model). In the fully adjusted models, none of the clot properties remained as independent predictors of CVD mortality.

The prothrombotic fibrin clot phenotype is distinguished by several characteristics, including stiff fibrin clots composed of dense fibre networks with low porosity and resistance to plasmin-mediated lysis (Collet *et al.*, 1996; Glover *et al.*, 1975; Lominadze *et al.*, 2010; Ryan *et al.*, 1999). The cross-sectional relationship between the prothrombotic phenotype and CVD has been researched extensively and results have indicated a strong association between prothrombotic clot properties and CVD events (myocardial infarction (MI), CAD, IS, PAD, venous thromboembolism [VTE]) in comparison with healthy controls (Bhasin *et al.*, 1991; Bridge *et al.*, 2014; Cieslik *et al.*, 2018; Collet *et al.*, 2006; Guimarães *et al.*, 2009; Kouvelos *et al.*, 2011; Meade *et al.*, 1993; Okraska-Bylica *et al.*, 2012; Pretorius *et al.*, 2011; Siniarski *et al.*, 2020; Skov *et al.*, 2014; Stępień *et al.*, 2009; Undas & Ariëns, 2011; Undas *et al.*, 2007; Undas *et al.*, 2010b).

As discussed in detail, in Section 2.5.2 of Chapter 2 (Literature), recent evidence (albeit limited), proposes a likely causal association between prothrombotic clot properties and CVD events/outcomes, including mortality. These prospective studies, however, investigated distinct CVD patient samples and were subject to a variety of inclusion criteria while being differentiated by sex, limiting their capacity to draw well-founded conclusions that could be extended to the broader public. However, the general observation made throughout the literature was that a

prothrombotic clot phenotype indeed contributed to an increased risk of thrombotic events as well as mortality (CVD and all-cause) (Bryk *et al.*, 2021; Celinska-Löwenhoff *et al.*, 2018; Cieslik *et al.*, 2018; Janion-Sadowska *et al.*, 2019).

In the fully adjusted model in the PURE cohort, none of the clot properties remained independent predictors of CVD mortality. These results question the independent, causal role of clot properties in CVD mortality. However, key differences between the PURE cohort and the available literature are that the previous studies that investigated the prospective association of clot properties with CVD, studied hospitalised patients with strict inclusion criteria and specific diagnoses while the PURE study investigated the contribution of clot properties on total CVD mortality (i.e., not defined/selected CVD conditions) in a free-living population. As a result, various additional factors present in the epidemiological setting such as rural/urban living conditions (one of the main predictors of mortality), could contribute to CVD mortality, possibly overshadowing the contribution of fibrinogen concentration and plasma clot properties. Lastly, limited to no research (especially prospectively) has been conducted on this specific population (Black Africans) and the loss of significance may be connected to ethnic characteristics (genetic or lifestyle factors contributing to haemostatic profile). Additional points of consideration are discussed in the limitations section.

## **5.5 Strengths of the study**

- The mortality data are novel for the PURE study. Even though the original baseline data was collected in 2005, the mortality data was not available until 2022. Thus, for the first time, associations can be made with all-cause and CVD mortality.
- The prospective study design used in this study is better equipped to analyse risk prediction, compared to cross-sectional studies that can only identify association. Even though causality cannot be proven, it provides new insights into the timing and order of events.
- Limited research has been conducted on the haemostatic profile of Africans, who are considered an under-studied population in terms of CVD aetiology. This study, therefore, provides novel findings regarding the haemostatic profiles of Africans.
- This study reports epidemiological data on a population level. Many other studies are restricted to randomised control cases in hospital settings with specific exposures/outcomes, restricting the generalisability of results.

