Vaal University of Technology

ESTABLISHING THE CORRELATION BETWEEN R353Q POLYMORPHISM AND HAEMOSTATIC MARKERS IN A BLACK ELDERLY COMMUNITY OF SHARPEVILLE GAUTENG PROVINCE SOUTH AFRICA

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This dissertation is being submitted in fulfilment of the requirements for the degree MTech in Biotechnology

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DEDICATION

I will like to dedicate this dissertation to my grandparents Mr. Wambo Frederick, Mrs. Magne Therese, Mr. Nzogoum Joseph, Mrs. Magne Denise and my coach Mr. Teboho Mofokeng, of blessed memories.

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ABSTRACT

Background: In a group of the elderlies (older person) in Sharpeville, Gauteng, South Africa, the majority live in poverty with a poor nutritional status. This makes them susceptible to develop infectious diseases as well as the risk of Chronic Diseases of the Lifestyle (CDL) such as cancer, diabetes, heart attack, obesity and hypertension. One of the most constant features of aging is the progressively elevated levels of coagulation factors such as FVII, fibrinogen, and impairment of fibrinolysis might play a role in the ageing process. These are associated with increased susceptibility to Cardiovascular diseases (CVD) commonly found. An association between elevated levels of FVII and R353Q polymorphism has been established as a risk factor for CVD. This genetic polymorphism R353Q characterizes the substitution in the exon 8 of the FVII gene of guanine-to-adenine, which results in the replacement of arginine (R) by glutamine (Q) in codon 353 of the *F*7gene.

Objectives: The aim of this study was to evaluate the prevalence of R353Q polymorphism in correlation with haemostatic markers within an urban elderly community in South Africa.

Method: This study was ethically approved, and it is an experimental research design on the prevalence of R353Q polymorphism in correlation with homeostatic status (Factor VII, Fibrinogen and PAI-1). The study was done in a black elderly population living in the Vaal triangle region of Sharpeville, attending a day care center, who gave consent to participate in the study. A purposely selected sample of 102 subjects, who met the inclusion criteria were used. The homeostatic status was measured by factor VII and fibrinogen measuring coagulation and PAI-1 measuring fibrinolysis.

Results: The prevalence of R353Q genetic polymorphism was established in 14.5% of the sampled population. The prevalence of the RQ (AG) genotype was determined in the sample population with 6.5 % of elevated factor VII levels, 7.8% of increased fibrinogen levels (coagulation) and 10.5 % of decreased levels of PAI-1. The R(A) allele, was detected in 1.3% of the sampled population of normal levels of FVII, fibrinogen and PAI-1. The dominant allele G(Q) was present in 76.3% of the sampled population. An imbalance haemostatic marker was established in the sampled population with 61% of elevated levels of factor VII, 70% of elevated levels of fibrinogen and 88% had a decreased level of PAI-1.

Conclusion: The prevalence of R353Q polymorphism was established in this sample population, having an imbalanced haemostatic status of hypercoagulation (factor VII and fibrinogen) and imbalance fibrinolysis (PAI-1), which are strongly associated to CVDs.

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GLOSSARY OF TERMS

Cardiovascular disease: is a disorder affecting the heart and arterial circulation supplying the heart, brain and cerebrovascular organs, whereby blood flowing to the lower periphery is obstructed, resulting in arterial disruption which connects the heart to peripheral circulation.

Cardiovascular risks: are factors which are strongly linked in describing the etiological characteristics or exposure of an individual to develop the disease at a faster rate.

Factor VII: is a vitamin K dependent serine protease glycoprotein produced in the liver, involved in the regulation of coagulation in the presence of tissue factor.

Fibrinogen: is a plasma protein involved in stabilizing the process of haemostasis and formation of a clot.

PAI-1: is an important regulator of fibrinolysis (clot dissolution), which inhibits the activation of plasminogen to plasmin with the presence of the tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA).

R353Q polymorphism: is a single nucleotide base that characterizes the substitution in the exon 8 of the factor VII gene of guanine-to-adenine, which results in the replacement of arginine (R) by glutamine (Q) in codon 353 of the F7gene.

ABBREVIATIONS

A	Arginine
a2-AP	a2-Antiplasmin
ADS	Assay Design Suite
ADP	Adenosine diphosphate
AI	Adequate Intake
AIDS	Acquired immune deficiency syndrome
APTT	Activated partial thromboplastin time
Arg	Arginine
AT	Antithrombin
ATP	Adenosine triphosphate
BFU	Burst-forming unit
CFU	Colony forming unit
CHF	Congestive heart failure
CDL	Chronic Diseases of Lifestyle
CRP	C-reactive protein
CSL	Center of Sustainable Livelihoods
CV	Coefficient variation
CVD	Cardiovascular disease
CVDs	Cardiovascular diseases
CVR	Cardiovascular risk
DIC	Disseminated intravascular coagulation
DMT1	Diabetes Mellitus type 1
DMT2	Diabetes Mellitus type 2
DNA	Deoxyribonucleic acid
g-DNA	Genomic deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
EAR	Estimated average requirement
EDTA	Ethylenediaminetetraacetic
EGF	Epidermal growth factor
EMP	Emperor Medical Co. Ltd.
ELISA	Enzyme Linked Immunosorbent Assays
ELT	Euglobulin Clot Lysis Time Test
et al	And others

FI	Factor 1
FIa	Factor 1 activated
FII	Factor 2
FIIa	Factor 2 activated
FIII	Factor 3
FIIIa	Factor 3 activated
FIV	Factor 4
FIVa	Factor 4 activated
FV	Factor 5
FVa	Factor 5 activated
FVI	Factor 6
FVIa	Factor 6 activated
FVII	Factor 7
FVIIa	Factor 7 activated
FVIIc	Factor 7 concentration
FVIII	Factor 8
FVIIIa	Factor 8 activated
FVIX	Factor 9
FVIXa	Factor 9 activated
FVX	Factor 10
FVXa	Factor 10 activated
FXI	Factor 11
FXIa	Factor 11 activated
FFQ	Food frequency questionnaire
FSAP	Factor VII activating protease
G	Glutamine
Gla	Gamma-carboxyglutamic
GIIb/IIIa	Glycoprotein IIb/IIIa (integrin αIIbβ3)
g/l	Gram per litre
GmbH	Gesellschaft mit beschränkter Haftung
HDL	High-density lipoprotein
HDL-C	High-density lipoprotein-Cholesterol
His	Histidine
HRP	Horseradish peroxidase

hs-CRP	Highly-sensitive C-reactive protein
HPLC	High Performance Liquid Chromatography
HPSCA	Health Professions Council of South Africa
HIV	Human immunodeficiency virus
HMWK	High molecule weight kininogen
HVR7	Hypervariable region 7
Hz	Hertz
IHD	Ischemic heart disease
IBL	Immuno - Biological Laboratories Co. Ltd.
IL-2	Interleukin-2
IL-6	Interleukin-6
IRQ	Interquartile range
Jak/Stat	Janus tyrosine Kinase/ Signal Transducer and Activator of Transcription
kb	Kilobyte
kDa	Kilodaltons
L	Liter
m	Meter
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization-Time of Flight
mcg/day	microgram per day
MI	Myocardial infarction
mL	Milliliter
mmHg	Milliliter of mercury
MRC	Medical Research Council
Met	Methionine
NCDs	Noncommunicable diseases
ng/ml	Nanogram per milliliter
Non-STEMI	Non-ST elevation MI
LDL	Low-density lipoprotein
LDL-C	Low-density lipoprotein-Cholesterol
LRP1	Lipoprotein receptor related protein 1
P	Peptide bond
P’	Peptide bond’
PAI	Plasminogen activator inhibitor
PAI-1	Plasminogen activator inhibitor-1

PCR	Polymerase chain reaction
PK	Prekallikrein
PPP	platelet pool plasma
PT	Prothrombin time
Q	Arginine
QC	Quality control
QA	Quality assurance
QCM	Quartz crystal microbalance
R	Guanine
RCL	Reactive center loop
RFLP	Restriction fragment length polymorphism
RT	Room temperature
RT-PCR	Real time- Polymerase chain reaction
RGD	Arg-Gly-Asp (sequence)
RHD	Rheumatic heart disease
R-*pNA*	Para-nitroaniline
RS	Reference Single nucleotide polymorphism
SA	South Africa
SANAS	South African National Accreditation System
SAP	Shrimp alkaline phosphatase
SAMRC	South African Medical Research Council
SSA	Sub Sahara Africa
SctPA	Single chain tissue plasminogen activator
ScuPA	Single chain urokinase plasminogen activator
Ser	Serine
SD	Standard deviation
SIRS	Systemic inflammatory response syndrome
SNP	Single nucleotide polymorphism
SPSS	Statistical Product and Service Solutions
SOWETO	Southern Western Township
SST	Serum separating tube
STATA	Statistics and Data
tPA	tissue-type plasminogen activator
TAFI	Thrombin activatable fibrinolysis inhibitor

TF	Tissue factor
TF-FVII	Tissue Factor-Factor 7
TFPI	Tissue factor pathway inhibitor
TMB	Tetra Methyl Benzidine
TNF	Tumor necrosis factor
TOF-MS	Time of light mass spectrometer
TPA	Tissue plasminogen activator
TXA2	Thromboxane A2
U/dL	Unit per deciliter
uPA	Urokinase-type plasminogen activator
VUT	Vaal University of Technology
VWF	von Willebrand factor
WHO	World Health Organization

CHAPTER 1

BACKGROUD AND JUSTIFICATIONS

1.1 INTRODUCTION

The blood haemostasis consists of platelets, blood coagulation factors, vascular system and fibrinolytic system. A haemostatic response to vascular injury depends on the interaction between the closely linked blood vessels, circulating platelets and blood coagulation proteins (Hoffbrand & Moss, 2011; Rifai *et al.,* 2018). Any change within the vascular circulation, coagulation cascade and fibrinolysis will result in either bleeding tendency or intravascular thrombus formation (Russo, 2012; Hall, 2016). Cardiovascular disease (CVD) is a global challenge leading to a high rate of mortality, loss of independence, with impairment of quality of life and social and financial sustainability (Labarthe, 2011, Seo & Choi, 2015). High blood levels of FVII have been associated with the risk of CVD with the influence of the R353Q polymorphism which modifies the level of blood plasma FVII (Turfan *et al.,* 2014). The modification of blood plasma FVII occurs when there is rupture of plaque, where the tissue factors are exposed to blood initiating the circulation of FVII. This process is considered as a major cause of CVD (Ben-Hadj-Khalifa *et al.,* 2013). The genetic polymorphism is a single nucleotide base that characterizes the substitution in the exon 8 of the FVII gene of guanine-to-adenine, which results in the replacement of arginine (R) by glutamine (Q) in codon 353 of the F7gene (Irish & Green, 1998; Shanker *et al.,* 2009; Mo *et al.,* 2011; Criado-Garcia *et al.,* 2011; Cheraghi *et al.,* 2013). This polymorphism result in a 20% decreased of FVII levels with 20% of individuals who are carriers of the Q 353 allele having low FVIIc and FVII antigen concentration as compared to those with R353Q homozygote (Azzam *et al.,* 2017). According to Ben-Hadj-Khalifa et al. (2013), the reduction of FVII levels has been demonstrated by a numerous case studies, whereby the Q allele was proven to be associated with CVD, but some other studies showed a weaker association between the reduction of FVII levels and Q allele as associated with CVD and some studies did not detect these findings.

As indicated in Figure 1, any imbalance within haemostatic system (coagulation factors FVII, fibrinogen and fibrinolysis PAI-1) has been proven to be associated to the development of CVD. With the presence of the genetic polymorphism R353Q which reduces the levels of FVII and thus, contribute to an haemostatic imbalance and a risk for CVD.

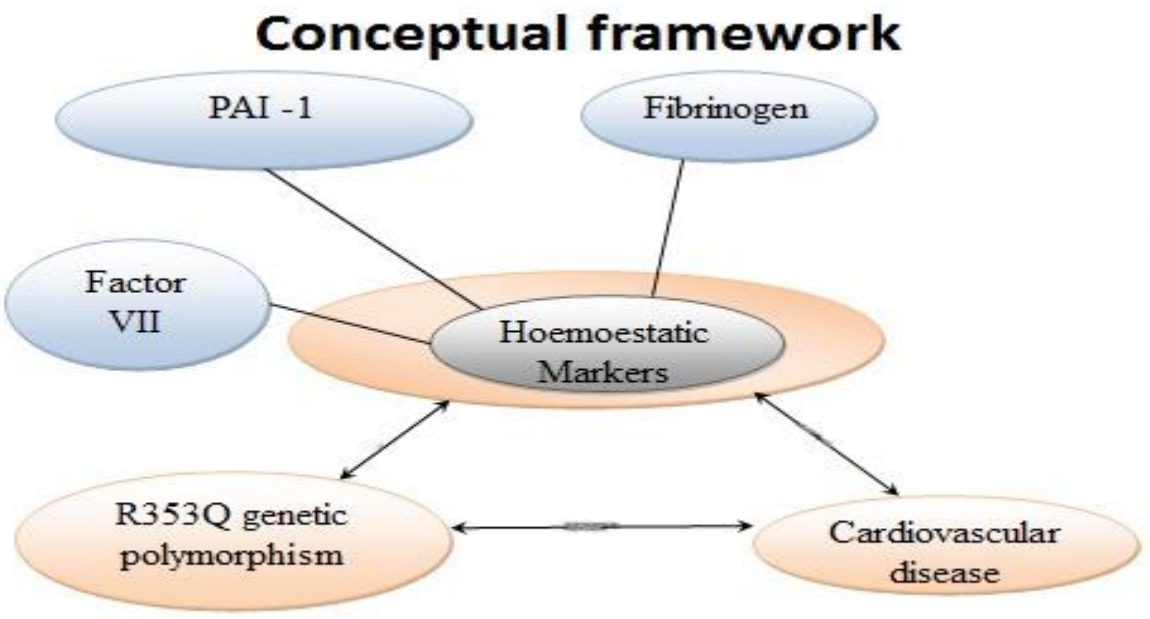

Figure 1: The different haemostatic markers which correlate with the R353Q genetic polymorphism in cardiovascular disease

The haemostatic markers for clot formation (FVII and fibrinogen) and clot dissolution or fibrinolysis (PAI-1) was evaluated in correlation with the genetic polymorphism R353Q of the FVII gene as CVR. FVII is synthesized in the liver as vitamin K dependent serine protease glycoprotein which regulate the process of coagulation. The presence of tissue factors (TF) in circulation binds to FVII forming TF-FVII complex (Böhm *et al.*, 2015). The TF-FVIIa complex following an activation with elevated levels of FVII has been found as a risk factors of CVD, with the modification of the FVll levels influenced by the genetic polymorphism R353Q (Turfan *et al*., 2014). Fibrinogen is an adhesive protein produced by hepatic cells and is involve in stabilizing plasma proteins of the process of haemostaisis and clots formation (converting fibrinogen to fibrin in the presence of thrombi forming a haemostatic clot) (Palta *et al.,* 2014). High levels

of fibrinogen has been identified as a risk factor of CVD, affecting the process of coagulation and platelet aggregation (Kattula *et al.*, 2017). PAI-1 is produced in the liver as a superficial serine protease inhibitor, which is very essential in the regulation of fibrinolysis (inhibiting the activation of plasminogen to plasmin) (Simone *et al.*, 2014). High and low levels of PAI-1 has been proven as an independent risk factors of CVD (Lange *et al.*, 2013; Phelan & Kerins 2014). This study was conducted to measure the haemostatic levels of FVII, fibrinogen and PAI-1 in correlation with the R353Q polymorphism.

1.2 PREVALENCE OF CARDIOVASCULAR DISEASE

In 2008, 17.3 million people died of CVD; with 80% of death case occurring in the middle low and middle-income countries. It is also estimated that by the year 2030, 23 million of the global population will die of CVD (WHO, 2013; Laslett *et al.*, 2012; Balakumar *et al.*, 2016; Boateng *et al.*, 2018).

1.2.1. Prevalence of Cardiovascular disease globally

CVD has been identified as the major cause of death globally (Aljefree & Ahmed 2015; Barquera *et al.*, 2015; Roth *et al.*, 2015; Balakumar *et al.*, 2016; Boateng *et al.*, 2018). In addition to CVD as the major cause of death in the world, non-communicable diseases are contributing to the death rate globally, which has suppressed communicable diseases as a life threatening and caused of death in the world (DeMaria, 2013; Balakumar *et al.*, 2016). Of these, CVD remains the highest leading cause of death, which accounts for about 17.3 million deaths per annum. This number is estimated to rise to 23.6 million by the year 2030 (Laslett *et al.*,2012; Balakumar *et al.*, 2016). According to Boateng et al. (2018), the death rate per annum due to CVD has accounted for 17.5 million and is projected to increase to 22.2 million by the year 2030. The prevalence of CVD is increasing at a greater rate in developed countries more than in developing countries.

This high mortality rate of CVD encountered in developed countries, is due to the prevalence of risk factors (hypercholesterolemia, hypertension, diabetes, smoking, unhealthy dietary changes and reduced physical activity) and related chronic diseases (Fuster & Kelly 2011; Seo & Choi 2015). There is a higher prevalence of risk factors for CVD in high income countries as compared to middle income countries, having a higher incidence rate of disease and mortality rate (Deaton *et al.*, 2011; Chugh *et al.*, 2014; Boateng *et al.*, 2018). Meanwhile, the mortality rate of CVD encountered in low and middle-income countries are due to the

prevalence of hypertension, smoking, diabetes and dyslipidemia (DeMarie, 2013; Rabkin, 2015; Sliwa *et al.*, 2016), with poverty and chronic diseases as a contributing factor, affecting both men and women in low and middle income countries (Fuster & Kelly 2011, Hill *et al.,* 2013).

The elderly of age ≥70 years in low to middle income countries are at higher risk of developing of CVD, accounting for about 50% of deaths reported (Keates *et al.*, 2017). The increased susceptibility to diseases is a burden in the ageing population, and age dependent disorders are at a higher risk of developing CVD, with hypertension as an independent risk factor for the development of CVD in the elderly population (Prince *et al.*, 2015).

1.2.2. Prevalence of Cardiovascular disease in Africa

The prevalence of CVD is increasing at an alarming rate, particularly in urban areas in low and middle income African countries (Njelekale *et al.,* 2009; Cappucio & Miller 2016). The predominance of infectious diseases and poor nutritional status as a major cause of CVD (Sliwa *et al.*, 2016). Another major burden attributed to an increased rate of CVD is the socio-economic costs which results to loss of productivity and high rate of poverty (Cappucio & Miller 2016). In Africa, hypertension (stroke and hypertensive heart disease) is the highest risk factor for CVD (Aljefree & Ahmed 2015; Roth *et al.,* 2015; Balakumar *et al.*,2016; Cappucio & Miller 2016; Sliwa *et al.*, 2016; Keates *et al.*, 2017).

The rising incidence of hypertension is attributed to some factors such urbanization (migration from rural to urban areas), adoption of western lifestyles such as smoking and alcohol abuse (Sani, 2007; Adeloye & Basquill 2014). However, rheumatic heart disease (RHD) in Sub Saharan Africa (SSA), has the highest prevalence in the world (15 to 20 per 1000 people), which is the most important form of acquired CVD in children and adolescents (Mocumbi, 2012; Zühlke *et al.*, 2014). The risk factors contributing to the development of CVDs are obesity, diabetes, hypertension, elevated lipid levels, high blood pressure, psychosocial factors, lack of physical activities, excessive intake of alcohol and poor nutritional status, high sugar levels, high cholesterol levels and smoking (Cappucio & Miller 2016). Obesity is the leading cause of hypertension and is the most common risk factor of CVD (Njelekale *et al*. 2009; Jiang *et al.*, 2016).

In SSA, the estimated number of deaths of CVD was reported to be 1 million in 2013 was 1, with 38% related to all noncommunicable diseases (NCDs) and in Africa 5.5% of death related to CVD (Keates *et al.*, 2017). In Nigeria, the prevalence of hypertension was found to range from 10% to 46.4% in rural areas

as compared to 8.1% to 42.0% in urban areas (Ejim *et al.,* 2011; Ofili *et al.,* 2015). According to studies conducted by Jingi et al. (2013), the prevalence of hypertension in adults was found to range from 20% to 25% in considering the disease survey data of 1991 whereby the qualifying blood pressure level was 140/90 mm Hg. High levels of hypertension has also been reported in Tanzania. This is common among obese individuals and the elderly population living in both urban and rural areas. Some studies in Ghana reveal the prevalence of hypertension is 4.5% in the rural areas and 8% to 13% in the urban areas. In Cameroon, the prevalence of CVD was found to be 54% due to hypertension, 26.3% as a result of cardiomyopathies and 24.5% for valvular heart diseases in urban areas.

1.2.3. Prevalence of Cardiovascular disease in South Africa

In South Africa (SA), the burden of CVD is increasing among all the age group, with hypertension and stroke estimated to constitute of 60% of chronic NCDs. CVD is predicted to be the prime contributory factor to morbidity and mortality rate, above the age group of 50 years. Approximately 196 people die per day of CVD, with 20% of these suffering from human immune virus (HIV) / acquired immune deficiency syndrome (AIDS) (Maredza *et al.,* 2011; Maredza *et al.,* 2015). Carriers of HIV/AIDS are at a higher risk of developing CVD (Fedele *et al.,* 2011; Mashinya 2015), with 18% of death rate caused by CVD (Rabkin *et al.,* 2015).

A triple cardiac threat was reported in Southern Western Township (SOWETO), which occurred with epidemiological transition such as infectious diseases, coronary heart diseases and HIV/ AIDS with the re-occurrence of tuberculosis. These risk factors contributed to an increased incidence of cardiomyopathy and heart failure. The burden of CVD is expected to increase with rapid transition of more than 150% in the next coming 20 years (White & Dalby 2008).

The major risk factors of CVD in SA are high cholesterol, obesity (40.1% in women and 11.6% in men), high blood pressure (50% strokes and 42% heart attacks), tobacco intake, decrease in physical activity and poor nutritional intake (Bryne *et al.,* 2016). These have contributed to an increased prevalence in Africa in both urban and rural areas. Hypertension as the most prominent cause of CVD was reported to range between 44% to 42% in one rural area of the Mpumalanga district amongst males and females respectively (Maredza *et al.,* 2011; Laurence 2016; Gaziano 2017). The prevalence of hypertension in South Africa has been strongly attributed to urbanization, more especially to the black population who are at a higher exposure risk and increasing the burden in developing CVD (McAloon, 2016). The common types of CVDs

in SA are congenital heart disease (CHF, rheumatic heart disease, Arrhythmia, coronary artery disease, acute myocardial infarction (MI), peripheral artery disease and HIV related CVD (Keates *et al.,* 2017).

The trend pattern of CVD death rate in SA is linked to several contributing factors, with the rise of chronic NCDs of epidemiological and demographic transition which have greatly reduced the fertility rate and the aging population of 60 years and above (Maredza *et al.,* 2011; Maredza *et al.,* 2015, Ntuli *et al.,* 2015). The elderly population of SA suffering from diabetes are at risk of developing CVD (Mutyambizi *et al.,* 2017). Hypertension (77.3%) has been proven as the highest risk factor for CVD in elderly population of age 65 years and above (Adeloye & Basquill 2014). According to the global ageing and Adult health in Low and middle-income countries, the prevalence of hypertension in the elderly population of SA of 50 years and above was estimated to range from 78% as compared of India with 32%. This prevalence of hypertension in SA is expected to increase in 60% within the ageing and growing population in the year 2025 (LIoyd-Sherlock *et al.,* 2014; Bowry *et al.,* 2015).

1.3 Context of the research

This study forms part of a multi-disciplinary study in collaboration with the Center of Sustainable Livelihoods (CSL) of the Vaal University of Technology (VUT). This research program was ethically approved by the Ethics Committee of the University of Witwatersrand, Johannesburg (M070126), with additional clearance that was submitted to the ethical committee of the Vaal University of Technology, Vanderbijlpark (20140827-1ms).

This study was carried out in a low socio economic free-living community of the black elderly population in Sharpeville, Gauteng, South Africa who are attending a day care centre daily. The majority of black South Africans have been subjected to urbanization which has resulted in an increased incidence of diseases of new lifestyle (Oldewage-Theron & Kruger, 2008). An increased risk of cardiovascular disease was reported by Oldewage *et al.* (2008) and Grobler (2015) in the same community. Therefore, the focused on a black urbanized elderly population.

1.4 Motivation

The genetic polymorphism R353Q has been reported to be associated with the variation in FVll levels in the coagulation process which may lead to the progression of CVDs (Cheraghi *et al.,* 2013). The carriers of the Q allele are more associated with lower levels of FVII than R allele carriers (El-Mitwalli et al., 2014). Studies on this polymorphism have been conducted in other populations across the globe (Shanker *et al.,* 2009; Criado-Garcia *et al.,* 2011; Turfan *et al.,* 2014; Bairova *et al.,* 2016; Azzam *et al.,* 2016). Although CVDs are also present in Africa being influenced by genetic factors and environmental factors (Ben-Hadj-Khalifa *et al.,* 2013), limited studies have been done on genetic polymorphisms that lead to their pathogenesis. This study is the first to be conducted on the R353Q polymorphism of the FVII gene in a black elderly population of South Africa.

1.5 Aim

The aim of this research was to evaluate the prevalence of R353Q polymorphism in correlation with homeostatic markers as a risk factor for cardiovascular disease within an urban elderly community in Sharpeville, Gauteng Province, South Africa.

1.6 Objectives

1) To establish the prevalence of R353Q polymorphism in the sample population

2) To determine the average fibrinogen concentration of the sample population

3) To evaluate the mean FVII concentration of the sample population

4) To determine the average Plasminogen activator inhibitor-1 (PAI-1) concentration of the sample population

5) To determine the vitamin K intake of the sample population

6) To determine the correlation between FVII, fibrinogen and PAI-1 of the sample population

7) To evaluate the correlation between dietary intake of vitamin K and haemostatic markers (FVII, fibrinogen and PAI-1) of the sample population

8) To determine the correlation between the genetic polymorphism R353Q and haemostatic markers (FVII, fibrinogen and PAI-1) of the sample population

1.7 Significance of study

This study will contribute to better understanding of risk factors associated with the development of CVD in an elderly population of South Africa.

1.7.1. Impact on Health Policy

This research will provide specific information about the impact of R353Q polymorphism on the homeostatic status of the elderly population of Sharpeville. This will also help the Heart and Stroke Foundation of South Africa and the Department of Health on strategic planning and management of CVD.

1.7.2 Scientific outcomes

Little or no studies have been conducted on the R353Q genetic polymorphism in South Africa. The results obtained from this studies will help in the better understanding of the different population allele's distribution among individuals with the Q allele having a lower level of FVII than those with the R allele.

1.7.3 V.U.T Capacity building

On completion of this research, a Master of Technology qualification will be obtained, contributing in building the research capacity in the department of Health Science at VUT.

1.7.4 Scientific capacity building

The information obtained from the association between the genetic polymorphism R353Q of the FVII gene and FVII coagulative activity levels as a risk factor for CVD, will help to further investigate other genetic polymorphisms associated with coagulation factors such as FII, FVIII, FIX and FX. These plasma proteins direct and control the process of coagulation by activating the surface membrane after being exposed to a tissue factor.

1.7.5 Community capacity building

This research will also provide information on how genetic and environmental factors influences health fundamental changes and diseases pattern within the Sharpeville elderly population, with poor nutritional

status. The feedback obtain will be given to the Sharpeville community for further recommendation on the pattern trend of the disease with regard to early disease management.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

The haemostatic system consists of blood coagulation factors, endothelium, platelets and a fibrinolytic pathway (Rifai *et al*., 2018). The response to vascular injury on the homeostatic system will depend on the interaction between the closely linked blood vessels, circulating platelets and blood coagulation proteins (Hall, 2016). Factor VII (FVII) plays an essential role in the activation of the extrinsic pathway, forming a complex with tissue factors when exposed and released by damaged or activated endothelium, or by the obstruction of atheromatous plaques (Ben-Hadj-Khalifa *et al.,* 2013). The release of tissue factors following a vascular vessel injury, will transform and activate FVII to FVIIa. This transformation of the extrinsic pathway of FVII plasma activity, has been proven as a risk factor for CVD (Bairova *et al.,* 2016). High blood levels of factor VII have been associated with the risk of CVD with the influence of R353Q polymorphism which modifies the level of FVII (Turfan *et al.,* 2014). The genetic polymorphism R353Q is associated with a 20% of reduction of plasma FVII levels. Moreover, 20% of the individuals who are carriers of the Q 353 allele have more reduced FVIIc and FVII antigen concentration than those with the R353Q homozygote (Azzam *et al.,* 2017). Therefore, individuals with Q allele may have an inherited degree of protection against CVD (Ben-Hadj-Khalifa *et al.,* 2013).

2.2 CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is a series of disorders affecting the heart and circulatory system, whereby the flow of blood and oxygen to the lower periphery is obstructed. The series of disorders include inflammatory processes, rhythm abnormalities, congenital defects, coronary artery disease, valve malfunctioning and heart failure (Labarthe, 2011; Zafar, 2015; Cohen & Hull 2015; Andrew, 2017). CVD disorder has become a global challenge because of impairment of life quality, causing loss of independence, financial and social sustainability leading to a high rate of mortality, which is common amongst various populations (Labarthe, 2011; Aljefree & Ahmed 2015). CVDs are strongly linked and influenced by risk

factors. These risk factors describe the etiological characteristics or exposure of an individual to develop the disease at a faster rate (Balakumar *et al.,* 2016).

2.2.1 Pathophysiology of Cardiovascular disease

The pathophysiology of CVD is occurs in the heart and blood vessels, resulting in cardiovascular dysfunction of one or more mechanisms, with detectable morphologic manifestations such as failure of the pump, obstruction to flow, regurgitant flow, shunted flow, disorder of cardiac conduction and rupture of the heart or a major vessel (Kumar *et al.,* 2010; Cohen & Hull 2015; Andrew *et al.,* 2017). This mechanism occurs when there is an inflamed endothelial cell that expresses the adhesion molecules, binding to macrophages, immune cells and other inflammatory cells. These macrophages now adhere to the injured endothelium, releasing numerous inflammatory cytokines and enzymes which further damage the vessel wall. Moreover, macrophages release growth factors which activate the smooth muscle cells to proliferate. The proliferation of these smooth cells produces collagen, which migrates to the fatty streak, forming fibrous plaque. The presence of these plaques may become calcified and will protrude into the vessel lumen obstructing blood flow to the tissues. This will result in ischemia and infarction (Huether & McCance 2012; Cohen & Hull 2015; Werner, 2016). Ischemia or Ischemic heart disease (IHD) is a disease of the heart characterized by the reduction of blood supply to the heart muscle, leading to blockage in the coronary arteries, due to atherosclerosis and thrombus (Choi *et al.,* 2009; Cohen & Hull 2015). Ischemia also causes functional disturbances of the heart such as impaired relaxation, which lead to diastolic dysfunction, impaired contraction and finally to systolic dysfunction. In addition, ischemia also causes myocardial stunning, which is a prolonged but reversible dysfunction following an acute ischemic activity, and myocardial hibernation. Which occurs when there is sufficient oxygen to maintain the viability of the myocardium, but not enough to support the normal function. If these conditions of ischemia are prolonged, death of the myocardial cells will take place (Kemp *et al.,* 2008; Werner, 2016). Ischemia can also result from low coronary arterial perfusion, shock associated with haemorrhage, severe aortic valve disease, leading to impaired coronary blood flow. Severe anaemic patients also stand a risk of getting myocardial ischemia. Moreover, impairment of flow has been associated with more than 95% of cases of stenosing coronary atherosclerotic plaque, which blocks the vessel lumen with 75% of atheromatous plaque. This leads to the development of ischemia, especially if the coronary collateral circulation is poorly established (Cross, 2013).

2.2.2 Etiology of Cardiovascular disease

Atherosclerosis is a disorder characterized by the hardening and thickening of vessel the wall, which is activated by the accumulation of lipid laden macrophages within the arterial wall. This results in the formation of plaque (Huether & McCance 2012; Hall, 2016). This plaque is composed of a raised lesion, having a yellow, soft and grumous core of lipid, which is covered by a fibrous white cap (Kumar *et al*., 2010; Cohen & Hull, 2015, Werner, 2016). The raised lesion is described an atherosclerotic plaque, which reduces blood flow and activates the formation of blood clots (thrombi) (Salvo, 2014). Atherosclerosis can also be described as a complex chronic inflammatory disorder, associated with the large and medium sized arteries, which results in an interaction between the arterial cell walls and white blood cells. This reaction causes an increased entrapment, uptake and deposition of lipids in the sub endothelium of arterial walls (Leon, 2009; Werner, 2016). This disorder is slow and progressive, which may start to manifest from childhood and eventually interfere with the rate of blood flow through the arteries of the brain, kidneys, heart, arms and legs. Thus, blood flowing to these organs could be obstructed (Steyn, 2007; Cohen & Hull 2015). The mechanism of Atherosclerosis commences when there is an injury in the endothelial cells that line walls of the arteries. The lesions progress pathologically from endothelial injury and dysfunction to fatty streak to fibrotic plaque, leading to complication of the lesion. This causes inflammation of the injured endothelial cells. The process of inflammation now mediates an initiating step and progression of atherogenesis (Huether & McCance 2012; Hall, 2016). The atherosclerotic lesion progressively narrows the lumen due to high grade plaque stenosis, causing acute atherothrombic occlusion, embolism of the distal arterial bed and ruptured abdominal atherosclerotic aneurysm (Cross, 2013).

2.2.3 Pathogenesis of Cardiovascular diseases

The pathogenesis of CVD is mainly attributed to atherosclerosis as the etiopathogenic cause of CVD, interrelate with genetic and environmental factors (Sayols-Baixeras *et al.,* 2014). Atherosclerosis commences with the progressive alteration of plaque involving the interaction of blood cells, vascular wall, lipoprotein and immune system leading to the development of CVD (Golia *et al.,* 2014). Risk factors such as obesity, diabetes, hypertension, inflammatory response, oxidative stress, endothelial dysfunction and sedentary life style are also associated with the development of CVD. These risk factors have different pathogenic pathways (directly and indirectly causing CVDs (Patel *et al.,* 2016). The pathogenesis of CVD in relation to oxidative stress and vascular inflammation causes injury to cells and stiffness of walls (arterial and vascular), elevating blood pressure (hypertension) resulting to CVD (Siti *et al.,* 2015). The extreme generation of reactive oxygen species causes the remodeling of oxidative damage and vascular dysfunction

(Zafar, 2015). Increased reactive oxygen species can also be caused by elevated levels of insulin, as the essential cause of oxidative stress, with obesity contributing to various vascular diseases (hyperlipidemia, hyperglycemia and hyperinsulinemia). These vascular diseases initiate the metabolism of insulin resistance as a risk factor in the pathogenesis of CVD (Patel *et al.*, 2016).