## 5.6 Limitations

While this data significantly added to the documentation of evidence with respect to the possible causal role of fibrinogen concentration and plasma clot properties to CVD mortality, the study had several limitations:

- The attrition rate of the PURE study was relatively high. Consequently, only baseline data collected in 2005 could be included in the analysis and prevented the use of follow-up data collected in 2010 and 2015. This prevented the study from identifying the possible contribution of changes in fibrinogen and plasma clot properties over time to incident CVD mortality.
- The use of a single data point at baseline and the long duration between data collection and events might have weakened the observed relationships. Most other prospective studies follow-up more frequently and thus have data collected closer to participants' deaths/events. Being an acute-phase protein, fibrinogen levels may vary over the short term due to acute infections, which may have implications for its role in long-term risk prediction.
- CVD classification and CVD mortality is classified via ICD-10 codes. Even though the codes provide sufficient information on broad groups for mortality the system remains broad on certain disease conditions e.g., stroke and the aetiology of diseases e.g., occlusive vs haemorrhagic.

## 5.7 Conclusion

CVD risk prediction has been mainly dependent on traditional risk models (e.g., the Framingham risk score and the Reynolds risk score) but these models alone have frequently failed to predict CVD in certain individuals (Hozawa *et al.*, 2007; Ridker *et al.*, 2007). Thus, to improve CVD risk prediction, novel biomarkers that can influence CVD onset/development/outcomes and include coagulation markers have been researched. Fibrinogen performs a vital role in blood coagulation as a key structural component of a thrombus (Danesh *et al.*, 2005; Kaptoge *et al.*, 2012; Koenig, 2003; Krobot *et al.*, 1992; Mora *et al.*, 2006; Pieters *et al.*, 2021; Wang *et al.*, 2017). The general agreement in the literature is that the prothrombotic clot phenotype presents as clots that form more quickly; have less porous, denser structures; have thinner and highly branched fibres; are stiffer than normal and are more resistant to lysis (Collet *et al.*, 2006; Fatah *et al.*, 1992; Sjøland *et al.*, 2007; Undas, 2017). The results of this study indicate that total fibrinogen and clot density contributed to an increased risk of incident CVD mortality before but not after adjustment for

known CVD risk markers, suggesting that they likely do not confer additional, independent risk on an epidemiological level in this Black South African cohort. While a (causal) relationship has been suggested in highly controlled case-control studies, the contribution of fibrinogen and plasma clot properties in the context of other CVD risk factors on an epidemiological level appears to be less prominent.

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## ANNEXURES

### 1. Annexure A:



Private Bag X1290, Potchefstroom  
South Africa 2520

Tel: 086 016 9698  
Web: <http://www.nwu.ac.za/>

**North-West University Health Research Ethics  
Committee (NWU-HREC)**

Tel: 018 299-1206  
Email: [Ethics-HRECAppl@nwu.ac.za](mailto:Ethics-HRECAppl@nwu.ac.za) (for human  
studies)

22 March 2022

## ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Health Research Ethics Committee (NWU-HREC) on 22/03/2022, the NWU-HREC hereby approves your study as indicated below. This implies that the NWU-HREC grants its permission that, provided the general 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: Fibrinogen concentration and fibrin clot properties as predictors of CVD mortality in Black South Africans**

**Principal Investigator/Study Supervisor/Researcher: Prof M Pieters**

**Student: HJ Jankowitz - 27313735**

**Ethics number:**

|   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N | W | U | - | 0 | 0 | 0 | 3 | 4 | - | 1 | 7 | - | A | 1 | - | 0 | 6 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

Institution Study Number Year Status Sub-study  
Status: S = Submission; R = Re-Submission; P = Provisional Authorisation;  
A = Authorisation

**Application Type: Sub-study**

**Commencement date: 22/03/2022**

**Expiry date: 31/03/2023**

**Risk:**

**Minimal**

**Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of an annual monitoring report and the concomitant issuing of a letter of continuation. A monitoring report is due at the end of March annually until completion of the study.**