2.3 CARDIOVASCULAR RISK MARKERS

Cardiovascular diseases are strongly linked and influenced by risk factors that contribute to the development of the disease at a faster rate (Andrew *et al.*, 2017). The risk factors of CVDs are classified as modifiable and non-modifiable (Basharat *et al.*, 2012; Andrew *et al.* 2017; Ahmari *et al.*, 2017).

2.3.1 Modifiable risk factors

Modifiable risk factors can be changed and prevented by adjusting lifestyle (Yarahmadi *et al.*, 2013). These modifiable risk factors include; abnormal serum lipids, hypertension, smoking, diabetes, obesity and irregular physical activities (Ouyang *et al.*, 2012; Ahamri *et al.*, 2017; Andrew *et al.*, 2017).

2.3.1.1 Hypertension

Hypertension has been established as a dominant risk factor for CVD (Landsberg *et al.*, 2013), with the presence of collagen stimulation, endothelial cell dysfunction and increased vascular stiffness (Wang & Bennett, 2012, Yeboah *et al.*, 2016) causing high blood pressure, affecting organs and vessels. As the diastolic pressure increases above 90 mm Hg and the systolic pressure increases to 140 mm Hg, hypertension (heart attack, stroke and death) can occur at this stage (Yeboah *et al.*, 2016; Andrew *et al.*, 2017). The elevation of blood pressure induces oxidative stress (on arterial walls), vascular dysfunction and thickening of ventricular wall, subsequently results to heart failure (Zafar, 2015; Koene *et al.*, 2016).

2.3.1.2 Smoking

Smoking tobacco or cigarettes have been strongly associated as a high risk factor of CVD (Koene *et al.*, 2016; Tyndall, 2017). The chemical constituents of cigarettes such as nicotine and carbon monoxide are known to be risk factors for cancer and CVD. Inhaled chemicals cause the oxidation of low density lipoprotein levels (LDL) in plasma triggering an inflammatory response, injuring squamous epithelium

cells, stimulating monocyte adhesion to arteries and vessel wall leading to the development of atherosclerosis (Perk *et al.*, 2012; Koene *et al.*, 2016; Andrew *et al.*, 2017).

Table1: Negative vascular effects of smoking (Erhardt, 2009)

Negative vascular effects	**Risk marker**
Vascular dysfunction	Increased vascular resistance Decreased vascular compliance Decreased nitrous oxide
Increased inflammation	Increased homocysteine levels Increased C-reactive protein (CRP) Increased fibrinogen levels Increased interleukin-6 (IL-6)
Progression of atherosclerosis	Increased low-density lipoprotein cholesterol (LDL-C) Decreased high-density lipoprotein cholesterol (HDL-C)
Increased plaque development	Increased triglycerides Increased Lipid peroxidation
Development of thrombi	Increased blood levels of catecholamines
Increased platelet activation and coagulation	Increased blood levels of fibrinogen
Increased plaque instability	Increased levels of metalloproteinases
Other potential mechanisms	Increased oxidized low-density lipoprotein cholesterol
Oxidative stress	Decreased nitrous oxide
Mitochondrial damage to heart muscle	Increased mitochondrial DNA damage

In Table 1, the pathophysiological effects of smoking as an increased risk of CVD in vascular dysfunction, inflammation, atherosclerosis, thrombosis, oxidative stress, mitochondrial damage, platelet activation and coagulation are summarized. The levels of nitric oxide are reduced at the initial stages of atherosclerosis due to smoking, producing carcinogens, irritants, oxidizing agents and proinflammatory stimuli. The suppression of apoptosis, enhancing angiogenesis and stimulating abnormal signaling pathways which leads to the development of cancer and CVD (Erhardt, 2009; Koene *et al.*, 2016).

2.3.1.3 Obesity

The life expectancy of obese individuals is reduced as they are at a higher risk of developing CVD (Mangge *et al.,* 2013; Félix-Redondo *et al.,* 2013). This is due to the fact that obesity is interlinked with metabolic risk factors such as inflammation, insulin resistance, low HDL cholesterol, small dense LDL and hypertension. Other risk factors affecting the levels of obesity leading to CVD are summarized in Table 2. These factors with adipocytokines results in metabolic regulation having a direct effect on artery wall and identified as a predictive risk factor for CVD (Upadhyay 2015).

Table 2: Cardiovascular risk factors associated with obesity CVD (Tzotzas *et al.*, 2011)

Risk factors	Levels in obesity	Modulators
Fibrinogen	Elevated	Visceral fat, inflammation, insulin resistance
High sensitivity CRP	Elevated	Visceral fat, inflammation, insulin resistance
Lipoprotein(a)	Unpredictable	Genetic factors
Small dense LDL	Elevated	Visceral fat, triglycerides, insulin resistance
Homocysteine	Probably elevated (weak association)	Dietary factors, adipose tissue, hyperinsulinism

The cardiovascular risk factors in Table 2 associated with obesity, increases the risk of developing CVD, with the deposition of adipose tissue, adipocyte dysfunction, visceral adiposity. Increasing the pro-inflammatory secretion of adipokine, affecting insulin resistance or diabetic complications and immune response. Stabilizing lipid metabolism and the angiogenesis of blood pressure, resulting to the development of CVD (Tzotzas *et al.*, 2011; Gondim *et al.*, 2015; Kim *et al.,* 2016; Patel *et al.*, 2016).

2.3.1.4 Diabetes

Diabetes as a CVR has a direct impact on vascular disorders such as nephropathy, retinopathy, stroke, coronary artery disease and peripheral vascular disease. These have an effect in the heart muscle resulting in both diastolic and systolic heart failure (Dokken, 2008; Zafar, 2015; Patel *et al.,* 2016; Andrew *et al.,* 2017). A lack of insulin will cause an increase in glucose levels in the blood causing damage to the arteries (Steyn, 2007; Zafar, 2015; Koene *et al.,* 2016). Additional cardiovascular risk factors (as summarized in Table 3) varies between diabetes mellitus type 1 (DMT1) individuals as compared with diabetes mellitus type 2 (DMT2) individuals. Whereby Diabetes type 1 individuals are at a higher risk of developing atherosclerosis as compared to individuals of Diabetes type 2. This is because of elevated levels of glycemia modifying the proinflammatory state (Ferranti *et al.,* 2014). Diabetes is a severe risk factor when combined

with other associating risk factors. With dyslipidemia which is more prominent in diabetes type 2 individuals. This increases the risk of CVD by increasing the levels of triglycerides, postprandial lipidemia and low levels of high density lipoprotein cholesterol (Upadyhyay, 2015).

Table 3: Comparison of cardiovascular risk factors between type 1 and type 2 diabetes (Ferranti *et al.*, 2014)

Risk factors	Diabetes type 1	Diabetes type 2
Hypertension	+++*	++
Cigarette smoke	++	++
Inflammation	++	++
High LDL-C	+	+++
Low HDL-C	0*, +	++
Triglycerides	No record	++
Microalbuminuria	+++	+++
Insulin resistance	+	+++
Poor glycemic control	+++	+++

***Ranges from 0 to 3 (+++)**

Diabetes as a risk factor for CVD is associated with the combination of other risk factors (Table 3). These associations are linked to the development of atherosclerosis, damage of microvascular vessels resulting, endothelial cells abnormalities and platelet function (which activates the production of several pro-thrombotic factors). These activation increases the risk of CVD, whereby individuals of diabetes type 1 are at greater risk as compared to diabetes type 2 individuals (Ferranti *et al.*, 2014; Zafar, 2015; Kim *et al.*, 2016; Andrew *et al.*, 2017).

2.3.1.5 Abnormal serum lipid

Abnormal serum lipid comprises of total cholesterol (LDL-C and HDL-C) and triglycerides. Additionally, other risk factors associated with abnormal serum lipid such as smoking, hypertension family history and diabetes are predictive risks of CVD (Upadyhyay, 2015). As lipids are transported in the bloodstream,

lipoproteins are form due to binding of bloodstream with specific apoproteins. This may cause modification affecting the circulation rate of lipids. Thus, risk factors of abnormal lipoprotein associated with CVDs are increased LDL-C, decreased HDL-C and increased abnormal lipoprotein levels (Kumar *et al*. 2010; Patel *et al.,* 2016). This diffusion of abnormal serum lipid into the circulatory system is the major cause of atherosclerosis, forming plaques in blood vessels. The presence of these plaques results in muscle wall thickness, obstruction of blood flow to the heart and causing heart failure (Sayols-Baixeras *et al.,* 2014; Siti *et al.,* 2015; Patel *et al.,* 2016; Andrew *et al.,* 2017).

2.3.1.6 Physical inactivity

Physical inactivity has been reported to be a risk factor for developing CVDs (Huma *et al.,* 2012: Kim *et al.*, 2016; Andrew *et al.,* 2017), in association with other risk factors such as obesity, stress, diabetes, hypertriglyceridemia, ageing, smoking, decreased HDL-C and hypertension (Kumar *et al*. 2011; Zafar, 2015). The association between physical inactivity and risk factors will lead to visceral fat accumulation with the infiltration of adipose tissue releasing adipokines. A low grade systemic inflammatory state will be established, leading to the development of atherosclerosis, tumour growth, insulin resistance and neuro-degeneration (Gleeson *et al.,* 2011; Koene *et al.*,2016). Additional, physical inactivity also favours weight gain, high blood pressure, glucose levels, inflammation and reduction of vascular wall, contributing to the formation of atherosclerosis (Gondim *et al.,* 2015; Koene *et al.,* 2016). Regular exercise or physical activity has been proven beneficial in the modification of CVR markers and other risk factors such as diabetes, obesity, hypertension and dyslipidemia (Blonde *et al.,* 2015; Gondim *et al.,* 2015).

2.3.1.7 Inflammatory markers

Inflammatory markers have been used as quantitative indicators for CVR and include, C-reactive protein, fibrinogen, interleukin-2 (IL-2), tumor necrosis factor (TNF), homocysteine, hypercoagulability, albuminuria, cystatin-C, elevated lipoprotein, leukocyte and monocyte counts (Arboix, 2015). C-reactive protein leads to atherosclerosis by the selective binding in serum with very low lipoprotein density, amplifying plaque formation, with the presence of antibodies which creates an immune response, inducing the adhesion molecules expression and synthesis of nitric oxide modulation (Biasucci *et al.,* 2013). A high level of homocysteine increases platelet adhesiveness, increases LDL-C oxidation, vascular wall deposition, endothelial cell lesion, direct activation of coagulation and vascular smooth muscle growth (Leal *et al.,* 2013). Leukocytosis is associated with CVD by inflammatory processes, which causes proteolytic and oxidative damage of endothelial cells, and connects microvasculature to activates

hypercoagulability resulting to myocardial infarction (Shivappa *et al.*,2015). Elevated levels Factor VII have been found to be associated with the increased risk of developing CVD (Campo *et al.*, 2013) together, with the presence of additional risk factors (smoking, gender and age) (Turfan *et al.*, 2014). Increased levels of fibrinogen cause muscle stiffness due to changes in viscoelastic properties of fibrin clots leading to CVD (Martinez *et al.*, 2013). The modulation of endothelial activities also increases the risk of CVD by promoting the proliferation and migration of muscle cells (Martiskainen *et al.*, 2014). Plasminogen activator inhibitor 1 (PAI-1), D-dimer and von Willebrand factor (VWF) levels are inflammatory markers associated with CVD. They catalyse the conversion of plasminogen to plasmin as regulators of endogenous fibrinolysis. The activation of coagulation and fibrinolysis with D-dimer levels as a marker, activates the fibrin clot formation and clot dissolution (Willeit *et al.*, 2013). PAI-1 increases the risk of atherosclerosis by decreasing fibrinolysis which inhibits the tissue type plasminogen activator (tPA) and urokinase type plasminogen activator (uPA) (Schenkein & Loos 2013). Elevated lipoprotein, albuminuria and Cystain-C are risk factors for CVD, by damaging vascular wall, forming the proatherogenic and prothrombotic complex, which intensifies the deposition of cholesterol in the arteries, leading to the formation of atherosclerotic plaque (Arboix, 2015). Interleukin-6 leads to the formation of atherosclerotic plaque which causes arterial wall stiffness resulting in CVD by the activation of endothelial synthesis of hepatic high sensitivity C-reactive protein, adhesion molecules and procoagulant effects (Shivappa *et al.*, 2015).

2.3.2 Non-modifiable risk factors

They are risk factors which cannot be changed. These include age, gender, genetics and race (Ouyang *et al*., 2010; Andrew *et al.*, 2017).

2.3.2.1 Age

Age increases the risk of CVD and death is more likely to occur with individuals of 65 years and above (Huma *et al.*, 2012; Keates *et al.*, 2017). As an individual gets older the risk of CVD increases, with the level of cholesterol and blood pressure which is higher in older people than younger ones leading to the development of CVD (Andrew *et al.*, 2015). Gradual loss of arterial elasticity is encountered with ageing leading to vascular stiffness and hypertension. This is due to increased collagen deposition and disintegration of the elastin lamellae, initiating vessel dilation and enlargement of the lumen (Wang & Bennett 2012; Ferruzzi *et al.*, 2016). The cardiovascular system undergoes structural, thermodynamic changes, oxidative stress and endothelial dysfunction with age. These changes contribute to the exposure of atherogenic risk factor causing CVD (Zafar, 2015).

2.3.2.2 Family History

Family history of chronic diseases, coronary heart disease and diabetes have been used as a diagnostic parameter for CVD, since these diseases have been proven to be strongly associated with CVD (Dhiman *et al.*, 2014). Inherited diseases are susceptible to cardiovascular disorder (atherosclerosis) with progression of vascular ageing (Wang & Bennett 2012). Individuals suffering from stroke are at a higher risk of developing CVD, because of the alteration of genes, genetic transmission of susceptibly to stroke, environmental factors, family related sedentary life style, and inherited predisposition of stroke (Arboix, 2015). Moreover, inherited genes transmitted from first and second generation promote complex gene disorders, with the environmental interaction which causes gene mutation, making them more susceptible for the development of CVD (Imes & Levis 2014) with the inheritance of weak blood vessels as a predisposing factor of Stroke. Other risk factors associated with CVD of family origin causes arteriosclerosis by stiffness of blood vessels, plaque formation and elevated blood pressure (Werner, 2016). Causing vascular tissue damage, disrupting cardiac muscles and reducing oxygen supply resulting in myocardial infarction (ischaemic necrosis) (Moroba *et al.*, 2015).

2.4 HAEMOSTASIS

Haemostasis is an efficient and complex process involved in balancing the circulation of blood throughout the body. This process maintains blood flow during its passage into the vascular system, waiting to form a clot when there is tissue injury preventing excessive bleeding (Palta *et al.*, 2014). Haemostasis could also be defined as a protective process involved in maintaining the stability of a compact high pressure circulatory system following vessel injury. When the vessel is damaged together with the effusion of circulating blood, the process of clot formation is rapidly activated (Kretz *et al.*, 2015). Three types of haemostastic responses are involved following a vessel rupture. These include primary haemostasis, which consists of blood vessel contraction with platelet plug formation, secondary haemostasis involves the activation of coagulation cascade, deposition and stabilization of fibrin, tertiary haemostasis also known as fibrinolysis, comprises of dissolution of fibrin clot and dependent on plasminogen activation (Kristianto 2012, Periayah *et al.*, 2017).

When blood vessels rupture's (vascular injury) as indicated in Figure 2, circulatory platelets at the site of the injury are exposed, leading to adhesion and activation of platelets, resulting in platelet aggregation forming a stable haemostatic clot (Moss 2013; Tosenberger *et al.*, 2015). This reaction takes place in two different stages, known as the primary and secondary stages.

The primary stage involves platelet adhesion to the ruptured blood vessel, with platelet activation mobilizing the availability of more platelets at the site of vessel injury, allowing the formation of a platelet plug to stop the excessive blood flow (Faxälv, 2009; Periayah *et al.*, 2017). Upon platelet activation, intracellular vesicles and dense granules are triggered, with the secretion of adhesive proteins (fibrinogen and VWF). Adenosine diphosphate and serotonin (second wave mediators) are also secreted from dense granules, which release thromboxane A_2 to retain and activate more platelets in thrombus formation. The second stage is a coagulation process, which is composed of platelet aggregation resulting in the formation of a haemostatic plug, which is activated when exposed to tissue factors following an injury (Faxälv 2009; Palta *et al.*, 2014). The presence of tissue factors initiates blood coagulation to activate the development of thrombin and fibrin under normal situations in maintaining the formation of a thrombus temporally and spatially (Furie & Furie 2008; Moss 2013; Long *et al.*, 2016). The effusion of blood into the tissues and vasoconstriction help in narrowing of blood vessels at the site of injury in order to reduced blood flow (Beck, 2009; Moss, 2013).

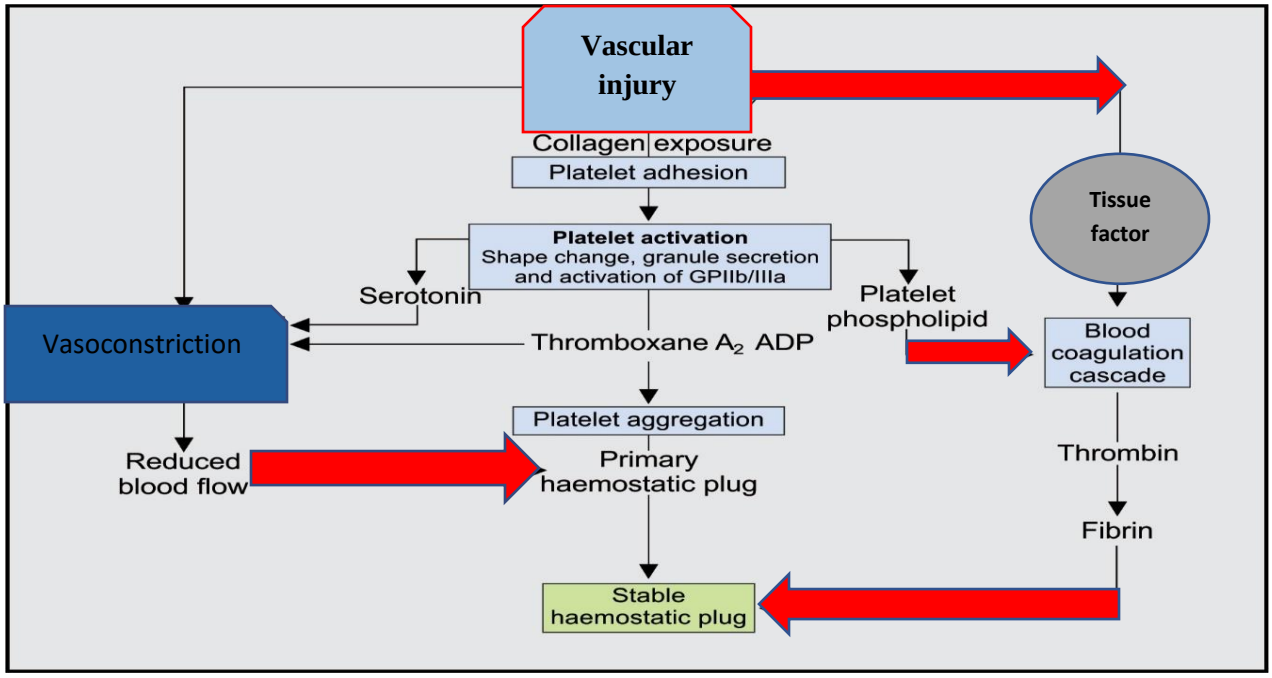

Figure 2: Illustrating the process of haemostasis (modified from Hoffbrand & Moss 2011)

2.4.1 Vascular System

The haemostatic response to vessel injury is very fast and occurs by localizing the site and adjusting the mechanism by vasoconstriction (Vascular spasm), platelet plug formation and the formation of a clot converting fibrinogen to fibrin (Subhasish *et al.,* 2010; Chapin & Hajjar 2015). Following an injury, vasoconstriction will take place to reduce and limit the amount of blood loss. This action is activated by vascular smooth muscle, platelets, endothelial cells releasing chemicals and local pain receptors activated by reflexes (Leticia *et al.,* 2014). The presence of platelets in vascular circulation is responsible for the myogenic contraction by releasing thromboxane A2 (TXA2), a vasoconstrictor substance formed by the direct damage of the vascular wall. The next step following vasoconstriction is the mechanical occlusion of a platelet plug at the site of injury (Subhasish *et al.,* 2010, Periayah *et al.,* 2017). The endothelial structure such as the basement membrane, collagen and microfibrils becomes exposed to the surface which is bound with VWF. This VWF in circulation binds to glycoprotein 1b, causing the initial monolayer of platelets adhesion (Leticia *et al.,* 2010), Palta *et al.,* 2014). Platelet adhesion becomes activated, and cytokines are released to the site of injury. Platelet aggregation is due to platelet factors which strengthen vasoconstriction and the activation of further platelets to bind to each other forming a platelet plug (Subhasish *et al.,* 2010, Periayah *et al.,* 2017). When platelets are being exposed to collagen fibres at the site of vessel injury, compaction of platelets will be formed, making them thicker to seal the injured vessel by releasing adenosine diphosphate (ADP) as a chemical messenger (Leticia *et al.,* 2010, Ghoshal & Bhattacharyya 2014). More so, the presence of protein-phospholipids as a tissue factor activates the last step of vasoconstriction by a series of reactions recognized as a coagulation cascade, which is a sequence of enzymatic reactions which causes the conversion of fibrinogen to fibrin forming a mesh that maintains the platelet plug and will be discussed in sub-section 2.4.3 (Subhasish *et al.,* 2010, Moss, 2013, Ghoshal & Bhattacharyya 2014; Chapin & Hajjar 2015).

2.4.2 Platelet

Platelets are described to be small non-nucleated cells which circulate in the vascular system, with the intension of stopping bleeding by the rapid formation of platelet plug at the site of injury (Faxälv, 2009, Ghoshal & Bhattacharyya 2014; Kretz *et al.,* 2015). Platelet ranges in concentration of 150 to 350 x 10^9/L, with an average lifespan of 7 and 10 days in circulation (Faxälv 2009, Ghoshal & Bhattacharyya 2014, Moreau *et al.,* 2015, Simak & Paoli 2017). Platelets are developed in the bone marrow as cells of the hematopoietic series known as megakaryocytes (Periayah *et al.,* 2017), which consist of large cells having multilobate nuclei which are obtained from endomitotic synchronous activities (DNA replication with lack of nuclear or cytoplasmic division). The origin of megakaryocytes exist in two stages, burst-forming unit

(BFU) and colony forming unit (CFU), which are all very sensitive to a different set of cytokines. When the CFU matures, it leads to the formation of identifiable megakaryocytes (Subhasish *et al.,* 2010; Machlus & Jr 2013, Woolthuis & Park 2016).

Although platelets do not have nuclei and cannot reproduce, they carry out many functional characteristics of the whole blood cells. They act as active factors in the cytoplasm as contractile proteins, with thrombosthenin which precipitates the contraction of platelets. In the endoplasmic reticulum and Golgi apparatus, platelets synthesize many different enzymes and store a large amount of calcium ions. Platelets form adenosine triphosphate (ATP) and adenosine diphosphate (ADP) in the mitochondria with the presence of systemic enzymes, which also complement prostaglandins causing tissue and vascular reactions. More so, platelets bring about the multiplication and cellular growth of vascular endothelial cells, vascular smooth muscle cells and fibroblasts with the presence of fibrin stabilizing factor, which facilitates vascular wall damage repairs (Periayah *et al.,* 2017).

Platelet activation and interactions with the sub-endothelial matrix, blood cells and other platelets are facilitated by the presence of functional receptors found on the surface of platelets and granules which fuses on the platelet membrane upon activation, releasing their content to the encompassing media. These granules are classified into larger α-granules, smaller dense granules and lysosomes. The α-granules containing an enormous variety of biologically active substances takes part in the process of platelet activation, platelet adhesion molecules, plasma coagulation factors and fibrinolysis proteins (Faxälv, 2009; Palta *et al.,* 2014). More so, α-granules also function as main storage in haemostasis by containing coagulation factors such as factor V, factor VIII, fibrinogen, VWF, plasminogen, PA1-1, a2-antiplasmin, with proteins membrane such as glycoprotein IIb/IIIa (GIIb/IIIa) and P-selectin (Batty & Smith 2010; Moreau *et al.,* 2015).

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2.4.3 Coagulation Cascade

Coagulation is the process that involves the formation of blood clots to prevent excess loss of blood from the vessels, organs and damaged tissues (Faxälv 2009; Moss 2013; Palta *et al.,* 2015; Periayah *et al.,* 2017). The coagulation cascade is a sequential process which is very complex and occurs in vivo (in the body) when blood vessels are injured. Coagulation can also occurs in vitro (outside the body) when coagulation testing is performed in the laboratory. The coagulation cascade involves three pathways, the intrinsic pathway, the extrinsic pathways, and the common pathway (Figure 2) (Palta *et al.,* 2014).

The extrinsic pathway (Figure 3) of the cascade occurs within membrane having a high content of phosphatidylserine. This pathway (extrinsic) begins when the tissue factor (TF) comes in contact with blood due to trauma or vessel injury. Circulatory blood contains FVII in a low concentration which binds to the tissue factor. This binding action of TF-FVII activates FVII (FVIIa). FVIIa binds to the factor X and activates it to FXa. FXa converts prothrombin (FII) to thrombin (FIIa). FX can be also activated by FIXa, a cofactor of FVIIIa in the intrinsic pathway. Thus, FIXa and factor Xa are the points of convergence between the intrinsic and extrinsic pathways (Ott, 2011; Moss, 2013; Long *et al.,* 2016). FXa complex with the cofactor prothrombinase (FVa) activates prothrombin to thrombin which results in the formation of a clot (fibrinogen to fibrin clot or FI/FIa complex). Thrombin (FIIa) will amplify coagulation further by activating the co FV, FVIII and FXI zymogen in the intrinsic pathway (Tanaka *et al.,* 2009; Moss, 2013; Sweeney, 2013; Palta *et al.,* 2015; Periayah *et al.,* 2017).

The intrinsic pathway begins with the contact activation pathway which initiates the formation of a primary complex on collagen by Prekallikrein, high molecular-weight kininogen, and FXII (Hageman factor). Prekallikrein is converted to kallikren (Moss, 2013; Palta *et al.,* 2014; Long *et al.,* 2016; Periayah *et al.,* 2017) This activates FXII to FXIIa. The FXIIa now converts FXI to FXIa. Factor XIa will activate FIX with FVIIIa as a co-factor to tenase complex of phospholipid membrane of activated platelets. This tenase complex converts FX into FXa which together with FVa form the prothrombinase complex. This FXa/FVa complex will increase the concentration of thrombin level, which will initiate the fibrin network formation (Faxälv, 2009; Moss, 2013; Long *et al.,* 2016).

In the final common pathway, thrombin will convert fibrinogen to fibrin, which is the building block of a haemostastic plug. In addition, this hemostatic plug will activate FVIII and FV with their inhibitory protein C, protein S together with the activation of FXIII. This will form covalent bonds that will crosslink the fibrin polymers formed from an activated monomer (Ott, 2011; Sweeney, 2013; Palta *et al.,* 2014; Periayah *et al.,* 2017).

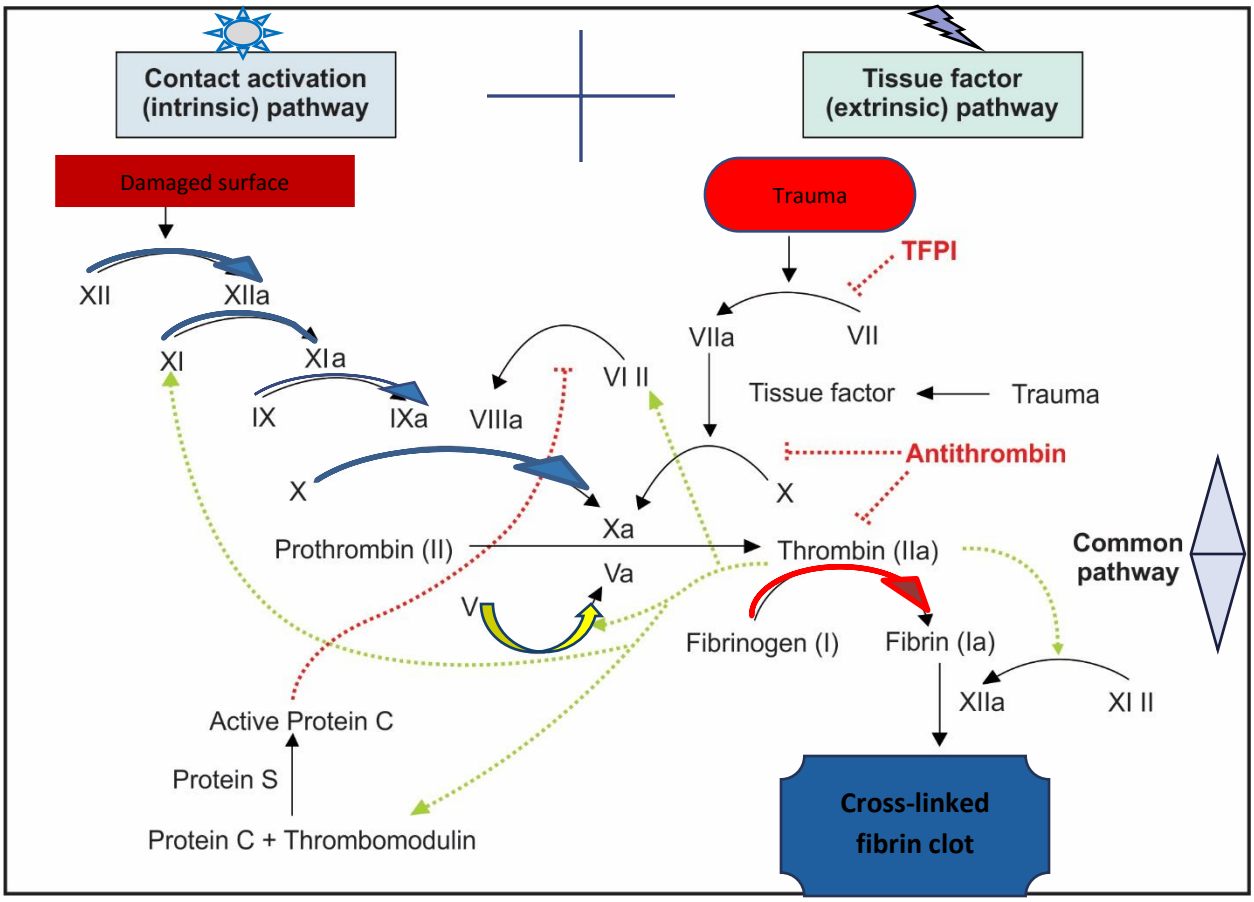

Figure 3: Steps involved in the coagulation pathway (modified from Peters *et al.*, 2013 & Sweeney 2013)

The coagulation pathway is composed of three pathways; extrinsic, intrinsic and common pathway. The extrinsic pathway begins with TF coming into contact with blood following an injury of a vessel. The presence of FVII in circulation is activated by TF forming the TF-VII complex to VIIa, which binds to FX to become FXa. This process of binding and activation continue till the conversion of prothrombin (FII) to thrombin (FIIa), with the convergence between the intrinsic and extrinsic pathways with FIXa and FXa (Long *et al.*, 2016). In the intrinsic pathway, the activation pathway begins, forming the primary collagen complex Prekallikrein. Converting to kallikren, which activates FXII to FXIIa to convert FXI to FXa till the formation of mesh framework of fibrin (FXa/FVa complex) (Periayah *et al.*, 2017). Thrombin converts fibrinogen to fibrin at the final common pathway, forming a haemostatic plug (Sweeney, 2013).

Table 4: Summarizes the primary coagulation factors, nomenclature, the pathways involved and their physiological functions

Clotting Names	Clotting Factors	Pathways	Functions
Fibrinogen	Factor I	Common	Serve as an adhesive protein forming the fibrin clot (Roghani *et al.*, 2014).
Prothrombin	Factor II		Assists tissue factor and contact activaton pathways by prothrombinase complex (Kaushansky *et al.*, 2010; Periayah *et al.*, 2017).
Tissue factor	Factor III	Extrinsic	Initiates the process of coagulation (extrinsic) by binding to factor VII (VIIa) (Ott, 2011; Moss, 2013).
Calcium	Factor IV	Common	It serves as a metal cation necessary for coagulation (Roghani *et al.*, 2014).
Labile factor	Factor V	Common	Cofactor in the activation of prothrombin by factor Xa (Hoffbrand *et al.*, 2011; Moss, 2013).
Stable factor	Factor VII	Extrinsic	Activates factor XI and factor X in its VIIa/TF complex, initiating the extrinsic pathway (Kaushansky *et al.*, 2010; Periayah *et al.*, 2017).
Antihemophilic factor A	Factor VIII	Intrinsic	When in it active form, factor VIIIa serves as a cofactor in the activation of factor X by factor IXa. A deficiency of VIII is caused by haemophilia A (Moss, 2013).
Antihemophilic factor B	Factor IX	Intrinsic	Activates factor X when activated by factor VIIa/TF complex or factor XIa (IXa) (Kaushansky *et al.*, 2010; Periayah *et al.*, 2017).
Stuart Prower factor	Factor X	Common	Activates prothrombin (prothrombinase complex) in its active form (FXa) with FVa (Hoffbrand *et al.*, 2011; Moss, 2013).
Antihemophilic factor C	Factor XI	Intrinsic	Activated by factor XIIa, thrombin on activated surface of platelets. More so, FXI is essential in the process of hemostasis (in vivo) and

			thrombosis (Kaushansky *et al.,* 2010; Moss, 2013).
Hageman factor	Factor XII	Intrinsic	Initiates the process of aPTT (Roghani *et al.,* 2014).
Fibrin stabilising factor	Factor XIII	Common	It's cleaved to thrombin when activated in presence of Ca^{2+}, crosslinking of fibrin to maintain fibrin plug, protects fibrin from fibrinolysis (Kaushansky *et al.,* 2010; Moss, 2013).
von Willebrand factor			Carrier of FVIII, linking platelets and subendothelium. Enables binding of protein (FVII) (Hoffbrand *et al.,* 2011; Periayah *et al.,* 2017).
Protein C		Common	Controls the process of coagulation by the inactivation of factor Va and VIIIa (Kaushansky *et al.,* 2010; Palta *et al.,* 2014; Chapin & Hajjar 2015).
Protein S		Common	Deactivates factor Va and FVIII as a cofactor of activated protein C (Palta *et al.,* 2014).
Thrombomodulin		Common	Bind with thrombin forming a complex; prevent the binding of protease to substrates and protease activated receptors. Binds to Protein C and serves as an anticoagulant (Hoffbrand *et al.,* 2011; Woolthuis & Park 2016).
Antithrombin III		Common	Serves as inhibitors is targeting protease (Kaushansky *et al.,* 2010; Sweeney, 2013).
Prekallikrein (PK)	Fletcher factor	Intrinsic	Participates in aPTT based at the beginning of the intrinsic pathway in the active form (Roghani *et al.,* 2014).
High molecular weight kininogen (HMWK)	Williams factor	Intrinsic	Serve as a coagulation protease inhibitor. Binds and inhibits factor VIIa/TF complex, activating FIX (Kaushansky *et al.,* 2010; Moss, 2013).

2.4.4 Blood Coagulation inhibiting factor

Regulatory factors of the coagulation cascade includes; inhibiting factors, blood flow and fibrinolysis. Thrombosis will occur if these regulatory factors are not activated. Tissue Factor Pathway inhibitor (TFPI) is present in plasma, synthesized in the endothelial cells and it the first inhibitor to react and accumulates at the site of injury due to platelet activation (Hoffbrand & Moss 2011; Cate *et al.,* 2017). TFPI regulates the process of haemostasis by binding to Xa which is present in plasma and, this action results in deactivating the enzymatic activity of Xa, forming the TEPI-Xa complex. The complex (TEPI-Xa) now combines with FVIIa (membrane associated tissue factor), hindering the enzymatic activity of FVIIa (Subhasish *et al.,* 2010; Periayah *et al.,* 2017). Another inhibitor involved in coagulation is antithrombin (AT). It is a protein which inactivates the enzymatic activity of thrombin, in conjunction with the adherence of heparin which intensifies the activity of AT (Faxälv, 2009; Palta *et al.,* 2014). Serine protease inhibitors present in circulation as proteins, act as pretentious substrates which are distinct enzymes, forming covalently linked complexes across the serine active site targeting enzymes together with the susceptible bond present in the inhibitor (Subhasish *et al.,* 2010; Chapin & Hajjar 2015). Protein C and protein S exist as inhibitors of FV and VIII as cofactors in the process of coagulation, with thrombin binding on the surface of endothelial cell receptor (thrombomodulin). This binding action leads to the activation of the vitamin K (independent serine protease protein C), capable of destroying the activated FV and FVIII preventing the formation of more thrombin (Hoffbrand *et al.,* 2011; Palta *et al.,* 2014; Cate *et al.,* 2017).

2.4.5 Fibrinolytic Pathway

The fibrinolytic pathway plays an important role in the breaking of clots and stabilizing an open vascular movement. This is achieved by the removal of fibrin from the vascular circulation through its degradation within the thrombus (Russo, 2012; Phelan & Kerins 2015; Chapin & Hajjar 2015). Thus, this pathway restores blood flow from occluded vessels. The mechanism involved in the removal and breaking of clots is known as fibrinolysis. Fibrinolysis is the process through which fibrin is broken, by ensuring the localization of fibrin clot formation and removal gradually as the vessel heals in order to restore normal blood flow (Batty & Smith 2010; Moss, 2013; Chapin & Hajjar 2015). This pathway (Figure 4) is activated by the conversion of the pro enzyme plasminogen to active proteolytic enzyme plasmin. Plasmin further breakdown fibrin into soluble fibrin degradation products, mediated by one of the two plasminogen activators, tPA and uPA (Krone *et al.,* 2010; Palta *et al.,* 2014).

tPA is a strong activator synthesized and secreted by the endothelial cells, with fibrin converting the zymogen plasminogen to its active form plasmin (Faxälv, 2009; Palta *et al.*, 2014; Phelan & Kerins 2014). tPA is also induced by the presence of histamine, adrenaline, thrombin, venous occlusion and exercise and results in the conversion of zymogen plasminogen to activate plasmin. Meanwhile uPA is commonly found in urine, and it is a single chain zymogen (pro-unikinase) which is expressed by macrophages, renal epithelial cells, endothelial cells and some tumour cells in activating either kallikrein or plasmin (Batty & Smith 2010; Duffy *et al.,* 2014; Palta *et al.*, 2014). uPA is independent of the presence of fibrin and converts plasminogen into plasmin. Plasminogen activators (tPA and uPA) occur as single chain precursor molecules, single chain tissue plasminogen activator (sctPA) and single chain urokinase plasminogen activator (scuPA), which take part in the enzymatic cleavage of plasmin (Faxälv, 2009; Chapin & Hajjar 2015).

The presence of inhibitors in plasma such as plasminogen activator inhibitors type (PAI-1 and PAI-2), alpha 2-antiplasmin (α2-AP) and thrombin activatable fibrinolysis inhibitor (TAFI) are associated with the regulation of the fibrinolytic pathway (Russo, 2012; Phelan & Kerins 2015). PAI-1 secreted from the endothelial cells and, acts as the main activator in the inhibition of plasminogen activity in opposition to non-fibrin bound tPA, uPA and plasmin. Alpha 2-antiplasmin is a serine proteinase, which function as an inhibitor of plasmin. TAFI is a non-serine proteinase, activated by a thrombin-thrombomodulin compound, which removes the binding site of lysine on fibrin for plasminogen and t-PA. Leading to the inhibition of fibrinolysis (Batty & Smith 2010; Palta *et al.,* 2014).

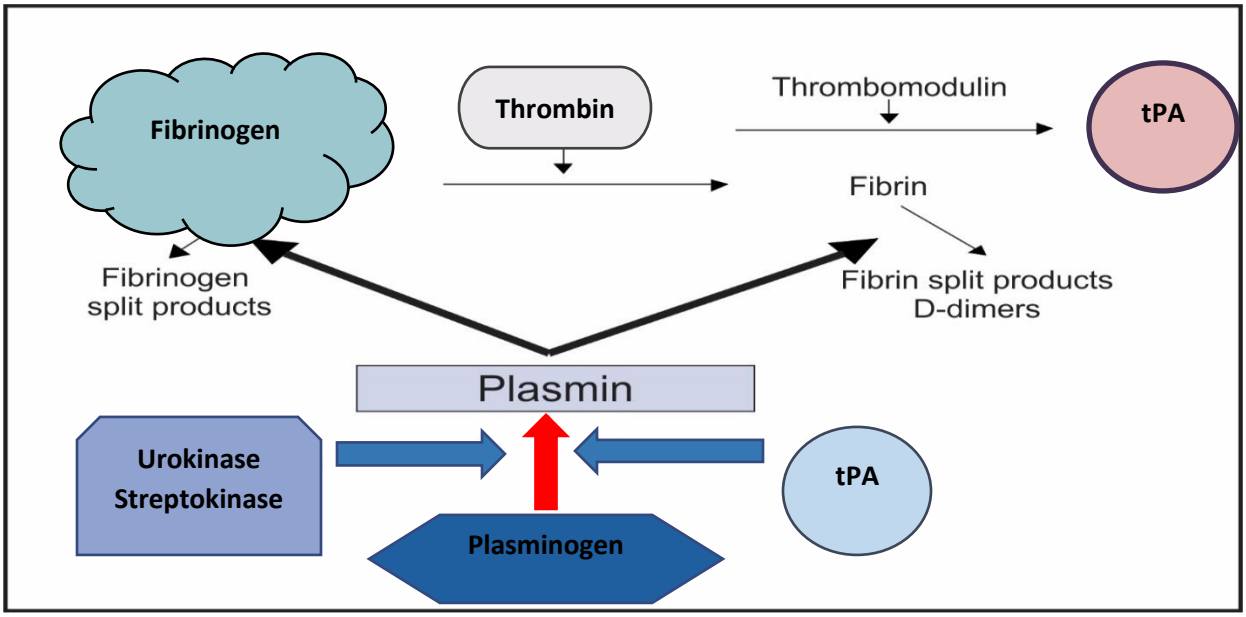

Figure 4: Fibrinolytic pathway (modified from Makar *et al.*, 2010)

The fibrinolytic pathway is initiated by the modification of plasminogen (pro-enzyme) to plasmin (active proteolytic enzyme), which degrades fibrinogen and fibrin into soluble degradation products. This reaction occurs in the presence of the plasminogen activators (tPA and uPA) and streptokinase. The presence of thrombomodulin with thrombin helps in the activation of t-PA, with thrombin as an important regulator of both coagulation and fibrinolytic pathways (Makar *et al.,* 2010; Chapin & Hajjar 2015). The fibrinolytic pathway participates in the development of arterial thrombosis by TAFI circulating in an inactive form. When TAFI is activated by thrombin/thrombomodulin complex, fibrinolytic activity is reduced by the removal of carboxy-terminal lysine residues from the slightly degenerated fibrin polymers resulting in a decreased binding between the fibrinolytic components plasminogen and tissue type plasminogen activator. Thus, TAFI modifies the fibrin clot to become more resistant to clot dissolution (Schmidt, 2011; Palta *et al.,* 2014). This is because of impaired clot dissolution resulting in thrombosis, with the production of auto antibodies. These auto antibodies are either directed against fibrinolytic receptor component or plasminogen activators leading to thrombosis (Chapin & Hajjar 2015).

2.5 FIBRINOGEN

Fibrinogen is a plasma protein involved in stabilizing the process of haemostasis and formation of clots (Levy *et al.,* 2012; Moss 2013; Palta *et al.,* 2014). This plasma protein is produced from hepatic cells, which are activated by thrombin forming fibrin macromolecules (Siegerink *et al.,* 2009; Martiskainen *et al.,* 2014). Fibrinogen macromolecules are composed of three polypeptide chains pairs known as alpha (α), beta (β) and gama (γ) (Figure 5) which are also involved in blood coagulation, thrombosis and defense mechanism (Vazquez *et al.,* 2011; Chapin & Hajjar 2015). These three polypeptide chains of the fibrinogen genes are positioned on the short arm of chromosome 1 (Kretz *et al.,* 2015). Fibrinogen is covalently bonded by coagulation factor XIIIa forming fibrin fibres, which are the principal components of clot formation (Siegerink *et al.,* 2009; Moss, 2013; Chapin & Hajjar 2015) (as described in 2.4.3).

2.5.1 Chemical structure

Fibrinogen has a rod-shaped structure with a long axis of 40nm, which is highly flexible and abundant in plasma glycoprotein (Khot *et al.*,2014). This rod shape structure exhibits a physiological pH of 5.2 of negative net charge with dimensions of 9 nm, x 47.5 nm, x 6 nm (Marucco *et al.,* 2013) and, size of the fibrinogen glycoprotein is 340-Kda. The chemical structure of fibrinogen is composed of two terminal global regions as indicated in figure 5 (D and E domain or region). These global regions consist of three polypeptide chains such as α, β and γ (Lord, 2011; Martinez *et al.,* 2013; Chapin & Hajjar 2015), varying

in diameter of 6.7, 5.3 and 6.7, respectively and connected to two terminal global regions of 1.5nm (Ciesla & Barbasz 2013). Polypeptide chains (α, β and γ) are connected to 29 disulphide bonds, which create a long dimer protein (αβ γ) of 45nm. At the end of the α helical coiled-coil is the point of binding of the three polypeptide chains (Park & Khang 2012; Martinez *et al.,* 2013). The γ polypeptide chain have a high binding affinity to thrombin activity, affecting clot formation structure (Allan *et al.,* 2012; Cronjé *et al.,* 2016).

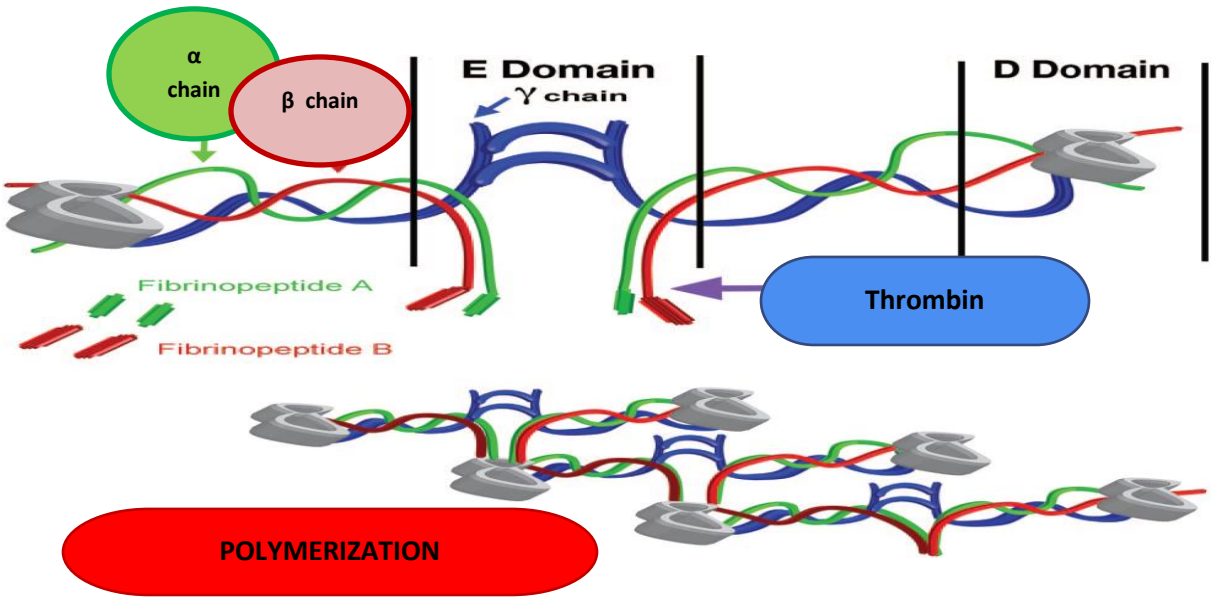

Figure 5: Structure of Fibrinogen (modified from Levy *et al.*, 2012)

As illustrated in Figure 6, the D domain is composed of the carboxyl termini consisting of polypeptide chains β and γ, known as β and γ nodules. The α polypeptide chain passes via the D domain, folding backward to connect to the coiled-coil configuration. The E domain is composed of the N terminus consisting of six chains, spreading from the center as two coiled coils. The coiled-coils are made of three chains, with each coil ending in the D domain globular region (Lord, 2011; Martinez *et al.*, 2013). Thus, the connection between the D and E domain depends on one of each of the three pairs of chains to be involved in the two coiled-coil regions (Averett & Mark 2009; Chapin & Hajjar 2015; Kattula *et al.*, 2017). The formation of fibrin is activated by thrombin (as described in 2.4.3), which releases fibrinopeptide A and fibrinopeptide B from the polypeptide chains α and β of the N terminal. The cleavage of fibrinopeptide A takes place before that of fibrinopeptide B. The cleavage of fibrinopeptide B occurs at a slower rate. The portion of the E domain known as the knobs of the N terminus sequences fuses into the hollow of the portion of the D domain (Hoffbrand *et al.*, 2011; Martinez *et al.*, 2013; Kattula *et al.*, 2017). When the cleavage of fibrinopeptide A occurs, polymerization is created into proto fibrils which roll unsteadily and extending

over fibrin units (Undas & Ariens 2011; Chapin & Hajjar 2015). This process of polymerization of proto fibrils integrates in the network of fibrin, which is flexible enough to permit, the cohesive mesh to be formed (Kollman *el al.,* 2009; Martinez *et al.,* 2013).

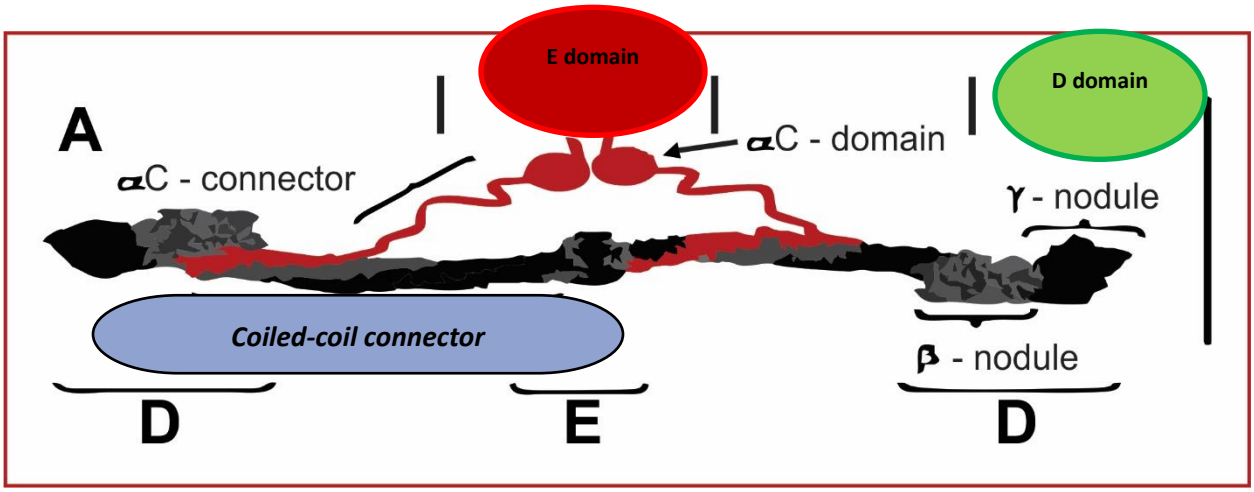

Figure 6: Coiled-coil configuration of fibrinogen (modified from Lord 2011)

When two terminal global regions combine, a coiled-coil configuration is formed. This connects the N-terminus to the central nodule by the disulphide bond to the E domain (Levy *et al.,* 2012; Marsh *et al.,* 2013). The D region is composed of β and γ polypeptide chains, meanwhile the E region is composed of α polypeptide chain, with the E domain containing six chains of the N-terminus (Figure 5) (Undas & Ariens 2011; Martinez *et al.,* 2013). The polypeptide chain α of the C terminus forms a smaller globular α C domain which extend apart from the molecule. At this point the polypeptide chains will each be involved in the two-coiled coil (Averett & Schoenfisch 2010; Duval & Ariëns 2016). This coiled region now establishes an α helical cord binding the D and E domains (Averett & Schoenfisch 2010; Hoffbrand *et al.,* 2011; Marsh *et al.,* 2013).

2.5.2 Physiology

The major role of fibrinogen is to facilitate the formation of a three-dimensional network of fibrin fibres, which is the main constituent of the structural basis of a clot formation (Moss, 2013; Tosenberger *et al.,* 2016). Fibrinogen as a plasma protein plays an important biological role in the stability of thrombosis and homeostasis. This is achieved by the conversion of fibrinogen to fibrin, leading to blood clot formation (as indicated in the extrinsic pathway of the coagulation cascade in Figure 3) (Hassan *et al.,* 2011; Wikkelsø *et al.,* 2013). Following a vascular injury, fibrinogen is very important in the stabilization of thrombi, inflammatory response and triggering foreign body reactions (Marucco *et al.,* 2013). The generation of

thrombi in plasma is increased by fibrinogen which binds with FXIII in circulation, increasing the formation of fibrin clot (Macrae *et al.,* 2016). The presence of fibrinogen and fibrin facilitates the conversion of plasminogen to plasmin (Chapin & Hajjar 2015). Fibrinogen also plays an important role in the mechanisms of homeostatic blood, influencing the process of red blood cell aggregation by determining the red cells deformities (Ghanbari-Niaki *et al.,* 2015). The polypeptide chains of fibrinogen regulate the production of clots, having thinner and branched fibres which increase the volume of clot stiffness containing a high affinity for thrombin binding sites, with inhibiting factors (FV, FVIII) and activated protein C sensitivity, protecting the degradation of thrombin as it acts as a sponge, with the presence of α2-macroglobulin and antithrombin (Duval & Ariëns 2016). Fibrinogen also plays a critical role in inflammation during the process of angiogenesis and wound healing. This occurs through the interaction of other cell types, endothelial cells and blood cells when in contact with extravascular space (Sörensen-Zender *et al.,* 2015). Furthermore, fibrinogen plays a pro-haemostatic function in controlling the distortion of blood in the aggregation of red blood cell formation (Ghanbari-Niaki *et al.,* 2015). Fibrinogen in circulation acts as an anticoagulant in thrombin activity inhibition, with the presence of gamma (γ) polypeptide chains which increases activated protein C plasma sensitivity in the formation of thrombin as well as in vitro analysis of activated partial thromboplastin time (APTT) (Omarova *et al.,* 2014).

2.5.3 Plasma levels

The normal plasma fibrinogen level varies between 2.0 and 4.5 g/L (Schlimp *et al.,* 2013; Waldén *et al.,* 2014). During situations of excessive bleeding experienced by traumatized patients, the recommended concentration for patients should be 1.5 to 2 g/l (Schlimp *et al.,* 2013). The moderate level of plasma fibrinogen produced daily by the liver is 2.0 to 4.5 g/l, at levels below 1 g/l, is an indication of fibrinogen deficiency (Wikkelsø *et al.,* 2013). At critical levels below 1.5-2 g/l, there is a higher susceptibility for perioperative and postoperative bleeding (Fries & Martini 2010; Waldén *et al.,* 2014). Furthermore, an increase level of 1 g/L above 4.0 g/L in plasma fibrinogen doubles the risk of CVD according to the conclusion made by the Fibrinogen Studies Collaboration 2007 (Grobler 2015).

2.5.4 Method of detection

The concentration of fibrinogen can be measured using analytical techniques such as turbidimetry on an automated coagulation analyzer, Thromboelastography and a Biosensor.

2.5.4.1 Turbidimetry

Plasma fibrinogen levels can be detected using turbidimetric techniques on automated coagulation analyzer (Klovaite *et al.,* 2013). The turbidimetric technique measures scattered light, which spread in all directions at different angles when in contact with the sample (Hou *et al.,* 2014). A photodetector is set at a specific angle as light is emitted through the sample, away from the incident beam. This method of detection is specific and utilizes the reduction of transmitted radiation as the particles are in suspension with the photodetector (Santos *et al.,* 2011; Bishop *et al.,*2013). As the plasma citrate sample is diluted in the buffer, highly concentrated thrombin is used for clotting activation (Kitchen *et al.,* 2009; Mohamed *et al.,* 2012; Solomon *et al.,* 2014). When light of specific wavelength passes across the plasma sample, the concentration is measured by the photodetector. Light scattering occurs as the fibrin strand is established, reducing the intensity of light descending on the photodetector, creating an increase in optical density which rises to a fixed variance. This action will stop the time, indicating the formation of a clot (Rodak *et al.,* 2012; Poon *et al.,* 2012; Rifai *et al.,* 2018). The clotting time is inversely proportional to the concentration of fibrinogen (Kitchen *et al.,* 2009; Oberfrank *et al.,* 2016). The modified Clauss method is commonly used in determining fibrinogen concentration (Schlimp *et al.,* 2013; Wikkelsø *et al.,* 2013). This technique is based on the preparation of a standard curve by the addition of diluted plasma standard of known fibrinogen concentration to a highly concentrated thrombin for clotting to take place. The clotting time of the diluted plasma is used to determine the concentration of fibrinogen from the standard curve, as the clotting time is inversely proportional to the concentration of fibrinogen (Ameri *et al.,* 2011; Poon *et al.,* 2012; Oberfrank *et al.,* 2016). The conversion of fibrinogen to fibrin polymer is achieved by the catalyzing reagent bovine thrombin when added to platelet poor plasma (Poon *et al.,* 2012; Solomon *et al.,* 2014). Owren buffer is used to dilute the platelet poor plasma in a 1:10 ratio. An inverse relationship between the clot formation interval and concentration of functional fibrinogen will be established. This is due to the concentration of the reagent and the diluted platelet poor plasma. The results obtained from the interval of clot formation are compared with the plasma dilution reference (Rodak *et al.,* 2012; Solomon *et al.,* 2014).

A. Advantages of Turbidimetry

- Turbidimetry techniques are accurate when analyzing coagulation end-point and it's mostly applied in haemostatic surveys laboratories (Yao *et al.,* 2013).
- On the basis of an analytical technique, turbidimetry analysis is sensitive (Bishop *et al.,* 2013).
- The modified Clauss method is very accurate, précised, reliable and is adjusted easily to automated coagulation analyzers (Ameri *et al.,* 2011; Solomon *et al.,* 2014).

B. Disadvantages of Turbidimetry

According to Hach (2013) the following are the disadvantages of turbidimetry;

- False positive result and a negative projection might be encountered when the instrument is susceptible to adjustment of pressure and changes in flow.
- Air bubbles generated from the agitation of suspended particles of higher density are difficult to measure and will lead to false positive results.

2.5.4.2 Thromboelastography

Fibrinogen levels can be determined by the application of a thromboelastography technique using a cup and pin assembly in measuring the viscosity of the clot (Fluger *et al.,* 2012; Wikkelsø *et al.,* 2013; Nowak *et al.,* 2017). The viscosity of the clot depends on the addition of specific coagulation activator with the tensile (viscoelastic) force created between the cup and pin. This viscoelastic force causes an interaction between the activated platelet glycoprotein receptor and fibrin polymerization. This interaction takes place during the generation of thrombin and by fibrinolysis during degradation of fibrin strands (Bolliger *et al.,*2012; Nowak *et al.,* 2017). When citrated blood is introduced into the cup, oscillation is set at 0.1 Hz frequency for 4^{o}C for 45 minutes. The pin is fixed in the cup and on addition of coagulator activator Kaolin, clotting is activated increasing the viscosity of blood. When clotting is formed, fibrin connects the pin to the cup changing the viscoelasticity to be transmitted to the pin generating an electrical signal. The electric signal for the torsion wire is plotted (Rodak *et al.,* 2012; Gautam *et al.,* 2016). A microprocessor detects the electric signal optically, tracing clot formation and degradation. A tracing curve is established after computerization of the thromboelastograph, consisting of coagulation components (plasma and platelet) with the reaction time (initial time of reaction still clot detection). Fibrinogen level is calculated by the transformation of the maximum amplitude value of platelet free plasma clot, which is directly proportional to the functional fibrinogen concentration (Fluger *et al.,* 2012; Tanaka *et al.,*2012; Kupcinskiene *et al.,* 2017).

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A. Advantages of Thromboelastography

- Rapid haemostatic clot stability, with reliable results and very sensitive to coagulation reactions (Bolliger, 2010; Galvez & Cortes 2012; Ghoshal & Bhattacharyya 2014).
- There is a correlation between the results obtained by the thromboelastrography method and standard method (Fluger *et al.,* 2012; Gautam *et al.,* 2016).

- The physiology of haemostasis is achieved as thromboelastography allows the integration of conventional coagulation analysis and platelet function (Galvez & Cortes 2012; Kupcinskiene *et al.*, 2017).

B. Disadvantages of Thromboelastography

According to Fluger *et al* (2012), Rodak *et al* (2012) and Kupcinskiene *et al.* (2017) the following are the disadvantages of thromboelastography;

- Limited to smaller concentrations.
- Short clotting time and small friable clots might not be detected.
- The prediction of other hypercoagulative markers might not be measured.

2.5.4.3 Biosensor

The application of a biosensor of quartz crystal microbalance (QCM) can be applied as an alternative method in determining the concentration of fibrinogen (Yao *et al.,* 2013; Oberfrank *et al.,* 2016). The detection reagent of fibrinogen is introduced into the detection cell. The calibration plasma of fibrinogen is added at different concentrations when there is an initial baseline frequency corresponding to the reagent of determination obtained (in vitro). There will be an immediate sharp frequency reduction due to the influence of sample fluid. As the calibration plasma is added, the time is recorded as start point (t_1). After 3 to 5 minutes, plasma coagulation will change the frequency and the time or end point will be recorded as (t_2) (Yao *et al.,* 2013). The point at which the timer stopped indicates the clotting time (Rodak *et al.,* 2012; Solomon *et al.,* 2014). The fibrinogen sample is placed onto a quartz crystal oscillator surface. Thrombin is added to start the reaction leading to agglutination, which causes a change into fibrous protein monomer and complex, creating an increase in the density and viscosity forming fibrin framework, thus increasing the mass load on the crystal surface. This reaction will result in a change in frequency. As the clotted blood diminished, the fibrous protein complex attaches to the crystal surface decreasing the mass load and increasing the crystal frequency for a short time frame. Upon deposition of the clotted blood, the crystal frequency decreases (Chen *et al.,* 2010; Oberfrank *et al.,* 2016). At this point the clotting time will be calculated by the start point and end point ($\Delta t = t2 - t1$) (Yao *et al.,* 2013).

A. Advantages of the Biosensor

- The use of QCM to determine fibrinogen levels have a real time output, label free and high sensitivity in detecting nucleic acid and protein.

- The results are obtained at a rapid time of less than 1 minute and correlates with the results obtained with standard method. The mode of operation is easy, it measures independently the sample transportation time and is very cost effective
- This method is rapid, accurate and affordable for fibrinogen identification. (Oberfrank *et al.*, 2016).

B. Disadvantages of the Biosensor

- The application of the biosensor with the influence of pressure on resonant frequency has an impact on the surrounding fluid, mass loading, hydrostatic pressure, adsorption at the surface and temperature (Seifried & Temelli 2015).

2.5.5 Fibrinogen as a cardiovascular risk marker

Increased fibrinogen plasma levels have been proven to be associated as an increased risk of CVD, such as myocardial infarction, coronary heart disease, ischemic stroke and renal disease (Lynch *et al.,* 2009; Curran, 2012; Shivappa *et al.,* 2015; Patel *et al.,* 2016). According to the Gothenburg Study, fibrinogen concentration have been found to be an independent risk factor for myocardial infarction and stroke (Ahmed *et al.,* 2013). Some Epidemiological and Framingham studies indicated that, as the level of fibrinogen increases, the risk of developing myocardial infarction and stroke also increases (Papageogiou *et al.*,2010; Kattula *et al.,* 2017). High level of plasma fibrinogen has also been involved with venous thromboembolism (deep venous thrombosis and pulmonary embolism) and identified as a risk factor (Klovaite *et al.*,2012; Kim *et al.,* 2016). High levels of fibrinogen have a direct impact on coagulation and platelet aggregation. This affects the structural properties of thrombus resulting in deep venous thrombosis and pulmonary embolism (Klovaite *et al.*,2012; Kattula *et al.,* 2017). Other risk factors associated with increased levels of fibrinogen include hypertension, diabetes mellitus and smoking. These risk factors have been identified as a CVR factors with young individuals at a higher risk of developing CVD. An elevated level of plasma fibrinogen is not only considered as an independent risk factor for myocardial infarction, but also predicts future coronary heart diseases (Papageogiou *et al.,* 2010; Kim *et al.,* 2016). According to Grobler (2015) other risk factors associated with increased levels of fibrinogen are seasonal changes, gender, race and environmental factors (pollution).

2.6 FACTOR VII

FVII is a vitamin K dependent serine protease glycoprotein produced in the liver and, involve in the regulation of coagulation with the presence of tissue factor (TF). FVII in combination with TF becomes

activated to form FVIIa and initiating the primary homeostatic balance. More so, FVIIa stimulates FIX and FX, generating an enormous concentrated thrombin burst (Suzuki *el al.*, 2012; Böhm *et al.,* 2015). FVII exists as a single chain zymogen (Turfan *et al*., 2014). Decrease levels of FVII are linked with bleeding disorders, although heritable FVII insufficiencies are unlikely the cause of bleeding tendency. But the prevalent origin of acquired FVII deficiency is due to a low level of vitamin K, decreased hepatic synthesis, disseminated intravascular coagulation (DIC) or warfarin treatment (Zantek *et al.,* 2013). Various levels of FVII activities can be influenced by either environmental factors or genetic factors. Environmental factors such as age, gender and smoking could result in a risk of CVD, with the presence of the genetic polymorphism R353Q (Turfan *et al*., 2014). Furthermore, there are some polymorphisms of factor VII gene, which result in an increased level of FVIIa and FVIIc as well as FVII antigen (Criado-Garcia *et al.,* 2011; Mo *et al.,* 2011; Azzam *et al.,* 2017). This includes three FVII promoter haplotypes, the first types is inserted in the 323 position of the 5' promoter region, with the allele A1 corresponding with the absence of the decamer and to the insertion of the A2 allele. In the codon 353 in exon 8 of FVII gene, the second polymorphism R353Q is situated at that position and thirdly the hypervariable region 4 of intron 7 (HVR7). This is a replicate of 5 to 8 variable numbers (Turfan *et al.,* 2014).

2.6.1 Chemical structure

FVII exist as a single chain molecule having a molecular weight of 55 kDa or 50000 daltons (Vadivel & Bajaj 2012; Böhm *et al.,* 2015; Tiscia *et al.,* 2017), secreted (when matured) as a single chain of 406 amino acids into blood circulation (Suzuki *et al.,* 2012). The half-life of this single chain is short, varying between 3-6 hours of procoagulant factor, having 10 Gla (Gamma-carboxyglutamic acid) residues (Kaushansky *et al.,* 2010, Greer *et al.,* 2014). The chain enzyme consists of light chain (N-terminal) and a heavy chain (C-terminal), connected by the presence of a distinct disulfide bridge. The heavy chain is made up of the catalytic domain (Figure 7) which is composed of a serine protease. Meanwhile the light chain contains the Gamma-carboxyglutamic acid (Kogiso *et al.,* 2011; Masden 2014; Tiscia *et al.,* 2017). The Gla is formed by the glutamic acid residues, which are modified (Böhm *et al.,* 2015) by the post translation addition of a carboxyl group to the gamma carbon by a carboxylase vitamin k dependent. The Gla (protein domain) has an affinity for phospholipid membranes having a negatively charged domain, which stimulates functional multiprotein complexes to accumulate at the surface (Hoffbrand *et al.,* 2011; Madsen, 2014). The FVII human gene is situated on the 13th chromosome (Figure 7), adjacent to the FX gene. The gene of FVII comprises of 8 exons and 7 introns, of 13 kb of size, having an arrangement which is very identical to other dependent factors of vitamin K (Kaushansky *et al.,* 2010; Traivaree *et al.,* 2017). The size of the introns ranges from 68 nucleotides to about 2.6kb and the exons vary in size from 25 nucleotides to 1.6 kb. The prepro leader sequence which is removed during processing is encoded by exons 1a, 1b and part of exon 2.