### General conditions:

*While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:*

- The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-HREC:
  - Annually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and
  - 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.
- The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-HREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.
- Annually a number of studies may be randomly selected for active monitoring.
- The date of approval indicates the first date that the study may be started.
- In the interest of ethical responsibility, the NWU-HREC reserves 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 NWU-HREC or that information has been false or misrepresented;*
  - *submission of the annual monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or*
  - *new institutional rules, national legislation or international conventions deem it necessary.*
- NWU-HREC can be contacted for further information via [Ethics-HRECAppl@nwu.ac.za](mailto:Ethics-HRECAppl@nwu.ac.za) or 018 299 1206

**Special conditions of the research approval due to the COVID-19 pandemic:**

**Please note:** Due to the nature of the study i.e. (statistical analysis of previously collected data), this study will be able to proceed during the current alert level, following receipt of the approval letter. No additional COVID-19 restrictions have been placed on the study except that the researcher must ensure that before proceeding with the study that all research team members have reviewed the North-West University COVID-19 Occupational Health and Safety Standard Operating Procedure.

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

Yours sincerely,



Digitally signed by  
Prof Petra Bester  
Date: 2022.03.22  
19:44:54 +02'00'

Chairperson NWU-HREC

Current details:(23239522) G:\My Drive\9. Research and Postgraduate Education\9.1.5.4 Templates\9.1.5.4.2\_NWU-HREC\_EAL.docm  
20 August 2019  
File Reference: 9.1.5.4.2

## 2. Annexure B

### **PURE study Information to community leaders for goodwill permission**

#### **Potchefstroom Campus**

#### **The PURE project**

#### **Information to communities**

Dear Participant

Thank you for being willing to help us in this very important project. We are sure that the project will contribute to improve health of all the people of the North West Province.

The aim of the project is to get enough information regarding the development of chronic diseases like Diabetes, Stroke, Lung disease and heart disease with urbanisation to plan appropriate health and nutrition intervention strategies.

For this study we need 2 000 subjects whom we can follow for 12 years. The baseline survey will be done from April 2005 to November 2005. The subjects must be from rural as well as urban communities. Therefore, 500 subjects from 4 different levels of urbanisation will be needed. Ganyesa and Tlakgameng were chosen for the rural and semi-rural areas because they are still under tribal law with a good infra-structure and stability. We also spoke to Chief M. Letlhogile and the mayor Mr E. Tladinyane and both gentlemen gave us permission to do the research in these two communities. Ikageng and the informal Ikageng were chosen as it is convenient and near the University. Cllr GG Megalanyane and Cllr Mahesh Roopa are informed about the study.

All the questionnaires will be filled out at your houses by trained research field workers who are from your communities. After a household survey and a family census on most of the households in your community to give us an overview of the total community, 250 men and 250 women from all four sites (Ganyesa, Tlakgameng, Ikageng, and the Informal Ikageng) will be asked to proceed with the study. These subjects should be

- Older than 35 years
- Healthy – which means that they must not be aware of any disease and do not take any chronic medication
- 

These 2 000 subjects will be asked to fill out the adult questionnaire, the food frequency questionnaire, the health questionnaire and the physical activity questionnaire. We will also make an appointment with each subject to take some measurements such as weight, height, skinfold thickness, ECG (test for heart abnormalities), lung functions, blood pressure, blood glucose, blood samples and a urine sample.

It is very important that we gather quality data and knowledge. Because HIV/AIDS is such a devastating illness and affects almost all aspects of health, it is necessary to know if HIV is absent before we analyse the data. Therefore we will ask questions about your HIV status which you are allowed not to answer.

It is also very important to us that you feel free to participate in this study and that you understand what the study is all about. The fieldworker will ask you to sign this form after you have read and understood it.