The 406 amino acids present is encoded by the remaining exons 2, 3 to 8, which is circulating in the blood as mature protein (Dario, 2012; Marder *et al.*, 2013).

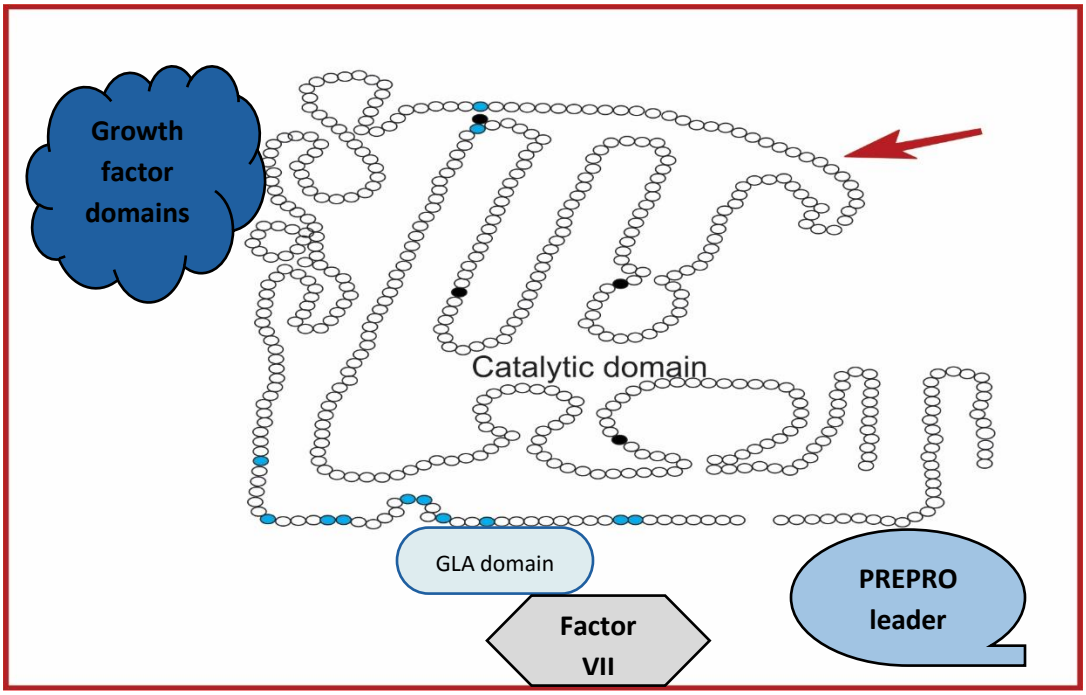

Figure 7: Schematic diagram of FVII Protein (modified from Kaushansky *et al.*, 2010)

The Growth factor domain is composed of Gla domain and prepro leader sequence (Suzuki *et al.*, 2013). The Gla domain consists of 1 to 38 residues (blue circles in Figure 7) of short hydrophobic segments of 39 to 45 residues (Vadivel & Bajaj 2012; Beeler *et al.*, 2018). These residues are formed due to the post enzymatic modification of glutamic acid in the endoplasmic reticulum by a specific γ glutamyl carboxylase. The prepro leader sequence is made of a single peptide with elements which controls the carboxylation reaction of glutamyl residues. The red arrow indicates the area where of cleavage of zymogen is converted to an active enzyme. The black circles are the active sites of the His, Ser and Asp residues of the catalytic domain (Kaushansky *et al.*, 2010; Marder *et al.*, 2013). The catalytic domain is made up of two domains folding of β barrel type which is antiparallel consisting of six β strands each. With the active site located in between the gap of the two domains (Napolitano *et al.*, 2017; Traivaree *et al.*, 2017). The formation of the covalent tetrahedral transition state is based on the enzyme distributions, whereby the hydroxyl group of the reactive serine (Ser) might provide a proton to the histidine (His) residue (Dario, 2012; Greer *et al.*, 2014).

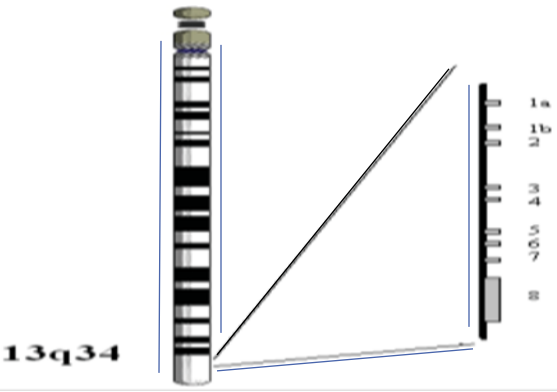

Figure 8: Human chromosomes 13 and arrangement of the FVII gene (modified from Dario, 2012)

The FVII gene is located on chromosome 13 (13q34), of 2.8kb opposite to FX gene, extending 12.5kb of 9 exons (Figure 8) (Tanaka *et al.,* 2010; Traivaree *et al.,* 2017). The exons 1a and 1b encode the prepro leader sequence with that of the 5' untranslated region (Kogiso *et al.,* 2011; Marder *et al.,* 2013).

The introns vary in size of 68 nucleotides to 2.6kb, with the exons ranging from 25 nucleotides to 1.6kb in size. The 2 exons with the 3-8 exons remaining, encode for the 406 amino acids which is circulating in blood as mature protein (Dario, 2012; Traivaree *et al.,* 2017).

2.6.2 Physiology

The essential role of FVII is to regulate the process of homeostasis by initiating and propagating the formation of clot, by the presence of tissue factor in circulation (Smith *et al.,* 2010; Boron & Boulpaep 2017). The formation of TF and FVII complex following a vessel injury leads to FVIIa, which activates FIX and FX (Kaushansky *et al.,* 2010; Napolitano *et al.,* 2017). More so, the process of homeostasis is also regulated in cases of TF independent mechanisms by recombinant FVIIa (rFVIIa) after activating FX binds to the phospholipids which are exposed outside activated platelets at the location of vessel damage (Pham *et al.,*2014). In the domain structure of human FVII, the Gla domain presents compatibility to the negatively charged phospholipid membranes (endothelial cells or activated platelets). This stimulates the multiprotein functional complexes to gather on the phospholipid membranes surface. The Gla domain which originates from two epidermal growth factors (EGF), are converted by the exons 4 and 5, with exons 6 which converts the activation peptide, with serine protease domain of 7 and 8 exons (Hoffbrand *et al.,*2011; Byskov, 2017; Napolitano *et al.,* 2017). FVII activating protease (FSAP) help in the interaction with the presence of the negatively charged polyanions (nucleic acids, heparin, polyamines or polyphosphates), in the activation of single-chain zymogen which are inactive to two active chain enzymes (Etscheid *et al.,* 2012; Kretz *et al.,* 2015; Byskov, 2017).

2.6.3 Plasma levels

FVII normal ranges vary from laboratory to laboratory, the majority classify it as normal or abnormal when its level is greater than 80 U/dL or less than 40 U/dL (Zantek *et al.,* 2013). A recent study conducted by Murray *et al.,* (2014), indicated that the normal FVII plasma level was found to be 50-100 U/dL. Elevated levels of FVII are an indicative risk for CVD, in addition with other contributing risk factors such as age, gender and smoking (Tufan *et al.,* 2014). Low levels of FVII or factor VII deficiency will result in a bleeding tendency (Suzuki et *al.,*2012; Zantek *et al.,* 2013; Faranoush *et al.,* 2015).

2.6.4 Method of detection

The plasma concentration of Factor VII can be determined by the Enzyme Linked Immunosorbent Assay (ELISA), Clot detection technique and Pulse chase analysis

2.6.4.1 Enzyme Linked Immunosorbent Assay (ELISA)

The application of immunoanalysis has been previously carried out in detecting FVII antigen and FVII activating protease (Criado-Garcia *et al.,*2011; Hanson *et al.,* 2012; Hyseni *et al.,* 2013; Böhm *et al.,* 2015, Antonio *et al.,* 2016). The basic concept of detection is the colour change of specific antibodies binding to antigens (Gan & Patel 2013). The polyclonal antibody of human FVII is pre-coated in the strip wells and plasma samples are being diluted for the binding of FVII antigen with the coated antibody, creating an enclosed binding complex and the unbound proteins are washed. Followed by the addition of Streptavidin-Horseradish Peroxidase (HRP) and allowed to react with the bounded complex. And washed to remove all the unbound HRP. A blue coloration is observed upon the addition of the colouring agent (Tetra Methyl Benzidine) which later changes to yellow (Byskov, 2017). This change in colour reaction is directly proportional to the amount of protein present. The addition of the stop solution (phosphoric acid) will stop the reaction and the absorbance will be measured at 450 nm wavelength (Grobler, 2015).

A. Advantages of Enzyme Linked Immunosorbent Assay

According to Hsueh & Hegerfeld-Baker (2011), Gan & Patel (2013) and Bystov (2017), the following are the advantages of Enzyme Linked Immunosorbent Assay;

- This application is beneficial for analysing antigens or antibodies, colorimetric results are easy to observe as well as measured using a spectrophotometer.
- The method is specific with samples having a higher concentration, with 90 samples analyzed at the same period for 2 to 3 hours.

- Smaller sample sizes are required, which is easy and flexible for research purposes.
- Colour strength is accurate for a sufficient period of time, reflecting the amount of primary antibodies present

B. Disadvantages of Enzyme Linked Immunosorbent Assay

- There is a greater chance for a falsely high positive result to occur due to non-specific binding of antibody or antigen on the plate (Gan & Patel 2013).
- The procedure is time consuming as there are many steps involved such as addition of reagents and washing with buffer (Nian, *et al.,* 2012; Lang *et al.,* 2013).

2.6.4.2 One stage clotting Assay

The activity of FVII can be determined by applying the technique of one stage clotting assay. Plasma deficient preparation of human recombinant tissue factor is added to the plasma of the patient (Suzuki *et al.,* 2012; Faranoush *et al.,* 2015). The basic principle of this assay relies on the sample capacity containing the factor of analysis. This factor deficient plasma under investigation must have a clotting factor of less than 1U/dL with all the other relevant clotting factors of normal levels. Thus, the measured clotting time of PT should be directly proportional to the amount of factors available in plasma deficient mixture with that of the test plasma or normal range (Key *et al.,* 2009; Makris *et al.,* 2014). As the plasma deficient is mixed in a 1:1 ratio with that of the patient plasma, the mixture of the Prothrombin time (PT) is influenced by the availability and quantity of the patient plasma. Thus, the standard curve prepared from PT values will determine the factor activity of the patient plasma, of 1:1 mixtures of factor deficient substrate with a reference plasma of serially diluted known factor activity (Riley *et al.,* 2006; Amiral *et al.,* 2017).

A. Advantages of one stage clotting Assay

- The method of clot detection is reliable, valid and produces accurate results
- The use of human tissue factor reagents is very sensitive for the analysis of coagulation factors (Key *et al.,* 2009; Mackie *et al.,* 2012; Amiral *et al.,* 2017).

B. Disadvantages of one stage clotting Assay

- The application of this technique might result in confusing and unpredictable results. Due to the use of different reagents, reducing the activities (Mackie *et al.,* 2012; Makris *et al.,* 2014).

2.6.4.3 Prothrombin time-base clotting assay

The application of prothrombin time-base clotting assay can be used indirectly to determine the levels of FVII defect or deficiency (Bain *et al.,* 2012; Kim *et al.,* 2015). As citrated platelet pool plasma (PPP), FVII deficient plasma, thromboplastin and calcium chlorides are brought into reaction; the polymerization of fibrin is generated, activating FVII plasma. The presence of calcium in the reaction activates the TF complex of FVII to be form FVIIa, following the activation and formation of FVIIIa, FIXa, FVa and FXa complex. This leads to the formation of a clot which is measured by a photo optical method. During the clotting time, the optical density of the plasma changes and the intensity is detected as light of specific wavelength passes through the plasma sample (Rodak *et al.,* 2012; Tripathi *et al.,* 2017). The clotting time recorded is known as the prothrombin time, and this time is compared against the reference range of factor VII test plasma (Kottke-Marchant & Davis 2012; Peters *et al.,* 2013).

A. Advantages of Prothrombin time-base clotting assay

- The application of multiple wavelengths increases the specimen quality, test menu flexibility, and the formation of clot (depending on the instrument) observed by graphical presentation (Rodak *et al.,* 2012; Kim *et al.,* 2015).
- The reagents used are sensitive when determining coagulation factors (Mackie *et al.,* 2012; Peters *et al.,* 2013; Tripathi *et al.,* 2017).

B. Disadvantages of Prothrombin time-base clotting assay

- Clotting time and small friable clots might not be detected. Results obtained might be affected by the presence of haemolysis, bilirubinaemia and lipemia interference.
- Indirect method of detection can be influenced by external factors (Rodak *et al.,* 2012; Peters *et al.,* 2013).

2.6.5 Factor VII as a cardiovascular risk marker

The complex TF-FVIIa upon activation has been found to be associated with venous thrombosis, restenosis, myocardial reperfusion injury and inflammatory diseases, in combination with the activation of coagulation. This might result in cancer progression, vascular remodeling and angiogenesis via the signaling pathways activation (Ott, 2011; Faranoush *et al.,* 2015; Naderi, 2015; Zeng *et al.,* 2016). More so, TF-VIIa complex has been found as a risk factor in activating the pathogenic mechanisms in cancer, which comprises of angiogenesis, cell survival, invasion and cell motility, which is the main causes of

thrombosis with individuals suffering from cancer, more specifically in ovarian clear cell carcinoma. This is due to the synthesis of FVII, which is generated in response to hypoxia, stimulating the release of micro-vesicles which is found in the TF-VIIa complex enhancing thrombosis as well as venous thromboembolism (Koizume *et al.,* 2012; Naderi, 2015). Moreover, TF-FVIIa complex has also been found to be associated with systemic inflammatory response syndrome (SIRS), by the activation of FX and FIX, which causes the conversion of prothrombin to thrombi. This mechanism reduces mortality by decreasing coagulation in response to sepsis which results in kidney failure, multiple organ dysfunction and shock (Hyseni *et al.,* 2013).

2.7 PAI-1

Plasminogen activator inhibitor-1(PAI-1) is an important regulator of fibrinolysis (as described in 2.4.5), which inhibits the activation of plasminogen to plasmin with the presence of the tPA and uPA (Koiou *et al.,* 2012; Crean *et al.,* 2012; Simone *et al.,* 2014). This regulation of fibrinolysis is determined by the interaction between plasma protein fibrinolytic and anti-fibrinolytic proteins, with fibrinolysis being determined by the enzymatic conversion of plasminogen to plasmin mediated by plasminogen activators tPA and uPA, with plasminogen activator inhibitor (PAI) regulating plasma antifibrinolytic activity (Hassanin *et al.,* 2013). PAI-1 belongs to a member of superficial serine protease inhibitors, which act as pseudo substrate activator for plasminogen (Phelan & Kerins 2014). In addition, PAI-1 as a family member of the serine protease inhibitors, is also an inhibitor of cell associated proteolysis and intravascular fibrinolysis. PAI-1 in blood circulation exists in two distinct forms; active form (having a short plasma half-life of 30 minutes) and inactive form (invisible). PAI-1 is produced in the liver, adipocytes, platelets, smooth muscle cells and brain. More so, PAI-I is secreted by cells when stimulated during pathological circumstances such as atherosclerosis, endothelial cells and inflammatory response (Huotari *et al.,* 2010; Simone & Higgins 2015; Simone *et al.,* 2015).

2.7.1 Chemical structure

Plasminogen activator inhibitor-1(PAI-1) is composed of a globular glycoprotein, having a molecular mass of 38 to 70 kDa, and it's made up of 379 amino acids in a single chain. This structure is known as a member of the serine protease inhibitors family (Buchan *et al.,* 2012; Craen *et al.,* 2012; Simone & Higgins 2015). The gene of PAI-1 is located on chromosome 7, which consist of 8 introns and 9 exons (Ismail *et al.,* 2011; Craen *et al.,* 2012; White *et al.,* 2015). PAI-1 glycoprotein is composed of 3 β sheets, A, B, and C, with 9

α helical domains labelled A to I. The domain is made of a marked reactive center loop (RCL), which is located in the carboxy terminus (C-terminus) (Simone *et al.*, 2014). The RCL contains residues of peptide bond P16 to P10', with the reactive site peptide bond 1 to peptide bond 1' (P1 to P1') which is situated in RCL with 30 to 40 amino acids resembling the normal substrate of the serine protease from the carboxy terminus. This serine protease can be differentiated by groups, which are the inhibitory and the non-inhibitory (Craen *et al.*, 2012; Simone *et al.*, 2014; Simone *et al.*, 2015). PAI-1 exists in active, latent and cleaved conformations (Figure 9). But PAI-1 is produced in an active but unstable form initially, which is converted into the latent form, with the latent form depending upon the insertion of the N-terminus of PAI-1 reactive center loop (RCL) into the β sheet A, which forms a new β strand. This establishes an unusual coil structure, with framework shift in the reactive site. Therefore, there is disruption between the Arginine 346 (Arg346) and Methionine (Met347) of the peptide bond P1 to P1', as a result prevent PAI-1 from merging with proteinases (Simone *et al.*, 2014).

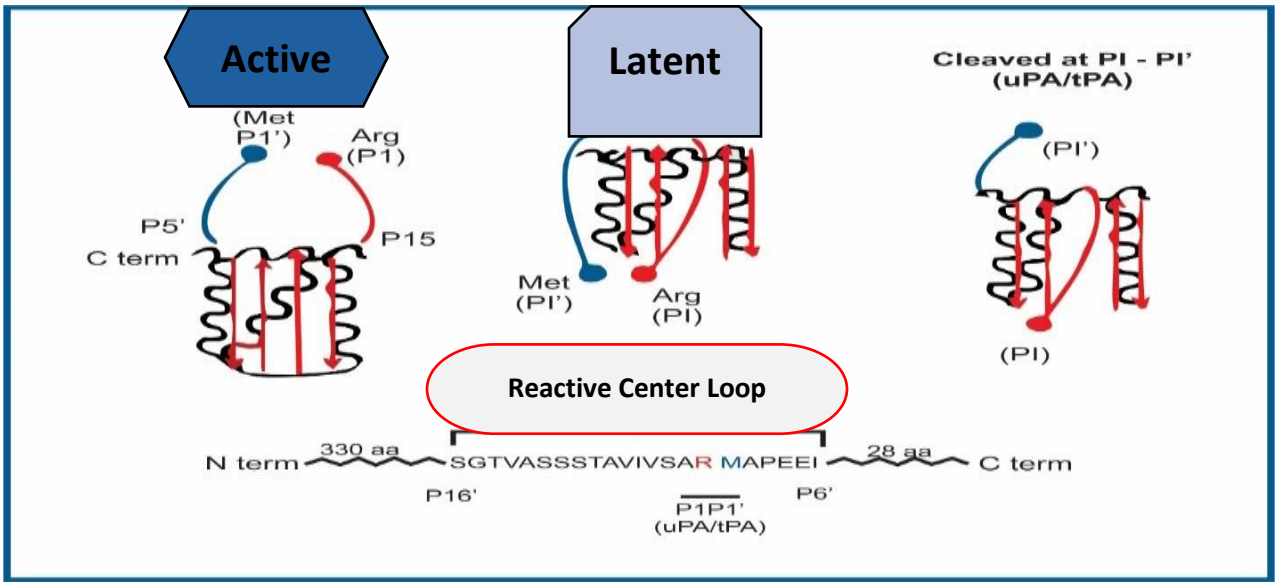

Figure 9: Active, latent and cleaved conformation of PAI-1(modified from Simone & Higgins 2015)

The active conformation of PAI-1 contains the C-terminus with the reactive center loop located at a hollow position, leading to the conversion of the latent conformation which implicates the side of the N-terminus in the reactive center loop to slide into the β sheet A(represented in red) forming a new β-strand 4A. Thus the P1 to P1' peptide with that of the extended loop by the C-terminus end of the reactive center loop are not inhibited by proteases. But the conformation of PAI-1 is reversible and can be stored away in the latent conformation, with anti-proteolysis being required for reactivation. PAI-1 in the substrate form can be

cleaved by proteases at the fissile bond between the Arg346 (P1 represented as red ball) and Met347 (P1' represented as blue ball) residues. The β-strand 4A formation distorts the P1 to P1' peptide when the N-terminus is cleaved at the end of the reactive center loops inserting into β-sheet A (represented in red), but this conformation is not reversible like that of the latent conformation which is unable to inhibit proteases (Simone *et al.,* 2014; Simone & Higgins 2015).

2.7.2 Physiology

The initial function of Plasminogen activator inhibitor-1 is to initiates the process of fibrinolysis, by inhibiting the activation of plasminogen to plasmin in the presence of tissue type and urokinase type of plasminogen activators (Koiou *et al.,* 2012; Simone *et al.,* 2014). PAI-1 as a member of the serine protease inhibitor plays a role in the activation of plasminogen as a pseudosubstrate (Phelan & Kerins 2014). PAI-1 in circulation helps in regulating the myoendothelial junction formation and might make some changes in the hetero-cellular signaling, interfering via the myoendothelial in opposition of vascular system (Herberlein *et al.,* 2010; Iacoviello *et al.,* 2013; Simone & Higgins 2015). PAI-1 plays an essential role in cellular motility by utilizing its anti-proteolytic and signaling functions. Cell motility is stimulated by PAI-1. The cooperation of low density lipoprotein receptor related protein 1 (LRP1), activates the Janus tyrosine Kinase/ Signal Transducer and Activator of Transcription (Jak/Stat) 1 signal motion. Cell locomotion can be modulated by PAI-1 when bonded with vitronectin through the tripeptide Arg-Gly-Asp (RGD) as a dependent interaction, stabilizing PAI-1 activity adequately prolonging its half-life. PAI-1 helps in promoting the process of endocytosis by regulating cell surface integrins when present, by using the Lipoprotein receptor related protein 1 (LRP1) (Simone *et al.,* 2014). Furthermore, PAI-1 controls tumour progression, tumour invasion and the formation of metastasis, most specifically with uPA in the plasminogen activator system. This control mechanism is achieved by the regulation of cell adhesion, migration and invasiveness. PAI-1 also controls the progression of tumour by its effect on angiogenesis and inhibits apoptosis when combined with cell cultures in the normal vascular smooth muscles cells with tumour cells (Craen *et al.,* 2012; Iacoviello *et al.,* 2013; Simone *et al.,* 2015; Simone & Higgins 2015).

2.7.3 Plasma level

According to Mayo Clinic (2014), the reference range for PA1-1 is 3 to 72 ng/ml. Increased PAI-1 plasma levels are commonly present in conditions such as obesity, early stages of impaired glucose tolerance, which causes diabetes type 2 and metabolic syndrome (Hassanin *et al.,* 2013). Alcohol has been confirmed to increase the plasma level of PAI-1 and tPA (Janssen *et al.,* 2014; Phelan & Kerins 2014). Elevated PA1-1

levels link to impaired fibrinolytic activity will leads to stoke and coronary heart disease (Iacoviello *et al.*, 2013). Low levels of PAI-1 or PAI-1 deficiency leads to hyperfibrinolysis with an abnormal bleeding tendency after a post-surgery or trauma (Mehta & Shapiro 2008; Simone *et al.*, 2014).

2.7.4 Method of detection

The plasma concentration of PAI-1 can be measured using Enzyme Linked Immunosorbent Assay (ELISA), Chromogenic assay and Euglobulin Clot Lysis Time Test (ELT).

2.7.4.1 Enzyme Linked Immunosorbent Assay (ELISA)

Enzyme linked immunosorbent assay has been used in determining the levels of PAI-1 (Lang *et al.*, 2014; Aziz *et al.*, 2014; Duffy *et al.*, 2014; Janssen *et al.*, 2014). The detection method is based on the specific antibodies binding to corresponding antigens, and a colour change is observed. The strip wells are coated with the anti-PAI-1 monoclonal antibody and soluble non-immune IgG. As the PAI-1 standards or plasma sample is added into the coated strip wells, a binding complex is created. As incubation is taking place, the binding of PAI-1 and antibodies is captured. The unbound conjugates were washed and Streptavidin-Horseradish Peroxidase (HRP) containing anti-PAI-1 IgG is added, enabling another bounding complex to be established. Followed by the washing of the unbounded conjugates, Tetra Methyl Benzidine (colouring agent) was added and a colour change was observed, which is directly proportional to the amount of PAI-1 present (Aziz *et al.*, 2014). Phosphoric acid (stopping solution) is added at the final stage and the absorbance was measured at the wavelength of 450nm (Grobler 2015).

A. Advantages of Enzyme Linked Immunosorbent Assay

- The change of colour indicates the presence of the antibody within a short period of time.
- The immunoanalytical technique is very specific and highly sensitive (Gan & Patel 2013; Aziz *et al.*, 2014).

B. Disadvantages of Enzyme Linked Immunosorbent Assay

- The various steps or stages involved in the immunoanalytical technique is time consuming, with the addition of different reagents.
- False positive results might be encountered (Nian, *et al.*, 2012; Gan & Patel 2013; Lang *et al.*, 2013).

2.7.4.2 Chromogenic Assay

The basic principle of chromogenic analysis is the effective use of synthetic substrates containing chromogen or chromophore (substance of coloured chemical), which are connected to amino acid of short specific residue for targeting the particular enzyme. Thus, there is the release of the chromophore during the enzymatic action, determined by spectrophotometry (Riley *et al.,* 2006; Peters *et al.,* 2013). The synthetic polypeptides amino acids sequence (valine-leucine-lysine) are digested to become, specific in enzymes activities (Rodak *et al.,* 2012; Mackie *et al.,* 2012; Yu *et al.,* 2017). The activity of plasmin can be analyzed by the application of synthetic the chromogenic substrate which releases paranitroanline (fluorophore or chromophore) during plasmin mediated digestion (Fjellström *et al.,* 2013; Amiral *et al.,* 2017). This chromophore binds covalently to the carboxyl end of the polypeptides amino acids during digestion. The exogenous plasminogen activator streptokinase is added with platelet poor plasma, the binding action will activates plasminogen (streptokinase-plasmin complex). This complex will form a reaction with the chromogenic substrates, releasing a colour. The colour intensity is directly proportional to the concentration of plasminogen (Mackie *et al.,* 2012; Rodak *et al.,* 2012; Peters *et al.,* 2013).

A. Advantages of Chromogenic Assay

- The use of chromogenic assay is advantageous as all the components are provided except for the blood sample.
- The wavelength of 405nm is used in modern analysis in targeting protease activity (Shaz *et al.,* 2013).
- The durability of the reagents has been applicable to many automated laboratories.
- The chromogenic substrate is selective and sensitive (Riley *et al.,* 2006; Makris *et al.,* 2014).

B. Disadvantages of Chromogenic Assay

According to Riley *et al* (2006) & Shaz *et al* (2013) the following are the advantages of Chromogenic Assay;

- Application of chromogenic assays are influenced by fibrin degradation products, heparin and lupus anticoagulants.
- Few analytical interferences flag are encountered with chromogenic assay
- The presence of fibrin degradation products and anticoagulants might influence the chromogenic substrate reaction.

- The concentration of sample, reagents and reaction condition can affect the selectivity of the chromogenic substrate.

2.7.4.3 Euglobulin Clot Lysis Time Test (ELT)

As plasma inhibitors of fibrinolysis are separated, plasminogen and plasminogen activators are released into the reaction. When the patient plasma is diluted with 1% acetic acid, a pH of 5.35 to 5.40 is established. A precipitate (euglobulin fraction) containing plasminogen, tissue plasminogen activator and plasmin are formed upon 30 minutes of refrigeration. After centrifugation, the supernatants are transferred and borate buffer and calcium chloride (thrombin reagent) are added to dissolve the precipitate. Upon addition of thrombin reagent, a timer is started (Rodak *et al.,* 2012; Minet *et al.,* 2015). The clot lysis time is calculated at the moment of clot formation still clotting disintegration (Pepperell *et al.,* 2014; Yu *et al.,* 2017).

A. Advantages of Euglobulin Clot Lysis Time Test

- This assay has been considered globally as a gold standard method to evaluate the plasma capacity of the fibrinolytic pathway (Rodak *et al.,* 2012; Schöchl *et al.,* 2012; Voelckel *et al.,* 2012; Dirkman *et al.,* 2014).
- The analysis is easy, simple and analysis can be re-run on frozen samples (Pepperell *et al.,* 2014).

B. Disadvantages of Euglobulin Clot Lysis Time Test

According to Schöchl *et al.* (2012) and Dirkman *et al.* (2014), the following are the disadvantages of Euglobulin clot lysis time test;

- Time consuming
- Labour intensive
- Inadequate reproducibility

2.7.5 PAI-1 as a cardiovascular risk marker

Increased levels of PAI-1 have been found to be associated as a risk factor for CVD (Lange *et al.,* 2013). Another significant causative effect of increased plasma level of PAI-1 is in the pathophysiology of diabetes, metabolic syndrome, septic coagulopathy, restenosis, atherosclerosis, myocardial infarction and vascular thrombosis which are all independent CVR factors (Simone *et al.,* 2014). Increase levels of PAI-1 plasma is a risk factor for the development of CHD, and it's also known as a risk factor for post bleeding tendency for cardiac surgery (Phelan & Kerins 2014). PAI-1 as a marker for insulin resistance has been

confirmed to be associated with the development of diabetes type 2 when increased in plasma circulation. Moreover, matrix degradation can be inhibited by elevated levels of PAI-1. When this happens, endothelial cell extensions and extracellular matrix will be prevented from growing (Heberlein *et al.,* 2010; Koiou *et al.,* 2012; Yarmolinsky *et al.,* 2015). Individuals suffering from metabolic syndrome and obesity are at a higher risk of developing atherosclerotic plaque disruption due to increased levels of PAI-1 (Phelan & Kerins 2014), with plasma PAI-1 antigen levels and activity which are found to be high in insulin resistant conditions, and obese individuals (Koiou *et al.,* 2012; Lalić *et al.,* 2015). According to the Northwick Park Heart Study, low fibrinolytic activity is an independent risk factor for developing CVDs (Phelan & Kerins 2014).

2.8 R353Q Polymorphism

The genetic polymorphism (R353Q) is a non-synonymous single base polymorphism in the exons 8 of FVII gene affecting FVII plasma level (Shanker *et al.,* 2009; Azzam *et al.,* 2017; Olson *et al.,* 2018). This polymorphism located in the catalytic domain which influences a decreased secretion determining a low plasma level of FVII antigen (Campo *et al.,* 2013).

2.8.1 Chemical Structure

R353Q polymorphism is an elementary nucleotide polymorphism which is represented by changing the guanine base to an adenine. When this change occurs, it leads to the substitution of arginine (R) to glutamine (Q) in the codon 353 of the FVII gene at the base position 135 (Figure 10) (Shanker *et al.,* 2009; Criado-Garcia *et al.,* 2011; Bairova *et al.,* 2016; Azzam *et al.,* 2017). This will trigger a missense mutation of the FVII gene in the codon 353, changing arginine (R) to re-establish to glutamine (Q) (Cheraghi *et al.,* 2013). The codon 353 in the exon 8 of FVII gene (Figure 11) is where the second haplotype is situated, with the last type varying in numbers between 5 to 8 repeated in intron 7 of the hyper variable region 4 (Turfan *et al*., 2014).

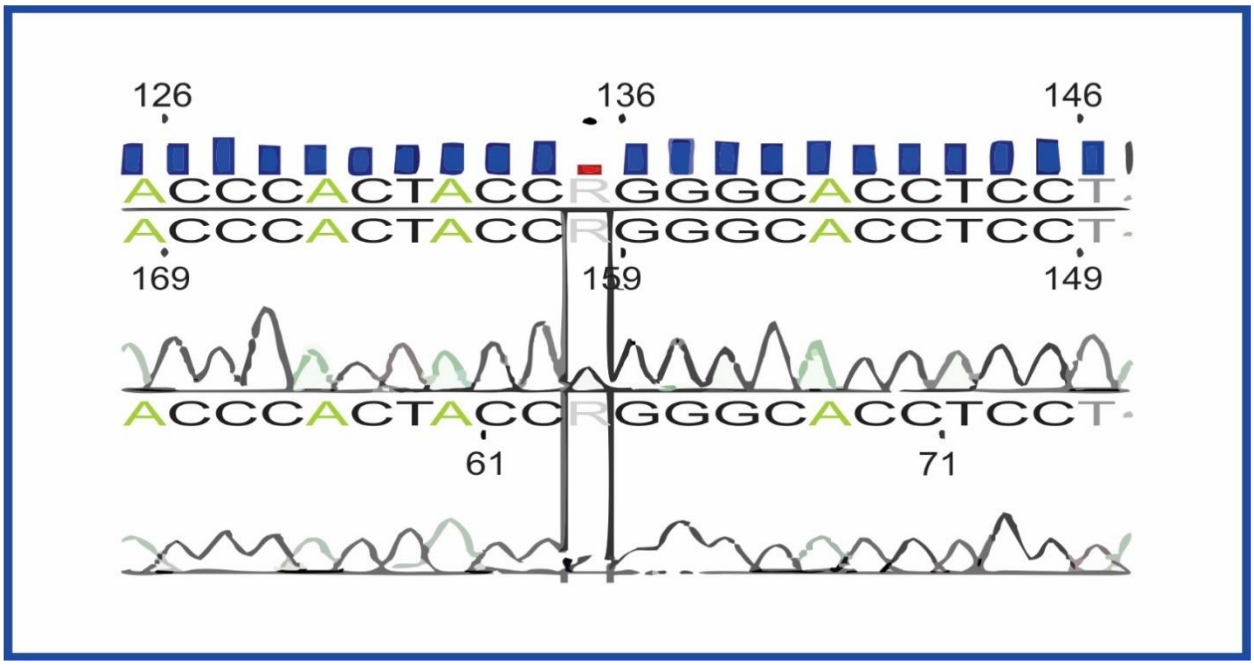

Figure 10: Showing the heterozygote sequence of RQ (AG) at base position 135 in FVII gene (modified from Shanker *et al.,* 2009)

A segment of the FVII gene is shown in Figure 8 as well as its function in the production of coagulation FVII proteins, which are involved in the process of coagulation, produced by the liver cells and circulating in an inactive form in the blood stream. Following a tissue or vessel injury, the inactive form of coagulation proteins are activated to initiates the process of clot formation (by the conversion of fibrinogen into fibrin). This stop in bleeding enables blood vessels to be repaired (Ben-Hadj-Khalifa *et al.,* 2013; Bairova *et al.,* 2016). The mature 406 amino acids are encoded by the gene of the FVII protein, which is composed of the light chain (N-terminal gamma-carboxyglutamic acid domain and two epidermal growth factor) and heavy chain (C-terminal serine protease catalytic domain) located on chromosome 13q34, containing 9 exons of 1a, 1b, and 2 to 8. (Bairova *et al.*, 2016; Azzam *et al.*, 2017; Kuperman *et al.*, 2017; Traiveree *et al.*, 2017). When heterozygote substitution occurs at base position 135, the amino acid arginine (A) is changed to R, restoring glutamine (G) to Q in the 353 codon of the FVII gene, thus, reducing the levels of plasma FVII (Shanker *et al.*, 2009; Mo *et al.*, 2011; Cheraghi *et al.*, 2013; Turfan *et al.*, 2014).

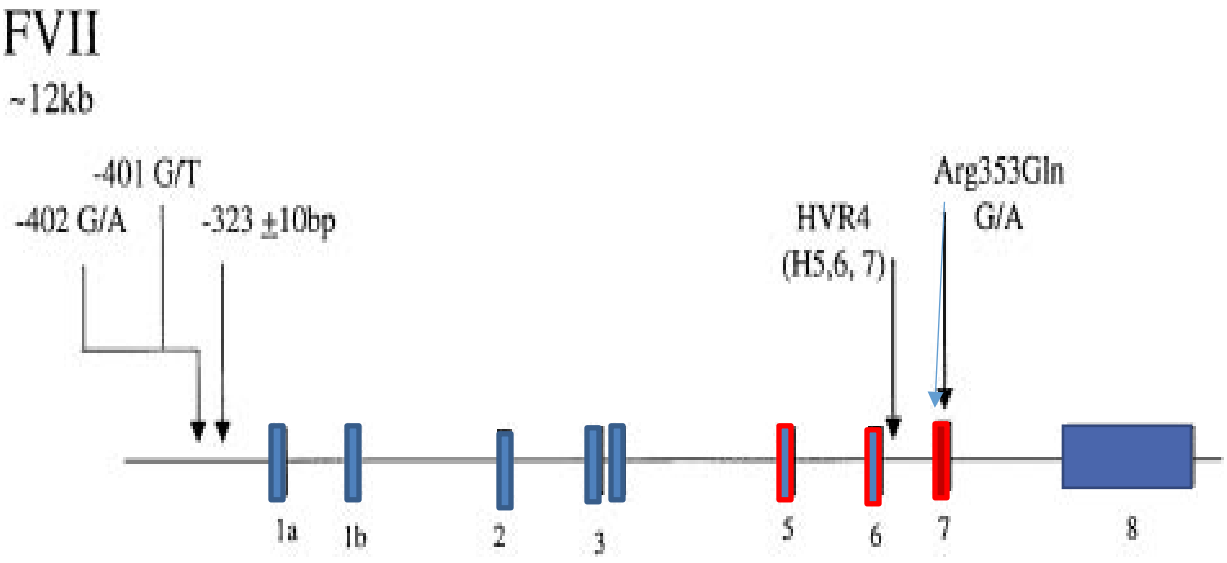

Figure 11 Structure of FVII showing location of the 353 codon (modified from Lane & Grant 2000)

As previously mentioned in 2.6, the codon 353 is situated in the exon 8 of FVII gene. The other two FVII promoter haplotypes are inserted in the 323 position of the 50 promoter region with the HVR7 replicating three alleles which are H5, H6 and H7 (Mo *et al.*, 2011; Turfan *et al.*, 2014).

2.8.2 Physiology

The genetic polymorphism R353Q has been identified to pre-dominate the plasmatic coagulation levels of FVII by reducing the plasma levels of FVII antigen as well as reducing it's secretion (Campo *et al.*, 2013). Following a vascular injury, plaque ruptures and TF are exposed in circulation with blood which initiates the movement of plasma FVII. This mechanism is regarded as the main cause of CVD (Criado-Garcia *et al.*, 2011; Napolitano *et al.*, 2017). The presence of genetic polymorphism (R353Q) reduces the level of plasma FVII from 20% (Mo *et al.*, 2011; Cheraghi *et al.*, 2013; Turfan *et al.*, 2014; Azzam *et al.*, 2017). These reductions in plasma levels of FVII have a direct impact in the process of coagulation, which increases the risk of CVD. Individuals with Q allele have a lower level of FVII as compared to those have the R allele in the population allele's distribution (Criado-Garcia *et al.*, 2011; El-Mitwalli *et al.*, 2014; Azzam *et al.*, 2017). More so, individuals with R allele are at a higher risk of developing thrombosis, meanwhile individuals with Q allele or 353Q having a low plasma and coagulant activity of FVII are associated with a protective mechanism for stroke and myocardial infarction (Mo *et al.*, 2011; El-Mitwalli *et al.*, 2014).

2.8.3 Methods of detection

The genetic polymorphism R353Q can be detected using the Polymerase chain reaction (PCR) and Real-time polymerase chain reaction (RT-PCR) (Lindman *et al.,* 2004; Shanker *et al.,* 2009; Criado-Garcia *et al.,* 2011; Cheraghi *et al.,* 2013; Turfan *et al*., 2014).

2.8.3.1 Polymerase chain reaction (PCR) or conventional PCR

The basic principle of this method depends on the replication of DNA in vivo, whereby double stranded DNA is uncoiled to a single stranded DNA. This process continues by duplicating and recoiling. Two repetitive cycles of denaturation of DNA is carried by increasing the melting temperature converting the double stranded DNA to single stranded DNA. The presence of primers is utilized to target the DNA during the annealing stage of two oligonucleotides, with the addition of nucleotide from primers using DNA polymerase as a stimulant in the presence of Mg2+ ions for the extension of the DNA chain (Somma & Querci 2010; Burtis & Bruns 2015; Rifai *et al.,* 2018). The reaction mixture to identify the FVII R353Q polymorphism contained 2 µL of DNA, 10 µM of each primer, 2.5 mM of each dNTP, 25 mM of MgCL2 and 5 U/µ of Taq polymerase with 10X KCl (MBI, Fermentas). The forward primer sequence for amplification was 5'GGGAGACTCCCCAAATATCAC-3', and reserve primer 5'ACGCAGCCTTGGCTTTCTCTC-3'.After amplification, the end products (digested using the *Msp I*) were visualized on 3% agarose gel stained with ethidium bromide (Green *et al.,* 1991; Cheraghi *et al.,* 2013).

A. Advantages of Polymerase chain reaction (PCR) or conventional PCR

- Polymerase chain reaction is beneficial as the technique is fast, cheap, simple to perform with the specificity of DNA amplification fragments and the generation of unlimited fragment of DNA copies (Joshi & Deshpande 2010, Burtis & Bruns 2015).
- The sensitivity of PCR yield quality results (Hajia, 2017).

B. Disadvantages of Polymerase chain reaction or conventional PCR

- The application of PCR is disadvantageous as quantitative data are not provided (Topcuoglu *et al.,* 2013).
- The method of detection also allows trace amounts of DNA contaminants to serve as templates which causes false positive results due to the amplification of incorrect target of the nucleic acid (Somma & Querci 2010; Burtis & Bruns 2015).

- There is a higher risk of contamination, which results in a prolonged detection time and low levels of target genome may occur leading to false negative results (Hajia, 2017).

2.8.3.2 Real-time Polymerase chain reaction (RT-PCR)

Real-time PCR implies the use of fluorescent technology in amplifying and monitoring specific target sequence of nucleic acid in a sample. As the amplification is in process, a fluorescent signal reaches a threshold level very quickly to correlates with the amount of original target sequence enabling quantification. The final product obtained can be distinguished by increasing the temperature. This will detect the melting point of the double stranded product (Seifi *et al.,* 2012; Burtis & Bruns 2015; Rifai *et al.*, 2018). The reaction mixture contains 150 ng genomic DNA, 3 mM of Magnesium Chloride (MgCl2), 200 µM of each nucleotide, 1 IU of Hot Start polymerase and the 1X reaction buffer (Dominion-MBL). The primers and probes were mixed (GATGCCCGTCAGGTACCACGTGCCC (C/T) GGTA GTGGGTGGCATGTGGGCCTCC) into the 1X buffer to a final volume of 10 µl (Taqman® -Applied Biosystems). DNA was denatured at 65^0C for 5 minutes, following with 40 cycles at 95^0C for 30 seconds of denaturation, a phase of primer-polymerase union (60^0C for 1 minute) and 72^0C for 30 seconds for extension (O'Hara *et al*., 1987, Lindman *et al*., 2004 & Criado-Garcia *et al.,* 2011, Burtis & Bruns 2015).

A. Advantages of Real-time Polymerase chain reaction

- The technique is sensitive, reproducible and the quantification of the amplicons are monitored (Paiva-Cavalcanti *et al.,* 2010; Debode *et al.*, 2017; Rifai *et al.*, 2018).
- The quantification rate of samples is fast, accurate and has a good quantitative detection, which permits the continuous monitoring of the amplification of DNA, limiting the use of gel electrophoresis (Topcuoglu *et al.,* 2013).
- The amplification and detection of nucleic acid stages are combined, for complex mixtures having specific sequences (Kashani &Malau-Aduli 2014).
- There is no end point amplification reaction, as the gene quantification occurs during the exponential stage. The DNA concentration can be increased by the optimization reaction of real time PCR (Smith & Osborn 2009; Rifai *et al.*, 2018).

B. Disadvantages of Real-time Polymerase chain reaction

- The use of ethidium bromide is very dangerous, and there is a risk of contamination. The novel miRNAs cannot be identified with lack of quantitative proportions (Paiva-Cavalcanti *et al.,* 2010; Marabita *et al.,* 2016).

2.8.4 R355Q polymorphism as a cardiovascular risk marker

The genetic polymorphism R353Q of FVII gene has been identified as a feasible instigating cause for developing myocardial infarction (Cheraghi *et al.,* 2013). The genotype 353R has been identified as a higher risk for developing thrombosis, meanwhile the 353Q genotype might have a protective mechanism against myocardial infarction, mesenteric thrombotic events and stroke within the younger population. This is because the genotype 353Q possesses lower levels of FVII and coagulant activity of FVII (El-Mitwalli *et al.,* 2014). In addition, the substitution of Arg353Gln will hinder the secreting factor VII from blood vessels to the cells, resulting to a decreased heterozygotes factor VII of 20% and homozygote of 40%. This occurs as a result of the mutability correlating the existence within these markers (Kaushansky *et al.,* 2010; Bairova *et al.,* 2016; Azzam *et al.,* 2017).

2.9 CONCLUSION

Blood coagulation factor, platelets, endothelium and fibrinolysis are the main principal constituents of the homeostatic system (Hoffbrand & Moss 2011, Rifai *et al.,* 2018). Any changes encountered in the coagulation cascade will initiates a homeostatic balance, activating a pathogenic risk with the presence of low grade inflammation resulting in CVD (Russo, 2012; Hall, 2016). Cardiovascular disease is a global challenge due to life quality impairment leading to loss of independence, financial and social sustainability, and causing a high mortality rate (Labarthe, 2011; Seo & Choi 2015). More so, risk factors such as smoking habit, dietary, overweigh/obesity and family history have been found to be strongly linked to CVD (Deaton *et al.,* 201; Ahamri *et al.,* 2017; Andrew *et al.,* 2017). Additionally, inflammatory markers such as highly-sensitive C-reactive protein (hs-CRP), fibrinogen, FVII and VWF have been proven according to the literature to be associated with CVD (Huether & McCance 2012; Arboix, 2015). The genetic polymorphism R353Q has also been identified as a CVR marker modifying the level of FVII plasma level (Turfan *et al.,* 2014). Factor VII is involved in the regulation of coagulation with the presence of tissue factor initiating a primary homeostatic balance (Suzuki *et al.,* 2012; Böhm *et al.,* 2015), with fibrinogen is involved in

stabilizing the process of haemostasis and clot formation (Levy *et al.*, 2012; Palta *et al.*, 2014). PAI-1 regulates the process of fibrinolysis, inhibiting the activation of plasminogen to plasmin with the presence of the tPA and uPA (Koiou *et al.*, 2012; Simone *et al.*, 2014).

CHAPTER 3

MATERIALS AND METHODS

3.1 INTRODUCTION

Hypercoagulability measured by increased fibrinogen, increased FVII and decreased fibrinolysis (measured by decreased PAI-1), are independent markers for cardiovascular risk (CVR). R353Q polymorphism regulating FVII has also been reported as a contributing factor for cardiovascular diseases (CVD). Grobler (2015) reported an increased CVR in the same population. Evaluating the prevalence of R353Q polymorphism in correlation with homeostatic markers within this elderly community, will contribute to a better understanding of risk factors associated in CVR in a black South African elderly. The methods followed to determine the prevalence of R353Q polymorphism and the coagulability status will be reported in this chapter.

3.2 ETHICAL CONSIDERATION

This is an ethically approved research project. Ethical approval was received from the Ethical Committee of the University of Witwatersrand, Johannesburg (M070126) (Annexure A), with additional clearance that was submitted to the Ethical Committee of the Vaal University of Technology, Vanderbijlpark (20140827) (Annexure B).

Guidelines were taken into consideration ensuring that the study was conducted according to the principles prescribed by the Belmont report (1979) with that of the Helsinki Declaration (2008). These ethical considerations as adopted by the Medical Research Council (MRC) of South Africa were applied. Good laboratory practice guidelines were applied in this study in compliance with the guideline of Health Professions Council of South Africa (HPCSA) and South African National Accreditation System (SANAS) guidelines.

The principles of the MRC were applied, they are as follows;

- **Autonomy**: Whereby the participant's human dignity is respected, and an informed consent form was signed by all the participants voluntarily.
- **Beneficence**: The results obtained from this study will help the participants and the community with a better understanding of the development of cardiovascular disease influenced by genetic

factors and environmental factors. Scientific information will be obtained on how these factors (genetic and environmental) affect the health fundamental changes and disease pattern with the elderly population of Sharpeville having a low insignificant nutritional status.

- **Non-maleficence**: Ensuring that there is no harm to the participants. The application of a safety protocol by a trained and qualified phlebotomist during the process of blood collection from the participants with the use of sterile syringes, cotton swaps and waste sharps container for disposal of biohazardous materials. This is to create an environment free of contamination and injury.
- **Justice**: Each participant was treated with care, respect and dignity. The scientific information and results obtained will be reported back to the sample population and department of health for follow up and recommendations.

3.3 SAMPLING STRATEGIES

Introductory visits to the day-care centre were done to explain the aims and objectives of the study to the participants and the centre management. An informed consent form written in English was distributed to participants. The content of the information consent sheet was explained and translated to the participants in their local language (Sotho) with the help of trained field workers who are competent in the language. This facilitated communication and approval was obtained from participants and consenting subjects on how the study was conducted with ethical precautions (Annexure C).

- Participation was voluntary and the participants as consenting subjects were well informed about the procedures and methodology of the study.
- All data or information obtained from individuals was kept confidential by using the subject numbers on their files and test tubes. This information was restricted to laboratory personnel (double blinded).
- Participants were treated with care and respect in order to avoid bias from the interviewer's.

3.3.1 Inclusion criteria

All the participants of age 60 years or above attending the day care centre in Sharpeville (Sharpeville centre for the aged) were included in the study. They all had the opportunity to participate in this study and gave their approval of consent for the study. No person suffering from chronic diseases was excluded.

3.3.2 Exclusion criteria

Subjects that could not provide substantial information or be able to complete the consent process (conditions like dementia) were excluded in this study. Individuals suffering from chronic diseases were not excluded from the study.

3.3.3 Sample size

According to Charan and Biswas (2013), the sample size of a cross sectional study is determined by estimating the prevalence of some parameters within the sampled population. The sample size of fibrinogen as an independent risk factor of cardiovascular disease was calculated according to the following formula.

$$n = \frac{Z_{1-\alpha/2}{}^2 p\,(1-p)}{d^2}$$

Where:

$Z_{1-\alpha/2}$ = 1.96 at 5% type 1 error (P<0.05)

p = 5% expected proportion in population = 0.05

d = Absolute error or precision (0.05)

$$n = \frac{1.96^2 \times 0.05\,(1-0.05)}{0.05^2}$$

n = 73 + 10% for non-responsive rate = 80

3.4 STUDY DESIGN

The study is an experimental research design that was conducted in a black elderly population in the Vaal triangle region of the Sharpeville community purposively selected and constituting samples of N= 102. The participants met the inclusion criteria, attending a day care centre, of equal and comparable age ≥60, socio-demographic status, black race, unemployed and pensioners.

3.5 DATA COLLECTION

3.5.1 Fieldwork

The data collected for this study was done under the supervision of Dr C.J Grobler with her research team in collaboration with the Centre of Sustainable Livelihoods (CSL) and formed part of a multi-micronutrient program in the day-care centre assessing CVR in a sample of elderlies of Sharpeville.

The research team was composed of the principal investigator, HPCSA registered medical technologists, HPCSA registered student medical technologists, a qualified phlebotomist and trained fieldworkers. The systematic data collection process was overseen by the principal investigator during the fieldwork. The qualified phlebotomist collected blood with the assistance of student medical technologists (ensuring that clinical and ethical protocols were applied).

The blood samples were collected in vacutainer collection tubes (fasted blood). The collection tubes were two 7 ml serum separating tube (SST), a 5 ml sodium citrate and two 5 ml of ethylenediaminetetraacetic acid (EDTA). On collection of data, the subjects were rotated between stations of proceeding sequences.

- **Station 1:** Each participant was allocated with a subject number on arrival at the reception desk with a file consisting of a control list (Annexure D), questionnaires and blood collection tubes in a Ziploc bag, all labeled with the corresponding participant's subject number. From there the participants were relocated to the next station.
- **Station 2:** Trained field workers completed the socio-demographic (Annexure H), food frequency (Annexure G), 24 hour recall (Annexure F) Health questionnaire (Annexure E) and anthropometric measurements (weight, height and waist circumference).
- **Station 3:** Blood pressure was measured using a TENSOVAL® MOBIL wrist monitor and clinical signs were observed by the phlebotomist before blood collection. The blood pressure readings were documented on the control list by the trained fieldworkers. A vacutainer system were used to collect fasted blood from subjects from the vena cephalica in two 7 ml SST, 5 ml sodium citrate and two 5 ml EDTA. After each collection, the blood tubes were placed in a cooler box of 8^0 C temperatures and were prevented away from direct contact from sunlight.
- On completion at station 3, subjects were requested to go back to station 1 (reception desk) for handing in their files and verification was done to ensure that all the subjects have attended all the stations. The control lists were checked, ensuring that all data collection processes were completed.

This study however only utilized the SST serum, citrated plasma and EDTA blood for the analysis reported.

3.5.2 Standardization and validation

Validity is the proportion of what is intended to be measured by the instrument (Leedy & Ormrod 2010; Varenne *et al.*, 2015). Validation or intended measurement (described in Table 5) depends on the instrument accuracy, precision, data acquired, and accuracy of response variable and application of the instrument.

Table 5: Types of instrument to measure validity (Vanderstoep & Johnston 2009; Leedy & Ormrod 2010; Heale & Twycross 2015; Royal 2016; Gygi *et al.*, 2017; Ko *et al.*, 2017)

Types of validation	Description
Face validity	It's the proportion to which an instrument truly measures what is intended to be measured.
Content validity	The degree in which an instrument fully represents the content area being measured.
Criterion validity	It is the extent in which results obtained from a measurement instrument correspond with other measuring instruments.
Construct validity	Measures the degree of diagnostic parameters not visible but predicted to have existed theoretically.
a) Convergent validity	The extent at which the assessment of other instruments are similar to recent measurements.
b) Discriminant validity	Where by the assessment instrument does not correlate with another measuring instrument.
Predictive validity	The proportion at which a measurement instrument correlates accurately to the targeted measurements of interest.

Reliability is proportion of consistency measured by the instrument (Vanderstoep & Johnston 2009; Ko *et al.*, 2017). The reliability of the instrument (as indicated in Table 6) used was determined by calculating the standard deviation, mean, coefficient of variation and standard calibration curve.

Table 6: Measurement instrument reliability determination (Vanderstoep & Johnston 2009 & Leedy & Ormrod 2010; Heale & Twycross 2015; Ko *et al.*, 2017)

Types of reliabilities	Description
Interrater reliability	An agreement made by two or more individuals evaluating the same sample.
Internal consistency reliability	The proportion of having the same or similar results within the same instrument.
Equivalent or parallel forms reliability	The degree at which two different measuring instruments having the same distinctive measurement principle produces similar results.
Test-retest reliability	The reliability of results obtained by the same instrument when measured at different times.

3.5.3 Quality assurance and quality control

The main purpose of quality assurance (QA) is to overlook and analyze the systematic quality of results obtained from the true test. Quality assurance incorporates the pre-analytical, analytical and post-analytical testing process (McPherson & Pincus 2011; Bishop *et al.*, 2013). This process enables the proper selection method monitoring the accuracy and precision of the analytical performance, with the daily use of normal and abnormal controls including the methods of validation (Burtis & Bruns 2015).

Quality control (QC) is a tool used to monitor the analytical performance of a test system (Garcia et *al.*, 2014). The use of QC is to ensure the validity of a method implemented. Apart from monitoring the analytical performance, QC also detects the following; analytic errors encountered during analysis and the prevention of the incorrect patients report values (Bishop *et al.*, 2013). The accuracy and reliability of the results reported were obtained by calculating the CV of the control values. These controls were commercially prepared having known values, together with each sample were analyzed using different measuring instruments. The SD was calculated and used to monitor the precision of the measuring instrument. The results obtained were accepted as accurate at 1SD values and CV below 15%. Any values obtained above 2SD were rejected.

3.5.3.1 Blood collection

The collection tubes were checked by verifying the labels with the corresponding subject number on the file of the participants (face validity). Fasting blood from participants was withdrawn by a qualified

phlebotomist. Blood was withdrawn from the vena cephalica of the participants by the phlebotomist using a vacutainer system and tourniquets. Participants were seated comfortably before the phlebotomist withdrew blood. The reliability of results was ensured by preventing haemolysis from occurring by gently mixing the tubes in a figure 8 motion containing anticoagulants (EDTA and Sodium citrate). This was to ensure even distribution of the anticoagulant in the blood tube. Double checking the subject number was done to make sure that the correct tubes for correct analysis have been collected, thus, ensuring internal consistency reliability of results.

3.5.3.2 Transportation

Internal consistency reliability of results was established by placing all samples collected in a cooler box containing ice packs. This was done to ensure that the temperature was maintained at 8^{o}C and the samples were protected against direct exposure from sunlight, to prevent red cells from lysing, which might affect the samples stability. Transportation of samples to the laboratory was done within 2 hours of collection. Face and content validity were applied to prevent haemolysis from occurring by avoiding direct contact with ice when placing in the cooler box.

3.5.3.3 Preparation of Serum

Upon arrival at the laboratory within 4 hours of collection, the samples were separated by centrifuging at 3000 rpm for 10 minutes at room temperature (RT). Following centrifugation, the sample separated (serum and plasma) were transferred into their corresponding aliquot test tubes of 2 ml with labeled numbers (content validity).

In order to minimize or eliminate or prevent errors such as mismatching, the labeled aliquot tubes were numerically organized before serum could be transferred into the aliquot test tube. Also face validity verification was applied, ensuring that the reciprocal sample number is the same with that of the aliquot tubes.

3.5.3.4 Storage

Samples transferred into aliquot tubes containing serum were stored at -80^{0}C. DNA extracts were stored at -20^{0}C before analysis to prevent contamination, with the daily monitoring of temperature and recording on temperature charts (content and criterion validity). The reliability of results was ensured by daily checking of the thermometer of the freezers and comparing it with the acceptable range applicable for freezers of -20 ± 2^{o}C or -80 ± 2^{o}C (internal consistency and equivalent or parallel form).

3.5.3.5 Blood analysis

The analyses of samples were performed under standard laboratory procedures in compliance with the South African National Accreditation System (SANAS) requirements, under the supervision of Dr C.J Grobler (Registered Health Profession Council of South Africa). Quality assurance, validity and reliability for each individual analysis will be discussed in 3.7.

3.5.3.6 Questionnaires

The 24-hour recall (Annexure F) and food frequency questionnaire (FFQ) (Annexure G) and 24-hour recall forms were completed by trained field workers to determine the nutritional intake of vitamins K.

The 24h recall questionnaire was standardized according to Oldewage-Theron et al. (2005). To ensure the reliability of results, the South African local food consumption tables (SAMRC FoodFinder® program) were used. The validity of the food intakes was obtained by comparing the results obtained from the 24-hour recall questionnaires against the FFQ questionnaire (Equivalent or parallel forms reliability). The data collected was compared with the estimated average requirement (EAR) reference and reported in mean and percentages.

3.6 MEASURING INSTRUMENTS

In this study, multiple measuring instruments were used for the collection of data. FVII and fibrinogen was determined using Sysmex CA 600 series (Siemens Healthcare Diagnostics products GmbH, Marburg, Germany). PAI-1 was measured using a semi-automated system EMP W206 microplate washer (Shenzhen Emperor Electronic Technology Co., Ltd, Nanshan, China), Rayto RT-2100C microplate reader (Rayto life and Analytical Sciences Co., Ltd, Nanshan, China) with internationally standardized Enzyme linked immunosorbent assays (ELISA) International kits (Immuno-Biological Laboratories (IBL) Co., Ltd (IBL International GmbH, Hambury, Germany). The genetic polymorphism R353Q was detected using the Polymerase chain reaction MassARRAY® System and iPLEX assays (Agena Bioscience, California, USA). Vitamin K intake was determined by the use of Food frequency and 24h recall questionnaires.

3.6.1 Sysmex CA 600 series

Sysmex CA 600 series (Siemens, 2007) Healthcare Diagnostics products GmbH, Marburg, Germany) is a fully automated blood coagulation analyzer measuring the clotting time that was used to measure the concentration of fibrinogen and FVII by employing the photo-optical clot detection principle. Other measuring principles employed by the Sysmex CA 600 series are chromogenic and immunologic detection.

The chromogenic detection technique measures the absorbance at 405 nm, on addition of a dye (para-nitroaniline) as the sample (blood plasma, reagent and substrate) are exposed to light. The change of light is detected by the instrument and the signal is transmitted to the photo diode which converts the intensity of detection to electric signals, enabling the optical density of the samples to be captured.

The immunologic detection method measures the absorbance at 575 nm. On addition of the stabilizing reagent with the antibody sensitivity reagent, the sample is exposed to light. This creates a change in light absorbance (due to the antigen antibody reaction). The detected signal is transmitted to the photodiode, converting the light to electrical signals. The microcomputer stores and calculates the transmitted light, determining the change in absorbance per minute.

The photo-optical clot detection principle utilizes four detectors to determine the light scattering at 660 nm caused by the clot formation. The application of light at 660 nm ignites the sample plasma/reagent mixture, which reduces the intensity of scattered light resulting in increased turbidity (fibrinogen changing to fibrin). As the process of coagulation is established, the intensity of the scattered light becomes stable and the coagulation curve is drawn by taking the time (X axis) and the intensity of scattered light (Y axis).

3.6.2 Enzyme Linked Immunosorbent Assays (ELISA)

The concept of ELISA is based on the immunologic technique detection of colour change of specific antibodies binding to antigens, whereby labelled antigens enzymes (alkaline phosphatase and glucose oxidase) and antibodies are utilized in detecting biological molecules (Gan & Patel 2013). PAI-1 ELISA IBL International kits (Immuno-Biological Laboratories Co., Ltd International GmbH, Hambury, Germany) micro wells were coated with anti-human antibodies in which sample or standard are added. These micro wells are protein specific in serum which binds to anti human antibody on addition with biotin-conjugate anti-human antibody, establishing a close binding complex which was previously formed. After incubation, proteins (unbounded) are all washed, followed by the addition of Streptavidin-Horseradish Peroxidase (HRP) to the bounded complex. A second incubation is done, followed by another washing in order to remove the unbounded HRP. A colouring agent (chromogen) substrate Tetra Methyl Benzidine (TMB) is added to react against the HRP, creating a change in colour. This is directly proportional to the amount of protein present. A stop reaction of 1M of phosphoric acid is added and the absorbance will be measured at a wavelength of 450 nm (Grobler 2015).

3.6.3 Polymerase chain reaction (MassARRAY® System and iPLEX assays)

The MassARRAY system (Agena Bioscience, California, USA) was used to detect the single nucleotide polymorphism (SNP) for R353Q at Inqaba Biotech, Pretoria, South Africa using the SNP Genotyping technique with iPLEX reagents and the MassARRAY system. This PCR system utilizes a simple, reproducible primer extension reaction chemistry (mass spectrometry) for the qualitative and quantitative SNP genotyping. This system is highly automated, sensitive and specific yielding more than 99.7% accuracy with the presence of MALDI-TOF mass spectrometry and iPLEX assays which replaces the polymorphism sequence length analysis with a SNP panel of interest, speeding up the genetic mapping. The genotypes and somatic mutation assays are designed using an online tool known as Assay Design Suite (ADS). This tool permits target sequence to be imported from the reference SNP (RS) numbers. Following DNA extraction, specific gene primers are amplified by the PCR, and the locus-specific alleles identified using the iPLEX single base extension (Figure 12). A single termination mix and universal reaction conditions were used for all SNPs. The extension of the primers is dependent to the template sequence, which results in an allele-specific in mass in between the extension products, enabling the data analysis software to differentiate between SNP alleles.

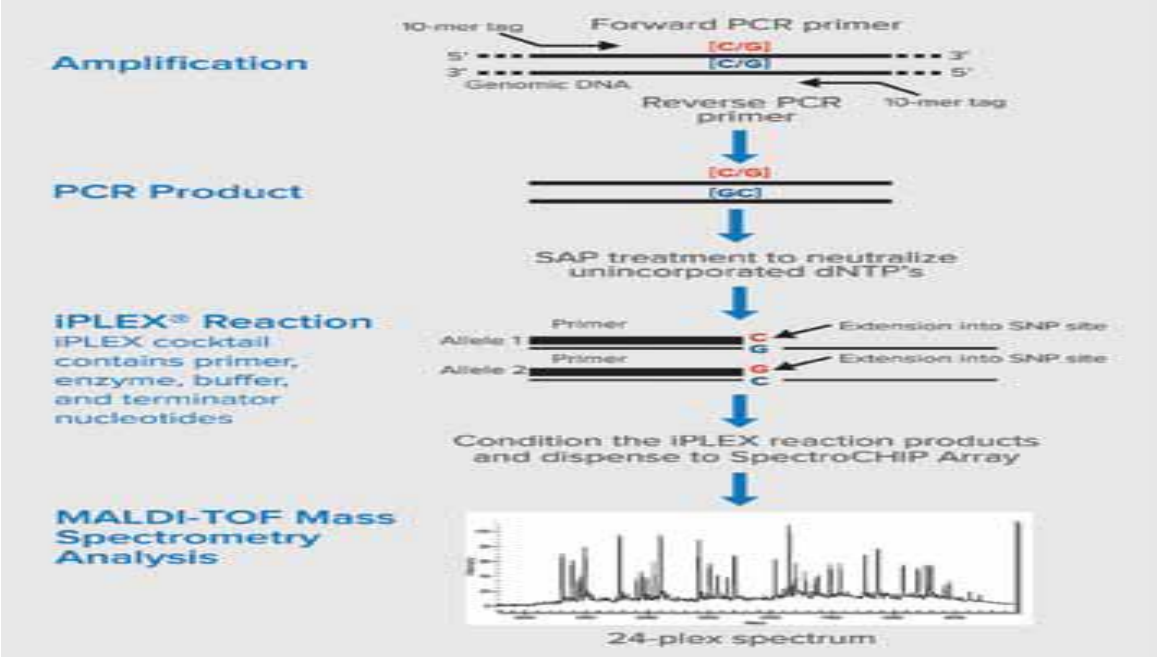

Figure 12: Showing the MassARRAY system workflow of genotyping

This method of genotyping begins with DNA amplification, PCR product, iPLEX reaction of primer, enzyme, buffer and terminator nucleotides which is dispense to the MALDI-TOF mass spectrometry analysis (Agena Bioscience,2018).

3.6.4 Questionnaire

The questionnaires (24-hour recall and food frequency questionnaire) were completed and standardized by trained field workers. Technical approaches were taken into consideration during the training session of field workers, with emphasis of preventing bias and leading questions by the interviewers. The 24-hour recall and food frequency questionnaires were formulated to contain positive and negative responses. The availability of trained field workers helps to facilitates the challenges which were encountered in answering the questionnaires. Due to the fact that participants might respond according to differences in their language, cultural and social status. The reliability of results was ensured by using the South African local food consumption tables (SAMRC FoodFinder® program) specific to vitamin K. The validity of the results obtained from the 24-hour questionnaire and FFQ questionnaire was ensured by comparing the data against the estimated average requirement (EAR) reference.

3.6.4.1 24-hour recall questionnaire

The 24-hour recall questionnaire (Annexure F) was used to obtain nutritional assessment information of foods consumed over the period of 24-hours. This questionnaire is fast and easy to administer in determining the daily quantitative food consumption patterns and the top 20 most consumed food items. According to Castell *et al* (2015) & George *et al* (2016), the 24-hour recall questionnaire is faster in determining the dietary intakes and suitable for low literacy populations, having a good responsive rate. The actual dietary food intake was determined by the trained field workers, requesting the subjects to recall the exact food consumed during the past 24 hours.

i) **Data collection procedure**

The collection of data for the 24-hour recall was carried out on the basis of individual (one on one) interviews between the subjects and trained field workers.

- Step 1: The subjects were asked to list all the foods they have consumed within 24 hours (food items and beverages).
- Step 2: After the subjects have listed the food consumed, probing questions were asked again in order to complete the subject's diet. Questions such as how was the food

prepared, estimation of portion size, brand name of the product (whereby low energy intake and high energy intake was compared).