North West Province

3<sup>rd</sup> Floor Tirelo Building  
Dr Albert Luthuli Drive  
Mafikeng, 2745  
Private Bag X2068  
MMABATHO, 2735

## NORTH WEST DEPARTMENT OF HEALTH

*Healthy Living for All*

Republic of South Africa

## OFFICE OF THE DDG

Enq: K. Mangonyane  
Tel: (018) 387 5833/5  
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[www.nwhealth.gov.za](http://www.nwhealth.gov.za)

Fax: (018) 387 5709

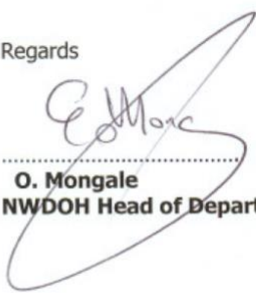
29 August 2005

Dr A Kruger  
North West University, Potchefstroom Campus  
North West Province

SUBJECT: Approval for Research- **Prospective Urban and Rural Epidemiological Study**

Approval is granted to conduct the above study in the North West Province, kindly make relevant arrangements with the management for suitable dates and times. Detail at the bottom of this letter has to be completed by you and returned to the Knowledge Management Directorate before your study may commence.

Regards

  
.....  
**O. Mongale**  
**NWDOH Head of Department**

KMx

Page 1  
1

29/08/2005



The NWDoH will be furnished with final research report by

*Please note that this is a prospective study over many years. We will submit a report 6 months after we did the baseline and again 6 months after each follow-up.*

...July 2006.....  
Submission date of the first report

...

A.Kruger



### 3. Annexure C



10/03/2022

To: Marlien Pieters

Reference: Unit records of data

Telephone: 012 310 6994

Email: [nozis@statssa.gov.za](mailto:nozis@statssa.gov.za)

**STATISTICS SOUTH AFRICA DATA USER'S AGREEMENT: UNIT RECORDS, MORTALITY and  
CAUSES of DEATH DATA for 2018  
Frequent Data User**

Statistics South Africa will make available unit record data compiled from death notification forms for the express purpose of calculating age standardized mortality rates.

The Data User must ensure that there is no misuse of the Data or breach of confidentiality and must agree to the following conditions:

1. The User(s) listed in bullet 8 agree that they will not attempt to use, nor permit others to use the data to establish the identity of any person included in any set.
2. The User(s) agree to keep the Data in a secure environment.
3. The Data may be used only by the named collaborative research team listed in bullet 8 and may not be shared with other Users.
4. The User agrees that any of the Data, or reliance by the User on any of the Data, is at the User's own risk, and that Statistics South Africa shall not be liable for any loss or damage howsoever arising as a result of such use.
5. The use of these Data in research communication, scholarly papers, journals and the like is encouraged, but the authors of these communications and documents agree to acknowledge/cite Statistics South Africa as the source of the Data, making it clear that the analysis and interpretation have been undertaken by the User. The User also agrees to submit to Statistics South Africa a copy of any research publication derived from these Data, for their information.
6. The applicant affiliated to University of Witwatersrand commits to retain data once the study related to the requested data is complete.
7. Non-adherence to the above conditions will:
  - i. Render the User liable for a fine which amount to **ZAR 500 000** (Five hundred thousand Rands).
  - ii. Statistics South Africa refusing to make available any datasets to the User and affiliated institution in future.

## 8. Signatures of Users:

- i. Name: \_\_\_\_\_  
 Organisation: \_\_\_\_\_  
 Signature: \_\_\_\_\_  
 Signed on: \_\_\_\_\_  
 Signed at: \_\_\_\_\_
- ii. Name: \_\_\_\_\_  
 Organisation: \_\_\_\_\_  
 Signature: \_\_\_\_\_  
 Signed on: \_\_\_\_\_  
 Signed at: \_\_\_\_\_
- iii. Name: \_\_\_\_\_  
 Organisation: \_\_\_\_\_  
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- iv. Name: \_\_\_\_\_  
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 Signed at: \_\_\_\_\_
- v. Name: \_\_\_\_\_  
 Organisation: \_\_\_\_\_  
 Signature: \_\_\_\_\_  
 Signed on: \_\_\_\_\_  
 Signed at: \_\_\_\_\_

Dr Christine Khoza  
 Chief Director: Health and Vital Statistics – Statistics South Africa

Date:

Ms Nozipho Shabalala  
 A-DDG: Population and Social Statistics – Statistics South Africa

Date:

Mr Risenga Maluleke  
 Statistician-General