- The quantity of each food consumed was documented by using food models as memory aids, spoons rulers and measuring spoons.
- The food groups (questionnaire) were rechecked to ensure that all the food groups were mention. In the case where the foods list record did not correlate with subject's record, probing questions were posed again. The 24-hour record of each subjects was compared.

ii) **Reliability**

The reliability of the 24 hours recall form was determined according to the questionnaire developed by Oldewage-Theron *et al* (2005). The dietary intake was analyzed by a registered dietician using the South African local food consumption tables (SAMRC FoodFinder® program). The estimated average requirement (EAR) was used to estimate the prevalence of inadequate intakes within the sample population. But the adequate intake (AI) nutrients level was estimated without using the EAR. The validity of the food intakes was done by comparing the 24-hour recall questionnaire against the food frequency questionnaire.

iii) **Statistical Analyses**

The data obtained from the 24-hour recall questionnaire (dietary intake and food consumption patterns) were captured on Microsoft Office Excel. The captured data was transferred and analyzed on SPSS version 12 and Food Finder program, respectively by a registered dietician. The means and standard deviations of the dietary intake and food consumption patterns were calculated. The values obtained were compared against the EAR of the subjects.

3.6.4.2 Food frequency questionnaire (FFQ)

The FFQ questionnaire is globally used as a measuring tool to study the association between diet and disease in a large-scale population (Sauvageot *et al.,* 2013). This questionnaire (Food frequency questionnaire) measures the dietary diversity of food consumed during a certain period of time (Jackson *et al.,* 2011; Mulligan *et al.,* 2014). The FFQ questionnaire is quick to administer and less time consuming. The daily food intake is well assessed using this questionnaire, which is very specific in identifying the food consumed such as fruit and beverage. This has been used on large scale studies, giving a higher estimate of some of the foods consumed (Cuenca, 2015). The food

group variety balances other nutrients varying from high, low and medium. The different foods group diversity includes the following; meat, eggs, dairy products, cereal, vegetables, other fruits, oil and fat diversity.

i) **Data collection procedure**

The FFQ collection of data was carried out by a one on one interview basis between the trained field workers and the elderlies (subjects). The subjects were asked to indicate which foods or groups of food they consumed specifically over a particular period, the portion size of food (large or small). This was done by ticking the corresponding food from the list of foods in the food list.

ii) **Reliability**

The reliability of this questionnaire (Annexure G) was standardized according to Oldewage-Theron and Kruger (2009), adapted from Matla (2008). All the food items having a dietary food patterns were listed with their local names, with majority of common food items included in the FFQ after having focus group discussions.

iii) **Statistical analyses**

Microsoft Office Excel was used to capture the data and analyzed on the SPSS (Statistical product and service solutions) version 12 program. The frequencies, means and standard deviations were calculated. The overall variety score, variety score across all nine food groups and variety score within every food group was calculated for a reference period of seven days to determine the different dietary diversity.

3.7 BLOOD PARAMETERS

3.7.1 Factor VII

The levels of plasma FVII were determined using the Sysmex CA 600 automated coagulation analyzer. The reagents of analysis consisted of FVII deficient plasma, Innovin, standard human plasma and owrens veronal buffer. The photo-optical clot method was used in determining the plasma level of factor VII.

3.7.1.1 Principle

Plasma deficient of the extrinsic pathway leads to prolonged thromboplastin time (PT). As FVII reagent is mixed with the plasma of patient (citrated plasma), thromboplastin time is tested. The results obtained are reported in percentage form and compared with the reference curve (dilution) of the standard human plasma or normal plasma. As the subject's plasma sample tubes were placed in the sampler rack at appropriate position, analysis of samples began. Each plasma sample was automatically aspirated at the aspiration position by the heated probe of required volume. The aspirated plasma samples (5 ul) were dispensed into a reaction tube in the samples tube rack by the heated probe. This reaction tubes rack containing the plasma samples were transferred by the heated probe into the incubation wells. Incubation took place at a specific time frame. The reagents (FVII deficient plasma, Innovin, standard human plasma and owrens veronal buffer) were aspirated and transferred by the heated probe into the reagent rack. The reagent rack containing the aspirated reagents was transferred and incubated in the heater probe for a specific time frame. Both samples (plasma and reagent) were transported by the sample catcher into the reaction tube and mixed by vibration. At this stage, thromboplastin reagent innovin (phospholipid protein extract of tissue) activates the coagulation cascade during the incubation time with plasma on addition of calcium chloride. Tissue factor and phospholipid initiates the coagulation cascade by the activating FX and FVII. When low concentration of FVII binds to tissue factor, activation occurs producing FVIIa. The FVIIa (activated) binds to FX and activates FX to become FXa (activated). The activated FX (FXa) with the presence of the cofactor prothrombinase now converts prothrombin to thrombin. This reaction leads in the formation of a clot. The reaction tubes were transferred to the detection wells with the illumination of red light for detection at a wavelength of 660 nm. As light is transmitted to the detector, the formation of clot is measured. Prolonged thromboplastin time (PT) depends on the presence of decreased clotting factors. Reactive thromboplastin will activate FX by FVII less rapidly as compared to unreactive thromboplastin. When this happens, PT will be prolonged due to the decrease in dependent coagulation factors (vitamin K).

3.7.1.2 Validation

The validation of FVII was determined by standard calibration curve generated by the automated coagulation analyzer Sysmex CA 600 using commercial FVII calibrators. Six calibrators were run in duplicates of concentrations varying from 0%, 11.4%, 22.8%, 45.5%, 91.0% and 136.5%. The reproducibility was determined by the two controls such as normal control (control plasma N) and abnormal or pathological control (control plasma P). These controls were run before analysis, after every hour interval and immediately when reagents were changed. The coefficient of variation was calculated, documented and used as an internal quality control referral. The control N values were 102%, 108.4%, 114.2%, and 114.8%.

The expected range for control N was 82 to 122%. The control P values were 36%, 37.3 %, 38.7%, and 39.8%. The expected range for control P was 27 to 45%.

3.7.1.3 Data analysis

The concentration of FVII was captured on Microsoft Office Excel. SPSS version 12 software program was used for descriptive statistical analysis. The mean and standard deviation of data captured was determined.

3.7.1.4 Normal ranges

The normal range in determining FVII concentration was between 70 to 120% (Schaefer *et al.*, 2015).

3.7.2 Fibrinogen

Fibrinogen plasma levels were determined by the photo-optical clot method using the Sysmex CA 600 automated coagulation analyzer. The reagents of analysis consisted of Dade Ci-trol 1, standard plasma and multifribren U. Multifibren U reagent was composed of bovine thrombin, fibrin-aggregation retarding peptide, calcium chloride, sodium chloride, hexadimethrine bromide, polyethylene glycol, tris, bovine albumin and sodium azide.

3.7.2.1 Principle

Fibrinogen concentration was determined by using the modified Clauss method (Ameri *et al.*, 2011; Kotze *et al.*, 2014; Cronjé *et al.*, 2016), where by citrated plasma was brought in reaction with excess amount of thrombin reagent (Multifibren U) resulting in coagulation. On addition of bovine thrombin (catalyzing reagent) to platelet poor plasma, the conversion of fibrinogen to fibrin polymer is established. As thrombin cleaves on the polypeptide chains (Aα and Bβ), fibrinopeptide A and B are released. This release leads to the polymerization of fibrin monomer forming a fibrin clot in an insoluble form (Kollman *et al.*, 2009; Hoffbrand *et al.*, 2011; Undas & Ariens 2011; Chapin & Hajjar 2015).

The coagulation reaction time is dependent on the concentration of fibrinogen in the specimen of analysis (Siemens Healthcare Diagnostics GmbH 2008). The rate at which the clot is formed is proportional to the concentration of fibrinogen (Adam *et al.*,2010; Oberfrank *et al.*, 2016), as the sample mixed is illuminated by the red light at a wavelength of 660nm. The intensity of light changes due to increased turbidity of fibrinogen forming a fibrin clot, establishing a coagulation curve on basis of clotting time and the intensity of light represented on the X and Y axis (Sysmex CA 500 series 2003). Bovine thrombin helps in the conversion of fibrinogen to fibrin clot (prothrombin activated to thrombin) by cleaving selectively to the Arg-Gly bonds. The presence of calcium chloride activates platelets, releasing growth factors, activating coagulation by converting fibrinogen to fibrin.

3.7.2.2 Validation

Fibrinogen was validated by determining the standard calibration curve generated by the automated coagulation analyzer Sysmex CA 600 using commercial a fibrinogen calibrator's kit. Six calibrators were run in duplicates of concentrations varying from 0.7 g/l, 1.1 g/l, 2.3 g/l, 3.5 g/l, 5.6 g/l and 8.2 g/l. The reproducibility was determined by the three controls (Control plasma N, Ci-Trol® 1, and Control plasma P). The normal control (control plasma N) and the abnormal or pathological control (control plasma P) were run before the samples analysis, immediately when reagents were changed and after every hour interval. The control values for Ci-Trol® 1 were 2.8 g/I and 2.9 g/I, with the expected range of 2.3 to 2.9 g/l. Control N values were 2.8 g/I, 2.9 g/I, 3.0 g/I, and 3.2 g/I, with the expected range of 2.2 to 3.4 g/I. The control P values were 1.0 g/I, 1.0 g/I, 1.0 g/I and 1.0 g/I, with the expected value of 0.6 to 1.4 g/I.

3.7.2.3 Data analysis

Fibrinogen concentration was captured on Microsoft Office Excel. SPSS version 12 software program was used for descriptive statistical analysis. The standard deviation and mean of data collected was captured and determined by using p-values. The mean concentration was compared with the concentration of the normal ranges.

3.7.2.4 Normal ranges

The reference range used for determining fibrinogen ranged between 1.8 to 3.5 g/l (Hassan *et al.*, 2018).

3.7.3 Plasminogen activator inhibitor-1 (PAI-1)

Human serum PAI-1 was analyzed by using the EMP W206 microplate washer (Shenzhen Emperor Electronic Technology Co., Ltd, Nanshan, China) Rayto RT-2100C microplate reader (Rayto life and Analytical Sciences Co., Ltd, Nanshan, China) and internationally standardized PAI-1 ELISA international kit (Immuno-Biological Laboratories Co.Ltd International GmbH, Hambury, Germany). This kit was composed of a microwell plate coated with human PAI-1 monoclonal antibody, anti-human PAI-1 polyclonal antibody (Biotin-conjugate), alkaline phosphate Streptavidin-Horseradish Peroxidase (HRP) (conjugated), tetramethyl-benzidine (buffer solution), colouring substrate Tetra Methyl Benzidine (TMB) and 1M phosphoric acid of stop solution (IBL International 2012).

3.7.3.1 Principle

The plasminogen activator inhibitor-1(PAI-1) was analyzed by using the IBL ELISA kit.

- During the first stage of incubation, human serum was added into the microwells coated with monoclonal anti-human PAI-1 antibody. Human PAI-1 complex was established on addition of biotin-conjugate polyclonal anti human antibody.
- In the second stage of incubation, the unbounded protein was washed away with the assay buffer. Streptavidin-Horseradish Peroxidase (HRP) was added to the bounded complex.
- Following the third stage of incubation, the unbounded HRP was washed and a HRP reactive substrate solution was added into the microwells.

Tetra Methyl Benzidine (TMB) substrate (colouring agent) was added against the HRP, which created a change in colour which is directly proportional to the concentration of PAI-1 present. A stop reaction was added (1M of phosphoric acid) and absorbance was measured at wavelength 450 nm. The serum PAI-1 concentration was determined by the standard curve created from the standard dilutions of PAI-1(IBL International 2012).

3.7.3.2 Validation

The method of validation was carried out by the application of seven standard calibrators supplied by the kit of varying concentrations. These calibrator's concentrations varied from 5 ng/mL, 2.5 ng/mL, 1.25 ng/mL, 0.625 ng/mL, 0.313 ng/mL, 0.156 ng/mL and 0.078 ng/mL, and were used in duplicates to produce a calibration curve used in determining serum PAI-1. Sample controls of known human PAI-1 concentration were run in duplicates. The calibration curve displayed a direct relationship between the absorbance at 450nm of the stop reaction (coloured end-product). The concentration of PAI-1 was determined with the high value of 5 ng/mL and low value of 0.078 ng/mL of the standard concentration. Results obtained from controls were compared with the reference values (previously mentioned). Any value obtained above 5 ng/mL and below 0.078 ng/mL calibrator's concentration and control range were considered as invalid.

3.7.3.3 Data analysis

Microsoft Office Excel was used to capture the raw data obtained from the concentration of PAI-1. The SPSS version 12 software program was used for descriptive statistical analysis, determining the mean and standard deviation of data using p-values.

3.7.3.4 Normal ranges

The normal range for PAI-1 is 1.2-286.0 ng/ml, having a mean level of 41.9 ng/ml (IBL International GmbH 2012).

3.8 R353Q Polymorphism

The detection of the single nucleotide polymorphism (SNP) R353Q was achieved by a PCR-based specific analysis technique (SNP Genotyping technique with iPLEX reagents and the MassARRAY system) R353Q primers and subsequent sequencing. The extraction of DNA was carried out by using the universal kit Quick-DNA™ from the Zymo Research Corporation.

3.8.1 DNA Extraction

The DNA was extracted from the EDTA blood samples was using the Quick-DNA™ Universal Kit (Zymo Research Corporation, Irvine, California, USA). This kit comprises of Proteinase k, BioFluid and cell buffer, genomic binding buffer, DNA Pre-wash buffer, g-DNA wash buffer, DNA elution buffer, Zymo-Spin™ IIC-XL columns and collection tubes. In a microcentrifuge of 1.5 ml tube, 200 µl of whole blood, 200 µl of BioFluid and Cell buffer and 20 µl of Proteinase K were added. The microcentrifuge tube was then mixed thoroughly using a vortex for 10 to 15 minutes. The reaction mixture was then incubated at a temperature of 55ºC for 10 minutes. This was followed by the addition of 420 µl Genomic binding buffer into the digested sample and mixed thoroughly for 10-20 seconds. The mixture in the microcentrifuge tube was then transferred into a Zymo-Spin™ IIC-XL Column in a collection tube and centrifuged at ≥ 12,000 x *g* for 1 minute. The collection tube was discarded and 400 µl of DNA pre-wash buffer was added into the spin column placed in a new collection tube. This was followed by centrifugation at 12,000 x *g* for 1 minute. The collection was later discarded and 700 µl of g-DNA wash buffer was added into the spin column and centrifuged at ≥ 12,000 x *g* for 1 minute. Following centrifugation, the collection tube was voided and 200 µl g-DNA was added in a new collection tube. This mixture was centrifuged of ≥ 12,000 x *g* for 1 minute and the flow through was discarded. The spin column was then transferred into a clean microcentrifuge and 50 µl of DNA Elution buffer was added directly on the matrix. The mixture was then incubated at room temperature for 5 minutes and centrifuged for 1 minute at a speed of ≥ 12,000 x *g* (in order to elute the DNA). At the final stage, the eluted DNA was stored at a temperature of ≤ -20ºC for later use in PCR reactions. Each DNA sample concentration was determined using the Shimadzu uv-mini-1240b spectrophotometer. The DNA sample was placed into a cuvette (10mm path-length) and the concentration was measured at an absorbance of 260 nm.

3.8.2 R353Q Amplification principle setup

The sequencing of R353Q was done at Inqaba Biotech, Pretoria, South Africa using the SNP Genotyping technique with iPLEX reagents and the MassARRAY system. The DNA samples were diluted from 50 ng/ul to 25 ng/ul and set for amplification prior to extension reaction of SNP of interest. The primers

sequence used were forward 5'-ACGTTGGATGTACTCGGATGGCAGCAAGGA-3', reverse 5'-ACGTTGGATGTGACCATGCCCGTCAGGTA-3' and extension probes 5'-ATGCCACCCACTACC-3' for each SNP of interest were designed online using Agena Assay designer software. The primers (forward and reverse) for PCR amplification were reconstituted at equal molar concentrations of 100 uM, with further dilution of working solution of 0.5 uM each concentration. The probes for iPLEX extension were reconstituted for equal molar concentrations of 500 uM. Multiplex PCR master mix excluding DNA was prepared, constituting of 1.8 ul water of HPLC grade, 0.5 ul 10X PCR buffer with 20 mM $MgCl_2$, 0.4ul $MgCl_2$, 0.1 ul dNTP mix, 1.0 ul Primer mix and 0.2 ul PCR enzyme yielding a total volume of 4.0 ul. A post PCR reaction clean up solution of shrimp alkaline phosphatase (SAP) was prepared (for removal of any remaining nonincorporated dNTPs from amplicons) which constituted of 1.53 ul of water (HPLC grade), 0.17 ul SAP buffer, 0.30 ul SAP enzyme yielding a total volume of 2.00 ul. The single extension (SBE) master mix (iPLEX reaction) was prepared (0.619 ul water of HPLC grade, 0.200 ul iPLEX buffer plus, 0.200ul of termination mix, 0.940 ul primer mix and 0.041 ul iPLEX enzyme), yielding a total of 2.00 ul.

3.8.2.1 R353Q PCR Amplification principle

The PCR amplification of target loci thermal cycling reaction was set according to the following stages. Denaturation (1 cycle) for 2 minutes at 94°C (initial denaturation) and final denaturation (45 cycles) for 30 seconds at 94°C. The annealing phase was set for 30 seconds at 56°C and 60 seconds at 72°C. The extension phase was set 5 minutes at 72°C (1 cycle) and held at 4°C for final stage. The prepared master mix (4ul) was transferred into the microplate wells and 1ul of DNA was added into each well. A non-template control was also included in the plate.

Following the PCR amplification stage, the amplicons were cleaned up using the post PCR SAP solution (Post-PCR SAP reaction clean up), where by 2 ul of the SAP solution was added to each well containing the PRC product according to the following cycles; 1 cycle at 37°C for 40 minutes, 2 cycles at 85°C for 10 minutes and final stage at 4°C.

At the iPLEX single base extension stage or iPLEX reaction, the primer extension master mix reaction of 2ul each was added to the SAP treated plate (for detecting single base polymorphism in the amplified DNA). The primer extension reaction thermal cycle was set conforming to the sequential cycles. Initial denaturation was set for 30 seconds at 94°C (cycle 1) and 5 seconds for 94°C (40 cycles). Annealing (5

cycles) was set at 52ºC for 5 seconds, extension for 80ºC for 5 seconds and final extension (1 cycle) at 72ºC for 30 minutes. The last step was held at 15ºC.

The primer extension reaction resin clean up step was done, following the iPLEX reaction, whereby the 15 mg resin and 41 ul of nuclease free water was added to each product (primer extension) to remove any salts present in the SBE plate.

3.8.3 R353Q Polymorphism detection principle

The detection of the extended products (R353Q) was done using a MassARRAY compact mass spectrometer software and Agena real time detection software. These softwares design automatically the PCR and MassEXTEND primers (for mutlplexed assays), controlling and preventing the combinations of primers and the extension of non-template products which could lead to non-specific extension.

As the SpectroCHIPS are placed into the mass spectrometer, a laser shot each spot under vacuum with a matric assisted laser desorption and ionization time of flight (MALDI-TOF) method. As the samples are completely vaporized and ionized by MALDI mass spectrometry, they are electrostatically transferred into a time of flight mass spectrometer (TOF-MS). This separates the matrix ions, detecting each sample depending on their mass to charge ratios.

3.8.4 Validation

To validate the detection of R353Q polymorphism in the population, the Sequenom RealSNP software (Sequenom, California, USA) was used, which ensured the production of any amplicons that only contain the target (R353Q) of the PCR reaction.

3.8.5 Data analysis spectrum

The data was analyzed by evaluating the chromatograms for strong peaks (amplification of the target) and baseline noise, after the amplification of R353Q polymorphism in the population.

3.9 Descriptive Statistics

Descriptive statistics is a standard of numerical (quantitative) analysis, which is used in summarizing the sample characteristics (Adams & Lawrence 2015). Statistically, normal distribution (bell curve) is a very important tool in data analysis whereby different statistics or data can be transformed from close to normal (Bartholomew, 2016).

For normal distribution to be determined, the dispersion of data or the proportion of distribution lies between the mean and standard deviation with 99% of the data lying between ± 3SD of the mean, 96% of the data lying between ± 2SD of the mean and 68% of the data lying between ± 1SD of the mean (Adams & Lawrence 2015; Bartholomew, 2016). For continuous and discrete distribution, the data will be reported as mean, median, mode and skewness (mean-median-mode inequality). The median will be used if there is a skewedness (positive or negative). Which is centrally located (median) between the mean and mode (Zheng *et al.,* 2017).

Histogram and cumulative frequency distribution graphs are used to determine normality displaying the interval ranking on the Y and X axis, with the frequency of each data represented and the points are connected to a straight line (Adams & Lawrence 2015; Bartholomew, 2016). The mean is obtained by adding all the data (observation) and dividing by the total number of data. The median is obtained by dividing the value of the data into half, where one set of data will be below or above the other set (Adams & Lawrence 2015).

The tools for the data analysis used in this study were Microsoft excel, SPSS version 12 and chromatogram software. This generic software explicitly rescinded all the manual errors and strains encountered in statistical calculations. Thus, reckoned the ideal values of the central measurement tendencies and measurement of dispersion used in this study, sketching desirable graphs for the each of the cross-examination tests of samples (FVII, fibrinogen, PAI-1, Vitamin K and R353Q polymorphism) over the sampled population.

CHAPTER 4

RESULTS

4.1 INTRODUCTION

In this chapter, the results obtained from the analysis of FVII, fibrinogen, PAI-1, dietary intake of vitamin K (questionnaires) and the genetic polymorphism R353Q are reported. The results are presented by means, standard deviations, medians, descriptive statistics and inferential statistics where applicable). Moreover, the data analysis tools used for this study were Microsoft excel, chromatogram and SPSS 12 software, which aided to achieved the ideal nature of the experiment in this study.

4.2 SAMPLE PROFILE

The number of elderlies attending the day care center in Sharpeville were 102 (refer to 3.4), of age >60 and older (pensioner) and race black (100%). Before the data were collected at the day care centre, the 102 elders signed the consent form for the study to be conducted.

The mean age was 73±9 years, varying between 60 to110 years, 85% females and 15% males, with the majority living in poverty, having poor diet or poor nutrition (Grobler, 2015).

Some challenges were encountered during the process of data collection, as insufficient blood was collected from some subjects. In case of insufficient blood collection (n=19), the data were imputed statistically. The results were reported as the sample size was statistically characterized.

4.3 HAEMOSTATIC LEVELS

The haemostatic levels (FVII, fibrinogen and PAI-1) of the sampled population were subjected to a normality test. The skewness values obtained from the different variables are as follow; -0.009 (FVII), 0.5 (fibrinogen) and 0.2 (PAI-1). According to Kim (2013), the skewness for a normal data distribution should be zero. Therefore, the mean and standard deviation was reported for FVII levels (Figure 12). For variables with positive skewed values, the median and the interquartile range (IQR) were reported for fibrinogen and PAI-1(Figure 15 and Figure 18). However, the tool analysis used was excel s and SPSS 12 software. This aided to achieved the ideal nature of the experiment in this study.

4.4 FACTOR VII LEVELS

The plasma concentration for factor VII was determined in 83 subjects out of the 102 subjects who gave consent as referred in 4.2. Following the normality test, the concentration of FVII levels were normally distributed (Figure 13). The tool analysis which was used to design the figure below was SPSS 12 software.

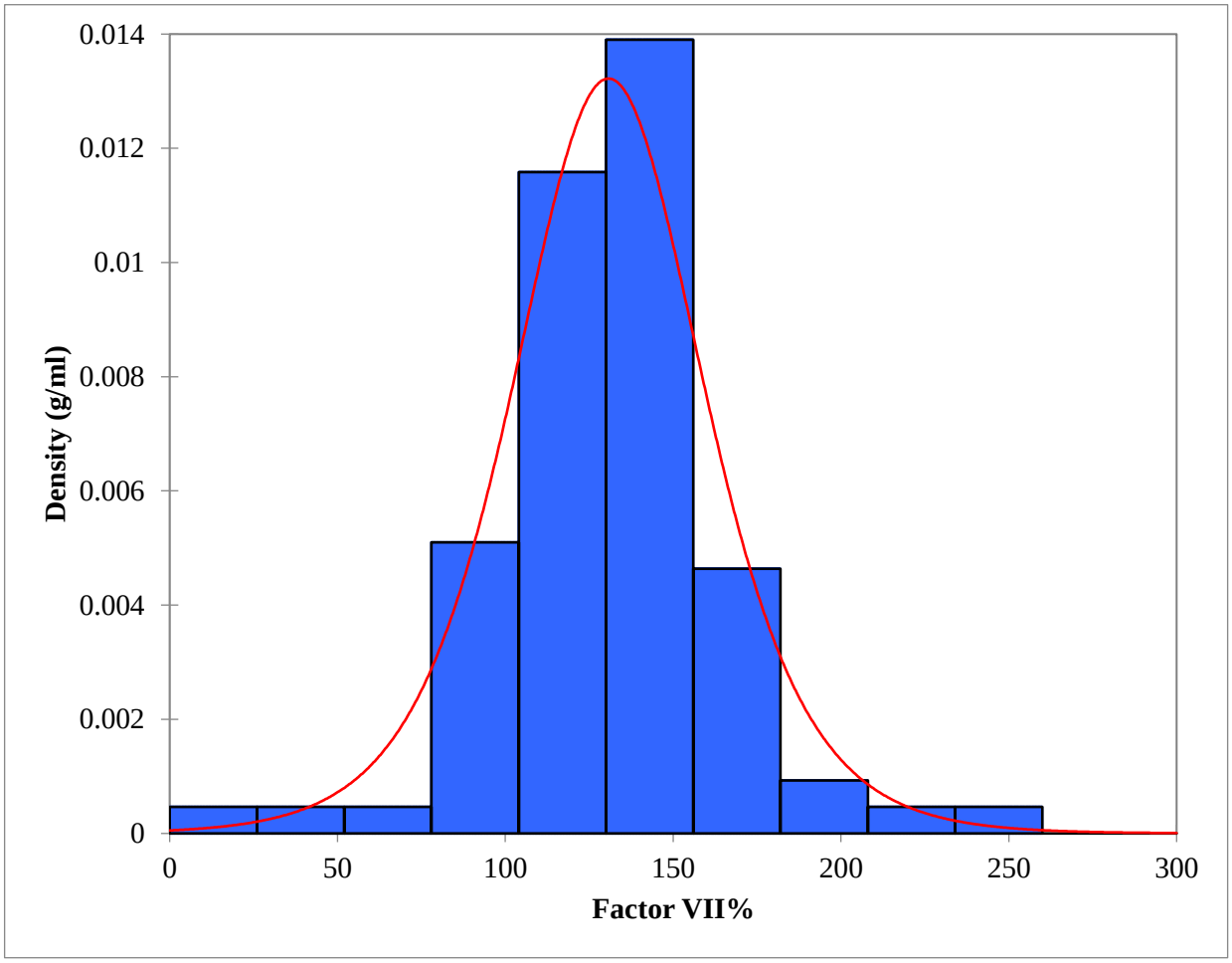

Figure 13: Normal distribution curve of factor VII concentration

The density plot value obtained from the normality test was -.0.009. This value is closer to zero and therefore the mean and the standard deviation were calculated and reported. The mean concentration of FVII was 130.9% and the standard deviation was 36.2 %. The normal value of FVII ranges from 70 to 120%. The high level of FVII is > 120% and low level is < 70% (Schaefer *et al.*, 2015). Thus, FVII levels were divided into three categories as Normal, high and low (Figure 14). In this sampled population, 61% (n=51) of the subjects had higher levels of FVII above the normal reference value, 35% (n=29) of the

subjects were within the normal reference value and 4 % (n=3) had a low level of FVII out of the 83 subjects. The figures (14 and 15) was design using microscoft excel program as the data tool analysis.

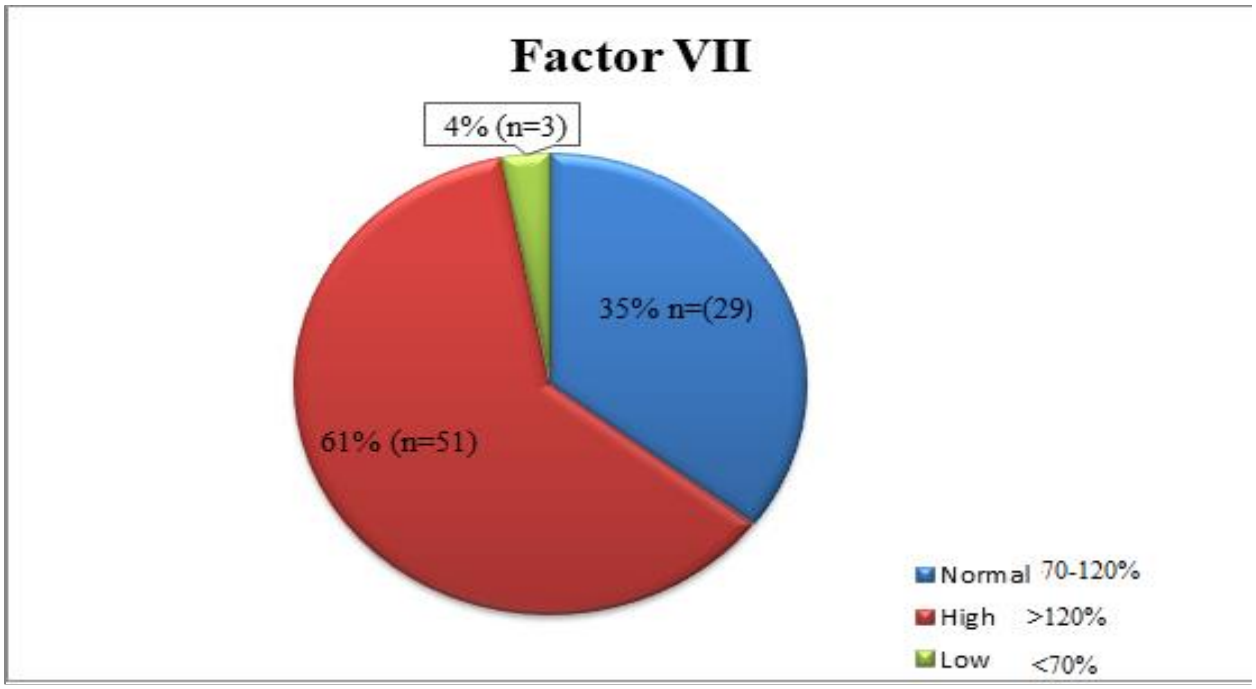

Figure 14: Factor VII plasma distribution of the sampled population

The analysis of abnormal FVII levels were repeated and in spite of that there were four outliers were encountered (Figure 15).

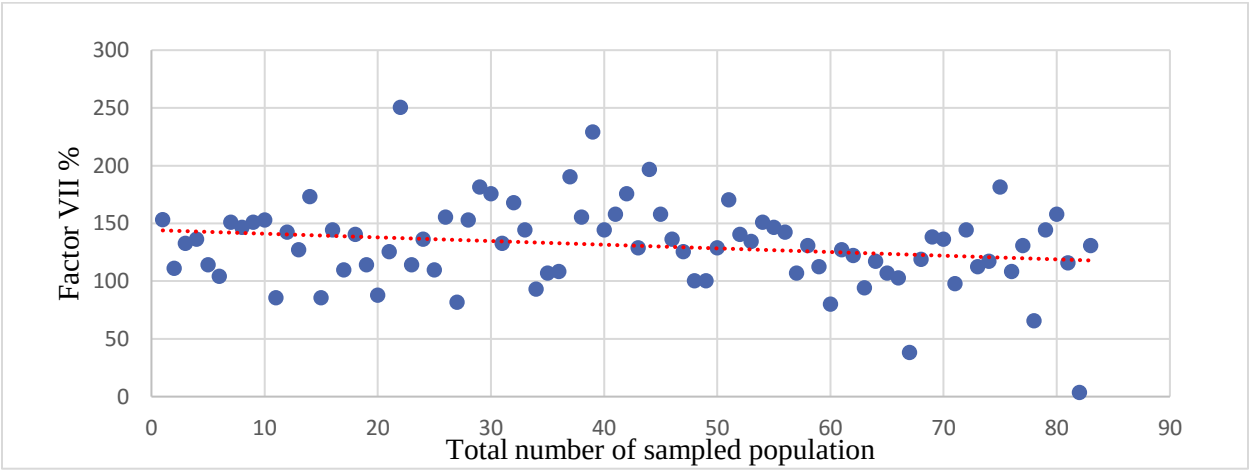

Figure 15: Scatterplot of FVII plasma concentrations

Two outliers were at the 200% region and the other two outliers were at the 0% region. The highest outlier was 250.5% and the minimum outlier was 3.8% as compared to the normal reference value of 70% to 120%.

4.5 FIBRINOGEN LEVELS

Fibrinogen concentration was determined in 83 subjects out of the 102 subjects who gave consent (as discussed in 4.2). The results obtained from the normality test for fibrinogen concentration indicated a positive skewed value (Figure 16) with skewness value of 0.5. The data analysis tool used to design figure 16 was SPSS version 12 software.

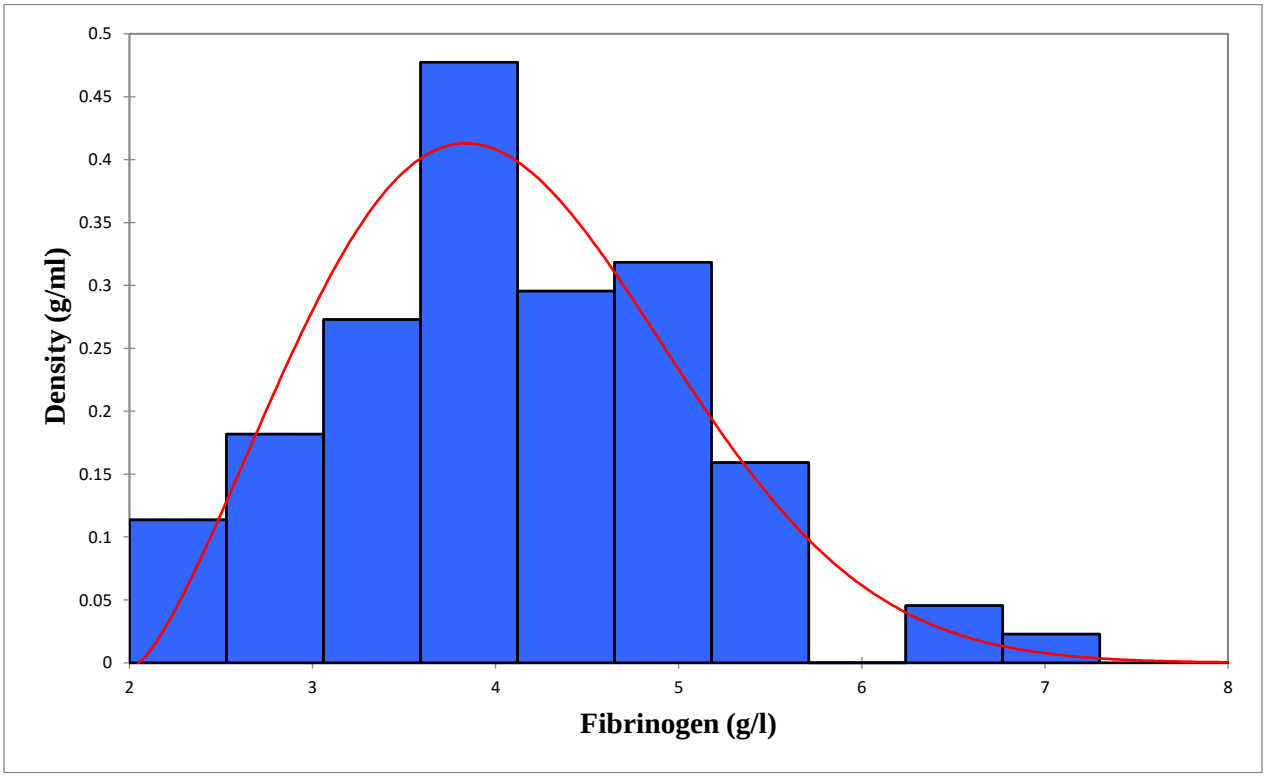

Figure 16: Skewed distribution of fibrinogen concentration levels

The plasma concentration of the sample population for fibrinogen was positively skewed, therefore the median and the interquartile range (IQR) was calculated. The median value was 4 g/l, with the IQR of 3.4 g/l (low) to 4.1 g/l (high).

Plasma fibrinogen levels were classified as high (hyperfibrinogenemia) when the value obtained was above the reference range, normal when the values was within the range of 1.8 to 3.5 g/l and low (hypofibrinogenemia) when the values obtained were below the normal reference of range (Figure 17) (Hassan *et al.,* 2018). The fibrinogen studies collaboration (2007) concluded that an increase of 1 g/l in plasma fibrinogen doubles the risk of CVD. Figure 17 was designed using Microsoft excel program.

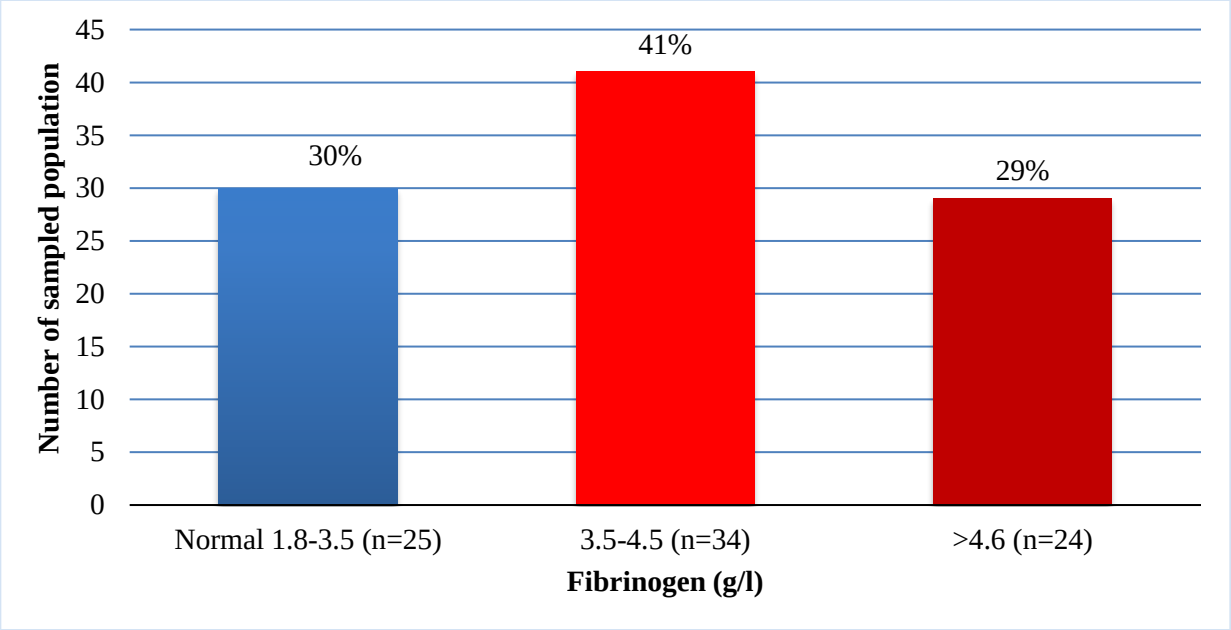

Figure 17: Fibrinogen plasma levels distribution of the sampled population

Although no subjects had a concentration below the normal reference range of 1.8 to 3.5 g/l, 30% (n=25) of the subjects were within the normal reference range for fibrinogen with 41% (n=34) of the sample population having fibrinogen level of 3.5 to 4.5 g/l and therefore doubled the risk of CVD. A total of 29% (n=24) had a fibrinogen level of >4.6 g/l and therefore four-times higher risk for CVD. The analysis for the determination of plasma fibrinogen levels was done on a repetitive basis and three outliers were encountered (Figure 18). Microsoft excel program was used to as the data tool analysis to design figure 18.

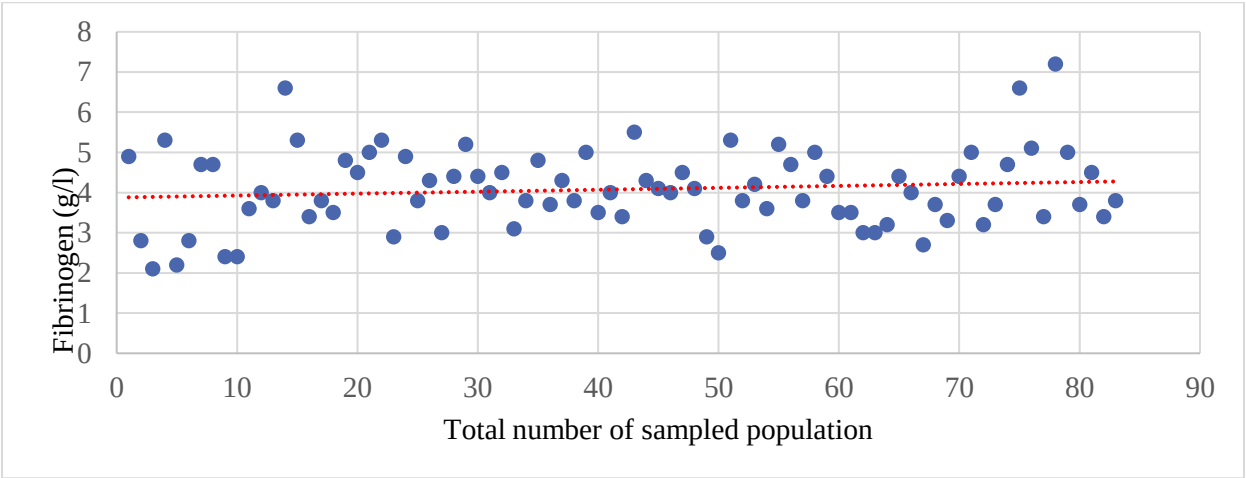

Figure 18: Scatterplot of the Fibrinogen concentrations

The two outliers were at 6 g/l region and one outlier at 7 g/l region. The upper limit was at 7.2 g/l and lower limit at 2.1 g/l as compared to the normal reference value of 1.8 to 3.5 g/l. As previously mentioned, no low levels of fibrinogen concentration were observed in the sampled population. The maximum limit indicated that 29% of the sample population are at double risk of CVD. Abnormal results were repeated and confirmed.

4.6 PAI-1 LEVELS

The concentration of PA1-1 was determined in 102 subjects who gave consent (as discussed in 4.2). The sampled concentration of the population following a normality test was skewed to the right as shown in the (Figure 19) with the skewness value of 0.2.

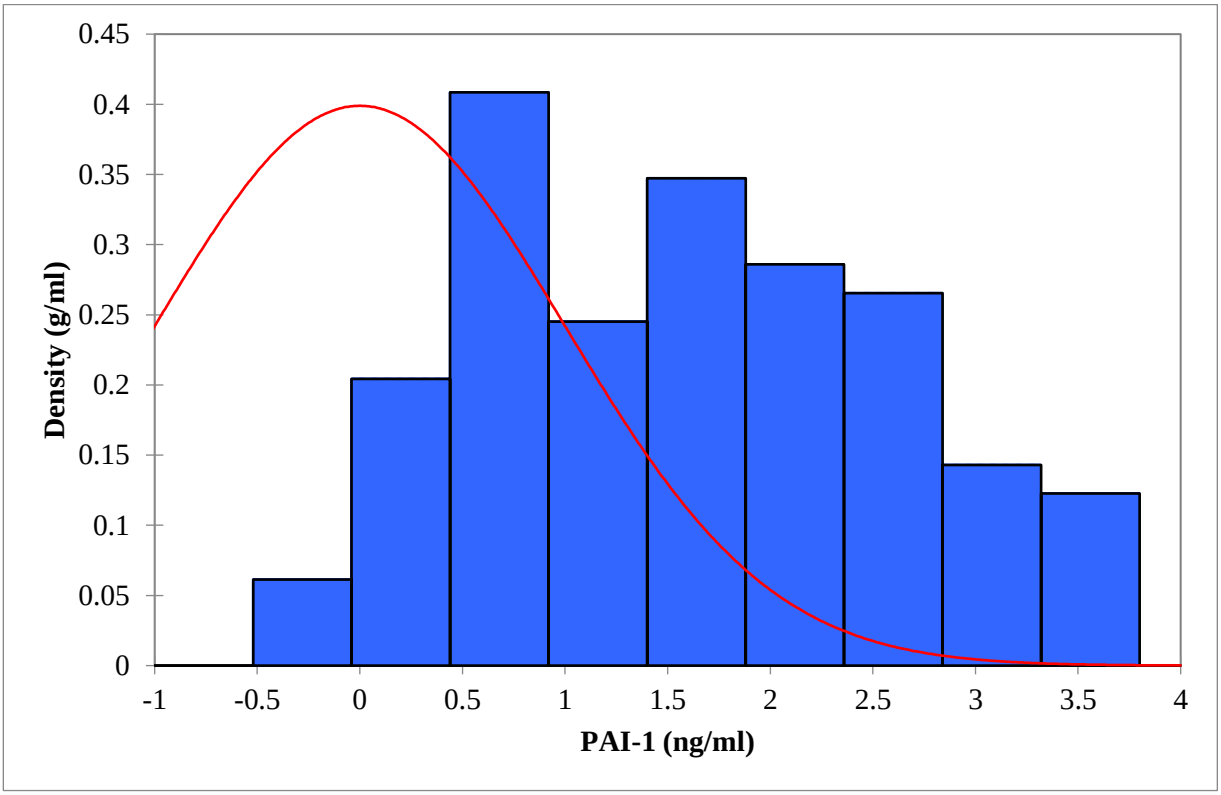

Figure 19: Skewed distribution of PAI-1 concentration levels

PAI-1 concentration of the sampled population was skewed to the right, therefore the median and the IRQ were calculated and reported. The median concentration levels of PAI-1 were 1.6 ng/ml, with the lower quartile value of 0.8 ng/ml and upper quartile value of 2.4 ng/ml. Figure 19 data analysis tool was SPSS 12 software.

The levels of PAI-1 concentration were categorized into 3 categories as high depending on if the value obtained was above the normal reference value, normal depending on if the value obtained was within the normal reference value of 3 to 72 ng/ml and low depending on if the value obtained was below the normal reference value (Figure 20) (Mayo Clinic 2014).

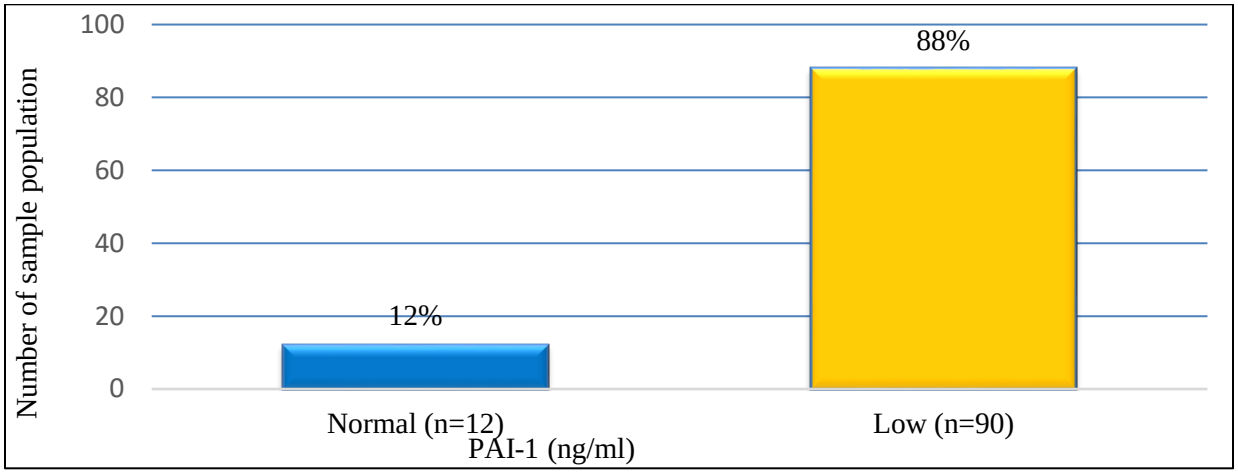

Figure: 20 PAI-1 plasma levels distribution of the sampled population

In this sampled population, there were no elevated levels of PAI-1 concentration above normal reference value. The minority of the subjects 12% (n=12) were within the normal reference level. A decreased fibrinolytic activity was encountered in 88% (n=90) of the sample population having low concentration of PAI-1. The determination of PAI-1 concentration was analysed on a repetitive basis and five outliers were observed (Figure 21). Figure 20 and figure 21 were designed using Microsoft excel program.

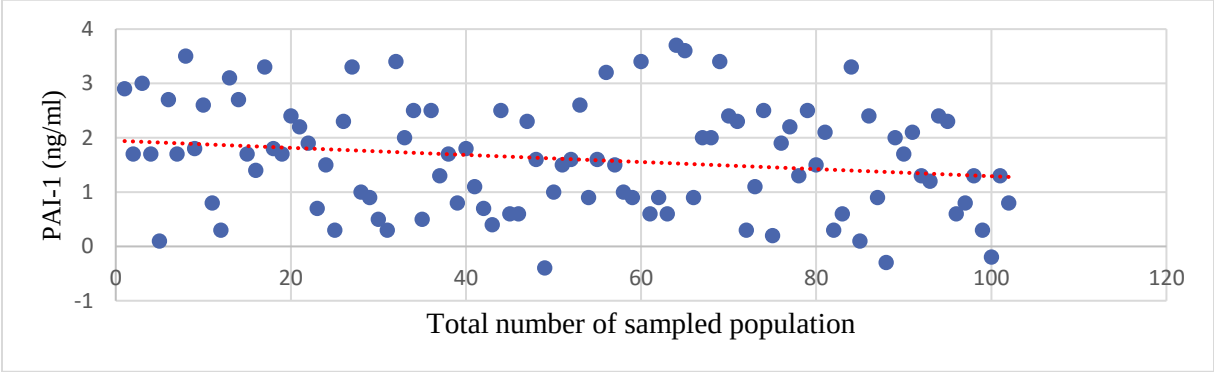

Figure 21: Scatterplot concentrations of PAI-1

Two outliers were at 3.5 ng/ml region with the maximum outlier at 3.7 ng/ml. Three outliers were at -0.5 ng/ml**.** The minimum outlier was at -0.4 ng/ml region as compared to the normal reference range of 3 to 72 ng/ml. Low levels of PAI-1 has been proven as an independent risk for CVD. Thus, 88% of this sampled population were at a higher risk of CVD.

4.7 VITAMIN K INTAKES

The recommended adequate intake (AI) of vitamin K globally is 120 mcg/day (males) and 90 mcg/day (females) (Bellows & Moore 2012; Bruno, 2016; Shea & Booth 2016; Fusaro *et al.,* 2017, Riphagen *et al.,* 2017; Palermo *et al*., 2017). According to Fusaro et al. (2017), the stratified intake of vitamin K was suggested to be 140 or 170 mcg/day for the age group of 18 to 59 and >60 years. In this sample population, 108 of the subject's daily vitamin K intakes were analyzed. The mean and standard deviation of vitamin K intake was 37.2 ± 0.7. Majority mcg/day of the sample population 94% (n=102) had lower levels of daily vitamin K intake, as compared to the recommended AI (Riphagen *et al.,* 2017). Only 6% (n=6) of the sample population had a normal AI for vitamin K (Figure 22).

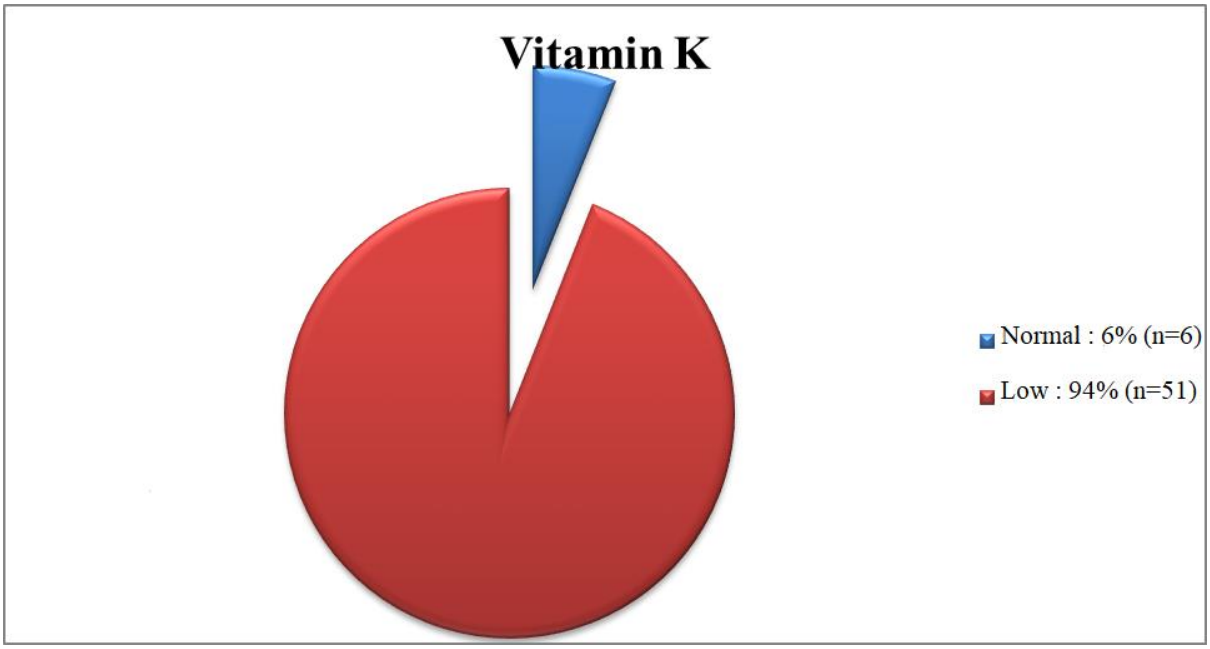

Figure 22: Vitamin K status of the sampled population

The data analysis tool used to design figure 22 was Microsoft excel program.

In this population, the majority (94%) had low daily intake of Vitamin K. Vitamin K deficiency has been proven according to other studies, to be common in the aging population as a risk factor of CVD (Bruno, 2016; Ballegooijen & Beulens 2017; Palermo *et al.*, 2017; Riphagen *et al.*, 2017).

4.8 PREVALENCE OF R353Q POLYMORPHISM

The genetic polymorphism R353Q was determined in 76 subjects out of 102 who gave consent for this study (as discussed in 4.2). The percentage of the sampled population for the detection of the genetic polymorphism R353Q was 74.5% (n=76). Of this percentage, 14.5% (n=11) of the subjects had the RQ genotype (heterozygous) which produced prominent peaks A and G at 4711 bp and 4730 bp respectively (Figure 23). The wild type allele Q (G) was detected in 76.3% (n=58) of the sampled population and the allele R (A) was detected in 1.3% (n=1) of the sampled population. There was no detection of any alleles and genotypes in 7.9% (n=6) of the sampled population (Figure 24).

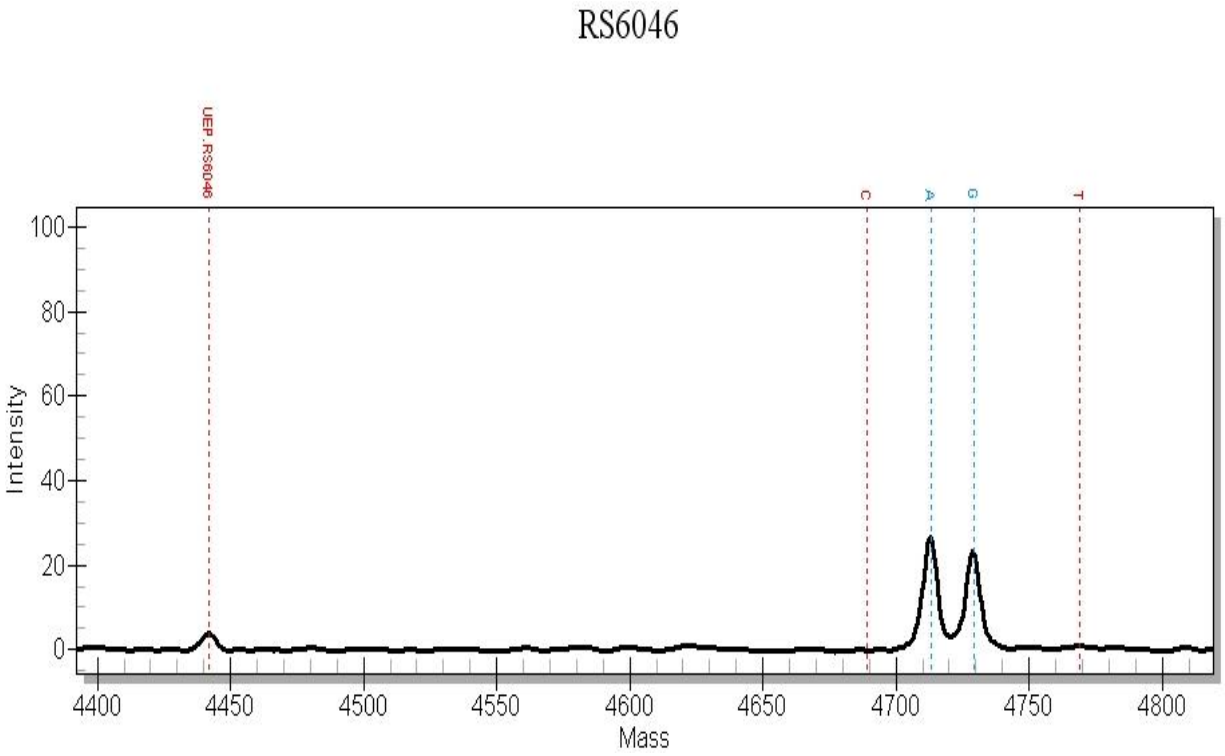

Figure 23: Chromatogram of the genotype AG at base pair 4711 and 4730, respectively.

A chromatogram for the sample RS6046 is shown in Figure 23 (designed using chromatogram software). The sequencing chromatograms following amplification displayed strong peaks and minimal baseline

noise. The chromatogram has two peaks A and G located at 4711 bp and 4730 bp, respectively. Peak A shows a higher signal intensity than G. The nucleotide G represents the dominant allele and A represents the co-dominance allele.

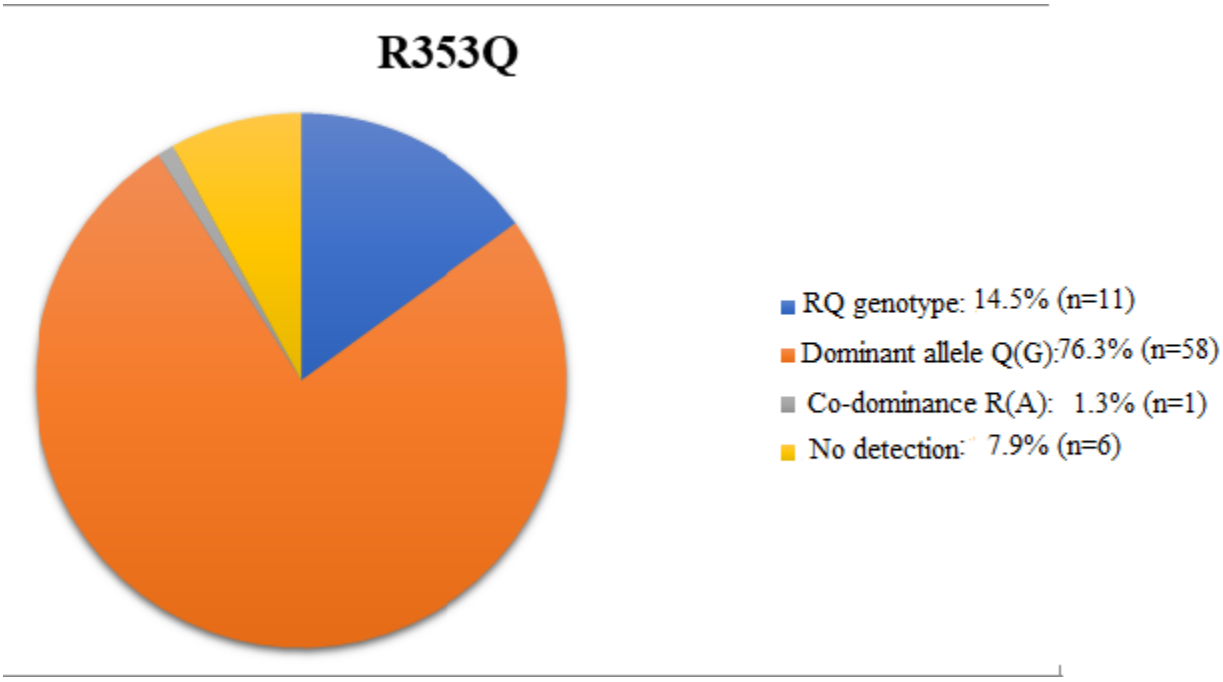

Figure 24 R353Q genetic polymorphism distribution of the sampled population

In this sampled population, only 1.3% was a carrier of A/R allele, 14.5% were carriers of the RQ genotype, 76.3% were carries of the G/Q allele and 7.9% had no detection of any alleles or genotype. The data tool analysis used to design figure 24 was Microsoft excel program.

4.9 CORRELATIONS

The correlation of data was done by using bivariate analysis (two or more variables in its relationship with one another, with respect to the overall quantity). The Pearson and Spearman rank correlation were used for correlating the different variables (Vitamin K, factor VII, fibrinogen, PAI-1 and R353Q polymorphism) with the p-value of 0.05 considered as significant.

4.9.1 Fibrinogen and factor Vll

Fibrinogen and FVII have been associated as independent risk factors to CVD when plasma levels are elevated (hypercoagulability) (Tofler *et al.,* 2017). The association between these two variables were measured and the Pearson (r) correlation was 0.1 (Table 7). There was no statistical significance established with these variables as the p-value was 0.113 (Figure 25). Microsoft excel program was used as data tool analysis.

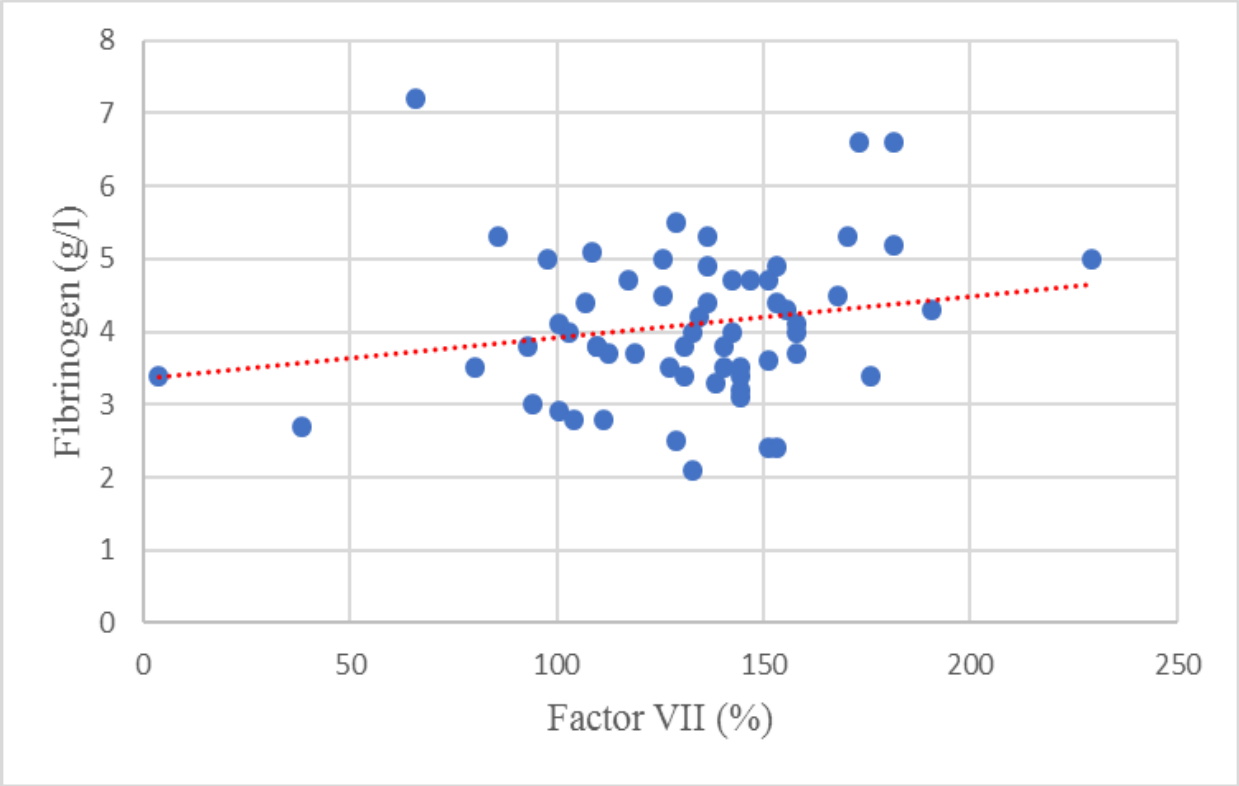

Figure 25: Fibrinogen and FVII correlations scatterplot

There is a weak strength correlation between these two variables, as the data are not uniformly spread. Neither does fibrinogen increases or decreases as FVII increases with the maximum outlier at 7.2 g/l and 6.6 g/l and minimum outlier at 2.1 g/l. In this sample population, 5% have extremely high levels of fibrinogen and thus, are at a higher risk of CVD. Therefore 2% of this sampled population having the concentration value of 2.1 g/l as compared to the reference value of 1.8 g/l to 3.5 g/l, have a less dense concentration of fibrinogen as compared to FVII.

4.9.2 Fibrinogen and PAI-1

Hypercoagulability and hypofibrinolysis have been strongly associated as a CVR (Pieters *et al.*, 2014). These two variables (fibrinogen and PAI-1) were computed and the Pearson correlation coefficient was used for bivariate analysis. The r value was 0.067, with the p-value of 0.611. No relationship was established between these variables (Figure 26). The figures (26 and 27) below were designed using Microsoft excel program.

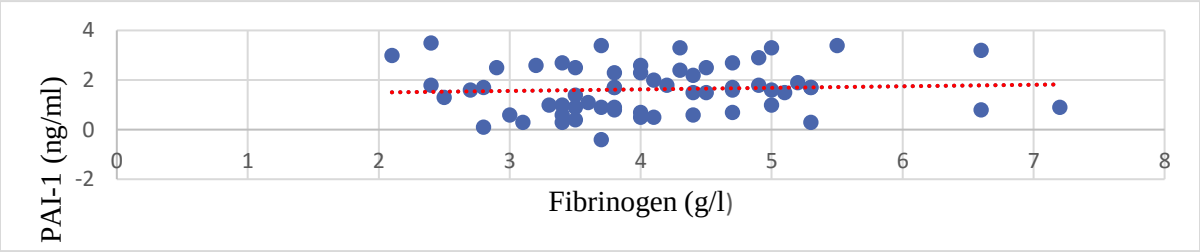

Figure 26: Fibrinogen and PAI-1 correlation scatterplot

There is weak strength relationship between these two variables, as there is no uniform spread between PAI-1 and fibrinogen data concentration. This indicate that there is no association between them as PAI-1 does not increase nor decrease as fibrinogen increases with the minimum outlier at -0.4 ng/ml having a less dense concentration of PAI-1 as against FVII.

4.9.3 PAI-1 and Factor VII

An association has been established between PAI-1 and FVII plasma levels as a risk factor for CVD (Aziz *et al.*, 2014). A positive weak correlation was established between PAI-1 and FVII variables. The r value was 0.267 (Table 7), with the p-value of 0.037 (Figure 27) as the p-value was less than 0.05.

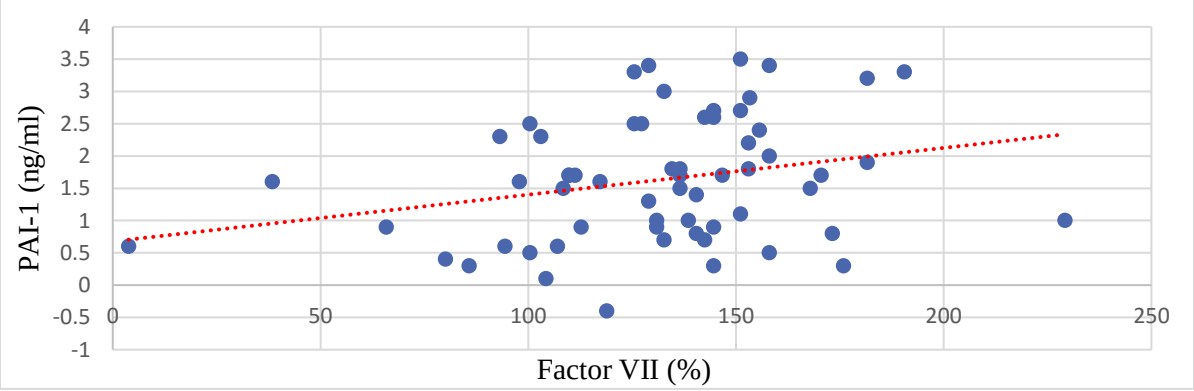

Figure 27: PAI-1 and factor VII correlation scatterplot

As PAI-1 increases, FVII also increases, indicating an association between these two variables. This correlation shows two outliers. The maximum outlier at 158% having a higher concentration of FVII, with 18% of the sampled population having higher levels of FVII, therefore are at a higher risk of CVD. Meanwhile 8% (minimum outlier) had extremely low levels of PAI-1.

Table 7: Summarizing the Pearson correlation between the homeostatic variables

		Factor VII	**Fibrinogen**	**PAI-1**
Factor VII	Pearson correlation coefficient	1	0.195	0.267*
	**		0.133	0.037
	N	61	61	61
Fibrinogen	Pearson correlation coefficient	0.195	1	0.067
	**	0.133		0.611
	N	61	61	61
PAI-1	Pearson correlation coefficient	0.267*	0.067	1
	**	0.037	0.611	
	N	61	61	61
****. Correlation is significant at the 0.05 level (2-tailed).**				

Table 7 shows the Pearson correlation between the homeostatic variables of FVII, fibrinogen and PAI-1. The correlation is significant at the 0.05 level (2-tailed). There was no association established between fibrinogen and FVII, as well as fibrinogen and PAI-1. There was a positive weak correlation between PAI-1 and FVII with the r value of 0.267.

4.9.4 Vitamin K and haemostatic markers

The correlation between Vitamin K and haemostatic markers (FVII, fibrinogen and PAI-1) was analyzed using the non-parametric Spearman's rank correlation, and the significance $p < 0.05$.

4.9.4.1 Vitamin K and Factor VII

Vitamin K is a very essential component for the synthesis of protein coagulating factors (Friedman 2016, Pacifici 2016; Shea & Booth 2016; Ballegooijen & Beulens 2017; Faria *et al.,* 2017; Fusaro *et al.,* 2017). FVII is a serine protease coagulation factor which is synthesized in the liver as a vitamin K dependent plasma glycoprotein (Suzuki *el al.,* 2012, Böhm *et al.,* 2015). In this sampled population, the correlation coefficient r was -0.027 (Table 8) giving a strength which is strong and negatively correlating these two variables (Figure 28) as compared to the P value of 0.005. Figure 28 was designed using Microsoft excel program as data tool of anaylsis.

Table 8: Summarizing the Spearman's rho correlation between Vitamin K and FVII

		Vitamin K	**Factor VII**
Vitamin K	Spearman's rho correlation coefficient	1.000	-.027
	Sig. (2-tailed)	.	0.867
	N	40	40
Factor VII	Spearman's rho correlation coefficient	-0.027	1.000
	Sig. (2-tailed)	0.867	.
	N	40	40

Table: 8 Shows the Spearman's rho correlation between vitamin K and FVII, with the correlating significance at 0.05 level (2-tailed). An association was established between vitamin K and FVII, having a strong negatively correlation strength with an r value of -0.027.

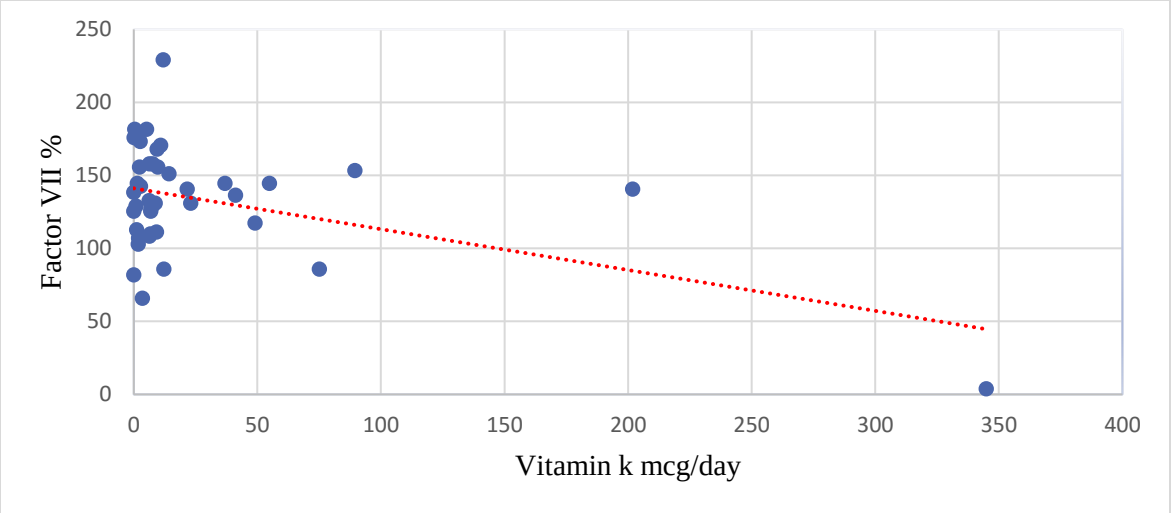

Figure 28: Vitamin K and Factor VII correlation scatterplot

As the vitamin K intake increases, FVII decreases, indicating an association between these two variables. The maximum outlier was at regions of 140.5 % and 229.2, respectively. The sampled population has a low daily vitamin K intake and are at a risk of bleeding and CVD. The minimum outlier was at region 3.8 %, having very high daily vitamin K intake of 344.91 mcg/day. This might be due to excess intake of vitamin K.

4.9.4.2 Vitamin K and Fibrinogen

The correlation coefficient obtained from these two variables (Vitamin K and fibrinogen) was positively weakly correlated with the r value of 0.126 (Table 9) which is closer to the p value 0.05. Three outliers were observed between these two variables (Figure 29).

Table 9: Summarizing the Spearman's rho correlation between Vitamin K and fibrinogen

		Vitamin K	**Fibrinogen**
Vitamin K	Spearman's rho correlation coefficient	1.000	.126
	Sig. (2-tailed)	.	0.431
	N	41	41
Fibrinogen	Spearman's rho correlation coefficient	0.126	1.000
	Sig. (2-tailed)	0.431	.
	N	41	41

Table 9 shows the Spearman's rho correlation between vitamin K and fibrinogen, with the correlating

significant at 0.05 level (2-tailed). The association established between these two variables was positively weakly correlated with the correlation coefficient of 0.126.

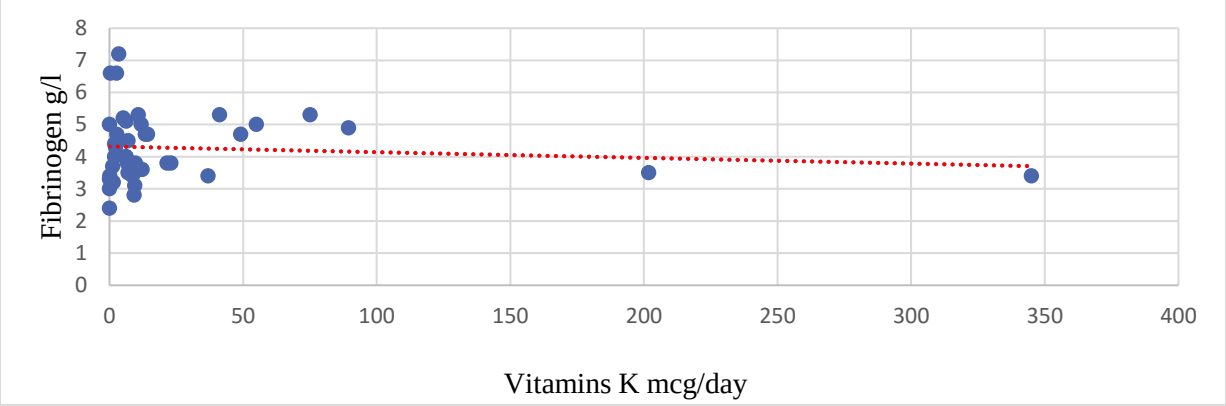

Figure 29 Vitamin K and Fibrinogen correlation scatterplot

There was no increase or decrease between vitamin K and fibrinogen with two minimum outliers observed at 3.4 g/l and 6.5 g/l regions, respectively. This is due to extremely low levels of vitamin K. The association established between these two variables are weakly correlated. Microsoft excel program was used to design figure 29.

4.9.4.3 Vitamin K and PAI-1

The correlation between vitamin K and PAI-1 in this sample population was weakly negatively correlated, with the correlation coefficient of -0.136 (Table 10). Two outliers were observed (Figure 30).

Table 10: Summarizing the Spearman's rho correlation between Vitamin K and PAI-1

		Vitamin K	**PAI-1**
Vitamin K	Spearman's rho correlation coefficient	1.000	-0.136
	Sig. (2-tailed)	.	0.391
	N	42	42
PAI-1	Spearman's rho correlation coefficient	-.136	1.000
	Sig. (2-tailed)	0.391	.
	N	42	42

Table 10 shows the Spearman's rho correlation between vitamin K and PAI-1, with the correlating significant at 0.05 level (2-tailed). A weak negative correlation was established between these two variables with the correlation coefficient of -0.136.

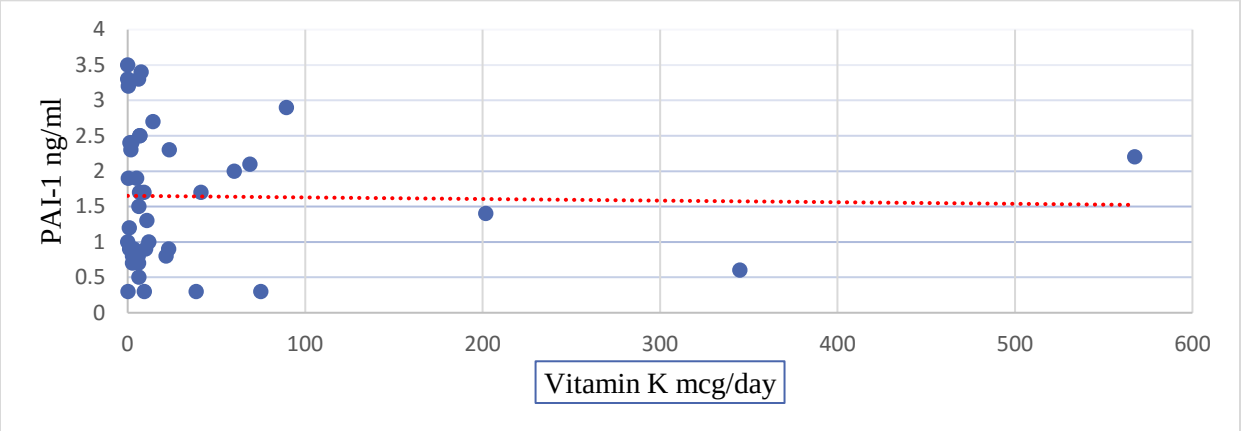

Figure 30: Vitamin K and PAI-1 correlation scatterplot

The association between these two variables is very weak as there is no increase or decrease of Vitamin K and PAI-1. Two outliers were observed (maximum) at 2.5 ng/ml region, having a high daily vitamin K intake (597.46 mcg/day). The minimum was at region 0.6 ng/ml of high vitamin k intake of 344.91 mcg/day. The association between these two variables were negatively correlated. Figure 30 was designed using Microsoft excel program.

4.9.5 Correlation between the R353Q polymorphism and haemostatic markers

The association between the R353Q polymorphism and haemostatic markers was analyzed using the non-parametric Spearman's rank correlation with the significance of $p < 0.05$.

4.9.5.1 R353Q polymorphism with FVII

The association between the genetic polymorphism R353Q and FVII was detected in 76 subjects out of 102 who gave consent as indicated in 3.4. The genotype AG (RQ) was detected in 6.6% (n=6) of the sample population with elevated levels of factor VII, 6.6% (n=5) with normal levels of FVII and 1.3% (n=1) of low FVII levels (Table 11). The dominant allele G (Q) was detected in 52.6% (n=40) of the sampled population with elevated levels of FVII, 23.7% (n=18) of normal levels and 1.3% (n=1) of low FVII levels. The co-dominant allele A (R) was detected in only 1.3% (n=1) of the sampled population with normal levels of FVII. There was no correlation between FVII and AG (RQ) genotype as the value obtained 0.095 (Table

11) was higher than the correlation significant level of 0.05. The correlation between FVII and the dominant allele G (Q) was statistically significant as the value obtained 0.010 was within the correlation significant level of 0.05. The correlation between FVII and the co-dominance allele A (R) was not statistically significant as the value obtained 0.242 was higher than the correlation significant level of 0.05.

4.9.5.2 R353Q polymorphism with fibrinogen

The correlation between the genetic polymorphism R353Q and fibrinogen was determine in 76 subjects out of 102 (as indicated in 3.5) who gave consent. The genotype AG (RQ) genetic polymorphism was detected in 7.9% (n=6) of the sample population with elevated fibrinogen levels and 6.6% (n=5) of the sample population with normal levels (Table 11). In this population, the dominant allele G (Q) was detected in 52.3% (n=42) with elevated levels of fibrinogen and 21% (n=16) with normal fibrinogen levels. The co-dominant allele A (R) was detected in 1.3% (n=1) of the population with elevated levels of fibrinogen. The correlation between the genotype AG(RQ) with fibrinogen was not statistically significant with the value of 0.241 higher than the p value 0.05. The correlation between the dominant allele G (Q) with fibrinogen was not statistically significant with the value of 0.369 which is higher than the correlating value of 0.05 (p-value). The association between the fibrinogen and the co-dominant allele A (R) was not statistically significant with the value obtained of 0.130 (Table 11)

4.9.5.3 R353Q polymorphism with PAI-1

The association between R353Q genetic polymorphism and PAI-1 was evaluated in 76 subjects out of 102 who gave consent (as referred to 3.5) AG (RQ) genetic polymorphism was detected in 10.5% (n=8) of the sample population with low levels of PAI-1. The dominant allele G (Q) was detected in 46% (n=35) of the population with low levels of PAI-1 and 9.2% (n=7) of the population with normal PAI-1 levels. The co-dominant allele was detected in 1% (n=1) of the sample population (Table 11). There was no statistical significant correlation between the AG (RQ) genotype with PAI-1. The value obtained 0.223 was higher than the p value of 0.05. The correlation between the dominant allele G (Q) with PAI-1 was not statistically significant as the value obtained 0.106 was higher than the p value of 0.05. The correlation between the co-dominant A (R) with PAI-1 was not statistically significant as the value obtained 0.709 was higher than the p-value 0.05.

Table 11: Association between the R353Q polymorphism and haemostatic markers

		R353Q Polymorphism			
Haemostatic Markers (n=76)		AG(RQ) Genotype (n =11)	G(Q) Dominant allele (n =58)	A(R) Co-dominant allele (n=1)	No allele detection (n=6)
FVII levels	High (>120 %)	5	40	0	1
	Normal (70-120 %)	5	18	1	5
	Low (<70 %)	1	0	0	0
	Statistical significance	0.095*	0.010*	0.242*	0
Fibrinogen levels	High (>3.5 g/l)	6	42	0	5
	Normal (1.8-3.5 g/l)	5	16	1	1
	Low (<1.8 g/l)	0	0	0	0
	Statistical significance	0.241*	0.369*	0.130*	0
PAI-1 levels	High (>72 ng/ml)	0	0	0	0
	Normal (3-72ng/ml)	0	7	1	0
	Low (< 3 ng/ml)	8	35	0	4
	Insufficient samples	3	16	0	2
	Statistical significance	0.223*	0.106*	0,709*	0

*> 0.05 Statistically not significant at 95% confidence interval.

The presence of the genetic polymorphism R353Q is associated with altering the levels of FVII, a homeostatic marker, which leads to the development of CVD (Lammertyn *et al.*,2014). The carriers of the Q allele (dominant) of the R353Q polymorphism have been reported to show low FVII levels and which also plays a protective role against MI (this finding has been reported to be contradictive by several studies) (Huang *et al.*, 2018). Meanwhile, the R allele (co-dominant) of the R353Q polymorphism increases the risk of developing CVD (El-Mitwalli *et al.*, 2014; Azzam *et al.*, 2017). In this study, it was confirmed that

52.6% (table 11) of the sampled population who are carriers of the Q allele had higher levels of FVII and 1% of R allele carrier had normal FVII levels.

According to previous studies conducted on the R353Q genetic polymorphism, it was reported that 78% of the sampled population were carriers of the R allele, 22% had the RQ genotype and 0% of Q allele was detected (El-Mitwalli *et al.*, 2014). Ben-Hadj-Khalifa et al (2013) reported that 4.4% of the sample population had the QQ genotype, 29.4% had the RG genotype and 66.2% had the RR genotype. Cheraghi *et al* (2013) reported that 74.1% of the sampled population were carriers of the Q allele and 25.9% were carriers of the R allele. Thus, individuals with Q allele have reduced levels of plasma coagulant activity of FVII, having a defensive mechanism for CVD. Meanwhile individuals carrying the R allele are at high risk of CVD. The R353Q reduces the levels of FVII, having a direct impact on the coagulation process which increases the risk of CVD (Mo *et al.*, 2011, Cheraghi *et al.*, 2013, El-Mitwalli *et al.*, 2014, Turfan *et al.*, 2014, Azzam *et al.*, 2017). This study confirmed that 1.3% of the sampled population having the R allele are at a higher risk of CVD. Individuals (14.5%) had the RQ genotype which reduces FVII levels as well as FVII secretion and disrupting the process of coagulation have been associated as the main cause of CVD. The majority of subjects in this sample population were carriers of the Q allele (76.3%) (figure 24).

CHAPTER 5

DISCUSSION AND CONCLUSION

Haemostatic markers have been found to be associated as a risk factor for CVD (Basu *et al.,* 2017; Tofler *et al.,* 2017). In this study of the elderly population of Sharpeville, 61% of the subjects had a higher level of FVII, 35% of the subjects had a normal of FVII and 4% had lower levels of FVII as compared to the reference value (70 to 120%). Thus, they are at a higher risk of developing CVD. The population in Turkey had a predominantly increased level of FVIIc and this population are at a double fold risk of developing CVD (Turfan *et al.,* 2014). According to the Framinghan Offspring study, there was a convincing evidence supporting the fact that an increased level of FVII results in the development of CVD (Tofler *et al.,* 2017). In America, an increased level of factor VII was established within the elderly population (predominately with the African American) to be associated as an independent risk factor for developing CVD (Tin *et al.,* 2015). This hypothesis was also supported by the first Northwick Park Heart Study. Which stipulated that elevated FVII concentration is associated as an independent risk factor CVD (Anderson *et al.,* 2014).

According to the studies conducted by Grobler (2015) on the same sample population, 68% of the elderlies had an increased level of plasma fibrinogen as compared to the reference value of 1.8 to 3.5 g/I. In this study, 41% of the sample population had increased levels of fibrinogen (3.5 to 4.5 g/l) above the normal value of 1.8 to 3.5 g/l and are at a higher risk of CVD while 29% of the sample population had increased levels above 4.6 g/l and are thus, at a greater risk of CVD. A similar study was conducted by Hamer et al. (2015) in Klerksdrop and Potchefstroom (North West Province South Africa) with the sample population composed of 86.5% urban Black and 89.1% White, of mean aged (47.5 ±7.8) and (47.5 ± 89.1%), respectively. Cardiovascular disease was diagnosed within the sample population with 9.0% Black and 11.4% white. There was a greater increased in fibrinogen levels in the Black race as compared to the White. A conclusion was established that this sample population is at a future predisposition to low grade hypercoagulability. This might contribute to a higher risk of CVD. Still in South Africa, according to studies conducted by Kotzé et al.(2014) within a Black Urban and Rural community in the North-west province of age varying from 35 to 65 years. From the data obtained, fibrinogen concentrations increased with age associated with several known CVD risks. In conclusion, a high concentration of fibrinogen is an independence risk factor for CVD in association with other CVD risk factors.

In London, it was concluded that the increased plasma levels of fibrinogen are strongly associated with CVD, with 30% of the sample population having a greater rate of mortality (Kengne *et al.,* 2012, Kim *et*

al., 2016, Kattula *et al.,* 2017). This was further supported by studies conducted in the North-West of London, with increased fibrinogen levels as an independent risk factor for CVD (Andenson *et al.,* 2014).

In Japan, 23% of the sample population of Tokyo had a higher level of fibrinogen with age varying from 30 to 80 years. Higher levels of plasma fibrinogen are strongly associated with hypercoagulability, which is an independent risk factor of CVD (Hattori *et al.,* 2015).

PAI-1 concentration levels have been associated as a cardiovascular risk factor, with either increased or decreased levels (Phelan & Kerins 2014). Increased levels of PAI-1 were found to be associated with CVD in the same sample population with 54% of the elderlies having an increased level of PAI-1 (Grobler ,2015). From the results obtained in this study in the same sample population, 88% of the elderlies ha a decreased level of PAI-1. Low levels of PAI-1 have been used as a prognostic for CVD (Patel *et al*., 2016). Thrombocytopenia has been associated with low PAI-1 levels as an independent CVR factor (Alvares-da-Silva *et al.,* 2014). Another study stated that, low levels of PAI-1 is a predisposed risk factor for CVD (Gram *et al.,* 2015). Global fibrinolytic activity is associated with CVD as a risk factor. Thus, it was suggested by other prospective studies the needs to investigate if low level of PAI-1 is associated with increased fibrinolysis (Lowe & Rumley 2014).

Vitamin K is a fat-soluble vitamin which is transported in circulation as lipoproteins and is very essential in the hepatic production of four coagulation factors (FII, FVII, FIX and FX), and thus establishing blood clotting process (Bruno, 2016; Pacifici, 2016; Ballegooijen & Beulens 2017). The carboxyl groups of the four coagulation factors are introduced into glutamic acid by vitamin K, yielding the gama-glutamic carboxyl (Gla) residues (Fusaro *et al.,* 2017; Riphagen *et al.,* 2017). FVII as a serine protease which is dependent to vitamin K synthesized in the liver (Suzuki *et al.,* 2012, Böhm *et al.,* 2015). The daily vitamin K intake in this sample population was found to be low in 94% of the sample population. This population is at a higher risk of bleeding due to vitamin K deficiency. This deficiency in vitamin K has been proven to be very common in the ageing population, due to vascular calcification, diabetes, hypertension and reduced endogenous production which are associated as risk factors of CVD (Vermeer, 2012; Bruno, 2016; Palermo *et al.,* 2017; Riphagen *et al.,* 2017). Thus, this population is at a higher risk of CVD. An association was established in this sample population between vitamin K and FVII, supporting the hypothesis of previous studies. A strong negative correlation between vitamin K and FVII was found with the correlation coefficient value of -0.027 as compared to the P value of 0.005. The association between vitamin K with fibrinogen and PAI-1 was very weakly correlated. Thus, the correlation was not established in this sampled population.

The genetic polymorphism R353Q is associated with 20% reduction of heterozygotes FVII and 40% reduction of homozygous FVII levels, therefore, hindering the secretion of FVII from blood vessels into circulation to cells (Kaushansky *et al.,* 2010; Mo *et al.,* 2011; Azzam *et al.,* 2017). The 353R genotype has been established to be associated with the development of CVD at a higher risk, as compared to the 353Q having a protective mechanism against the development of CVD (Criado-García *et al.,* 2011; Mo *et al.,* 2011; Ben-Hadj-Khalifa *et al.,* 2013; El-Mitwalli *et al.,* 2014). In this population, 6.6% was detected with the FVII genetic polymorphism R353Q with increased levels of FVII, 7.9% of increased levels of fibrinogen and 10.5% of low PAI-1 levels. The R(A) allele was detected in only 1% of the sampled population of normal haemostatic levels (FVII, fibrinogen and PAI-1). The dominant allele G(Q) was detected in 52.6% of high levels of FVII, 52.3% of elevated fibrinogen levels and 46% of low PAI-1 levels (Table 11. In this study, the correlation between FVII levels and Q allele was statistically significant at 0.010, which lies between the correlation significant level of 0.05. The insufficient samples of PAI-1 levels (27.6%) was inferred as insignificant. The test analysis of PAI-1 denotes that more that 72.3% of the sample population correlated against FVII, fibrinogen, RQ genotype, G allele and R allele were insignificant based on the correlation values as referred to table 11. This implies that the remaining insufficient samples will follow the same trend as the present one thus, being insignificant. The genetic polymorphism R353Q has been established as a CVR factor, which has been detected in the sampled population standing at a higher risk of developing CVD.

In conclusion**,** an alteration in coagulation factors (increased levels of FVII and fibrinogen) and fibrinolytic system (decreased level of PAI-1) have been established to be strongly associated with the development of CVD. With the presence of the R353Q genetic polymorphism in the reduction of FVII levels as a risk factor of CVD. Vitamin K deficiency has been proven to be associated as an independent risk factor for CVD. The 353Q genotype has coagulation activities, lower levels of FVII and having an inherited degree of protection against CVD as compared to the 353R genotype with a higher risk of developing CVD. This 353Q genotype has been established to have a higher significant frequency in individual suffering from acute myocardial infarction. This population is at a higher risk of developing CVD, with the AG (RQ) genotype detected and with the increased levels of FVII and fibrinogen as well as a decreased level in PAI-1 which has been established as the CVR factor. More so, other haemostatic markers such as fibrinogen and PAI-1 levels have been found in this study to be associated with R353Q polymorphism.

CHAPTER 6

SURMMARY AND RECOMMENDATIONS

6.1 INTRODUCTION

The elderly population of age 60 years and above are at a greater risk of developing CVD especially in low middle-income countries. The ageing population and age dependent disorders are at a higher risk of developing CVD, increasing the burden susceptibility of diseases (Prince *et al.,* 2015). These diseases are strongly influenced by genetic and environmental factors, attributing the development of diseases at a very rapid rate (Sayols-Baixeras *et al.,* 2014; Balakumar *et al.,* 2016). The progressive elevated levels of some coagulation factors such as factor VII, increased platelet activity, fibrinogen and impaired fibrinolysis are associated with ageing leading to CVD (Sepúlveda *et al.,* 2015) with the influence of R353Q polymorphism which modifies the level of blood plasma factor VII (Turfan *et al.,* 2014). One of the constant risk factors associated with CVD are infectious diseases and poor nutritional intake, in conjunction with low-socio-economic status. Other risks factors which have been established to be associated with CVD are chronic diseases of the life style (Sliwa *et al.,* 2016). The sample population had some increased coagulation factors, and therefore at a higher risk of developing CVD.

6.2 RESEARCHER'S CONTRIBUTION

This study was part of a multidisciplinary program being directed by the Center of Sustainable Livelihoods (CSL), in conjunction with the Department of Health Science at the Vaal University of Technology (VUT). This study aimed to evaluate the homeostatic markers as a CVR factor among an elderly community attending a day care centre. The researcher was part of the project and participated in the data collection. The researcher was responsible for the analyses of fibrinogen, factor VII, PAI-1 and R353Q polymorphism under standardized environment. The results obtained were statistically analyzed and reported by the researcher with the help of a statistician. The researcher consulted a nutritionist on the vitamin K intake and the results were reported. The researcher did various literature reviews on the different parameters and compiled the dissertation.

6.3 LIMITATION OF STUDY

This study was limited to a homogenous elderly, Black, South African community living in the Vaal region, Gauteng, South Africa. Secondly, the sample size was purposively selected (as the sample population has been identified by previous studies to be at a higher risk of CVD). The third limitation of this study was the gender distribution of the sample, which was uneven with 85% females and 15% males. The fourth

limitation was that sufficient blood in some collection tubes could not be collected from some the subjects. Lastly, this study only looked at genetic polymorphism R353Q as an influential factor for these coagulation factors (FVII, fibrinogen and PAI-1).

6.4 MAIN FINDINGS

6.4.1 Problem and settings

- Cardiovascular disease globally is the highest leading cause of death, with a death rate of 17.3 million of death per annum which has been estimated to highly increase to 23.6 million by the year 2030 (Laslett *et al.,* 2012; Balakumar *et al.,* 2016).
- The prevalence of CVD is higher in developed countries as compared to developing countries, which is also increasing significantly (Boateng *et al*., 2018) especially in urban areas of low and middle income countries in Africa (Njelekale *et al.,* 2009; Cappucio & Miller 2016).
- The predominance of infectious diseases, insufficient dietary intake, low socio-economic costs (resulting in poverty) and loss of productivity have been strongly supported as the major cause of CVD (Sliwa *et al.,* 2016, Cappucio & Miller 2016).
- Hypertension and stroke are the highest risk factors for CVD in Africa, which has been estimated to constitute of 60% of chronic NCDs (Maredza *et al.,* 2011, Aljefree & Ahmed 2015, Roth *et al.,* 2015, Maredza *et al.,* 2015, Balakumar *et al.,* 2016, Cappucio & Miller 2016, Sliwa *et al.,* 2016, Keates *et al.,* 2017).
- Individuals who are carriers of HIV/AIDS are at a higher risk of developing CVD (Fedele *et al.,* 2011; Mashinya 2015). In South Africa, 196 people die per day approximately of CVD with a minority of 20% suffering from HIV/AIDS (Maredza *et al.,* 2011, Maredza *et al.,* 2015).
- The elderly of age 60 years and above are at higher risk of developing CVD, with an increase in susceptibility of diseases burden in the ageing population and age dependent disorders (Adeloye & Basquill 2014). The prevalence of hypertension in the elderly population of South Africa has been estimated to range from 78%. This prevalence is expected to increase within the ageing and growing population in 2025 by 60% (LIoyd-Sherlock *et al.,* 2014; Bowry *et al.,* 2015).
- CVD is also influenced by genetic factors and environmental factors (Ben-Hadj-Khalifa *et al.,* 2013) with R353Q genetic polymorphism having a direct effect in the process of coagulation, reducing the levels of FVII. This genetic polymorphism R353Q has not been investigated in South Africa and limited studies were conducted in Africa.

6.4.2 Literature

- Cardiovascular disease (CVD) is an abnormal condition whereby the heart and arterial circulation supplying the heart, brain and cerebrovascular organs are affected thereby, obstructing blood from flowing to the lower periphery resulting in interrupting the arterial connecting the heart to peripheral circulation (Labarthe, 2011; Andrew, 2017).
- This condition (CVD) has been established to be strongly associated with other risk factors, categorized as modifiable and non-modifiable risk factors which describes an individual etiological characteristic of developing the disease at higher rate (Deaton *et al.,* 2011; Basharat *et al.,* 2012; Ahmari *et al.,* 2017).
- The heart and blood vessels are involved in the pathophysiology of CVD. This leads to the malfunctioning of various mechanisms and morphologic manifestations (Kumar *et al.,* 2010; Cohen & Hull 2015).
- Coagulation factors, endothelium, platelets and fibrinolysis are constituents of the homeostatic system. The interaction between them in response to vascular injury will depend on the closely linked blood vessels, blood coagulation proteins and platelets circulation (Hoffbrand & Moss 2011; Rifai *et al.,* 2018).
- With FVII as a vitamin K dependent serine protease glycoprotein. Which is responsible for the regulation of coagulation, combined with tissue factor for activation and initiation of primary homeostatic balance. Vitamin K is also responsible for yielding the gamma-glutamic carboxyl (Gla) residues by the introduction of carboxyl group into glutamic acid (Fusaro *et al.,* 2017; Riphagen *et al.,* 2017).
- As FVII is activated, other coagulation proteins such as FIX and FX are stimulated to induce excessive concentration of thrombin burst (Suzuki *et al.,*2012; Böhm *et al.,* 2015).
- Elevated levels of FVII have been identified as a risk factor of developing CVD. In conjunction with other risk factors such as smoking, gender and age, altering FVll levels which results to CVD (Campo *et al.,* 2013; Turfan *et al.,* 2014).
- The genetic polymorphism R353Q modifies the levels of FVII and has been identified as a risk marker for developing CVD by reducing the 20% of heterozygotes FVII and 40% of homozygous factor VII. This disrupts the plasma circulation of FVII secretion into the cells from blood vessels (Kaushansky *et al.,* 2010; Mo *et al.,* 2011; Cheraghi *et al.,* 2013; Turfan *et al.,* 2014).

- With the 353R genotype at a higher risk of developing CVD, meanwhile the 353Q genotype have a defensive mechanism in contrast of developing CVD (El-Mitwalli *et al.,* 2014).
- Another important coagulation protein which is involved in stabilizing the process of homeostasis and clot formation is fibrinogen (Levy *et al.*,2012; Palta *et al.,* 2014).
- Increased levels of fibrinogen have been identified with the presence of atherosclerosis as a risk factor of CVD (Tousoulis *el al.,* 2011, Kim *et al.,* 2016).
- Decreased levels plasminogen concentration have been confirmed as a risk factor of CVD (Papageorgiou *et al.,* 2010; Palta *et al.,* 2014).
- The mechanism of fibrinolysis is regulated by another important coagulation factor known as PAI-1, with the presence of t-PA and u-PA as plasminogen activators determining the process of fibrinolysis by enzymatic conversion of plasminogen to plasmin (Hassanin *et al.,* 2013).
- PAI-1 as a cardiovascular risk factor is associated with high and low levels concentrations (Phelan & Kerins 2014).
- Elevated levels of PAI-1 decreasing fibrinolysis by inhibiting t-PA and u-PA (uPA) (Schenkein & Loos 2013). Meanwhile Low levels of PAI-1 concentration initiates hyperfibrinolysis (Simone *et al.,* 2014).

6.4.3 Results

- The prevalence of this polymorphism R353Q was detected in 14.5% of the sample population as a CVR, 76.3% of the dominant allele G(Q) and 1.3% of R(A).
- The PAI-1 concentration was determined, with 88% of the sample population having low level of PAI-1.Thus, this sample population has an impairment fibrinolysis and therefore at a higher risk for CVD.
- FVII concentration levels of the sample population was found to be elevated with majority (61%) of the sample population having higher levels of FVII and therefore at a higher risk for CVD.
- The fibrinogen concentration of the sample population was determined with 41% having a plasma fibrinogen levels of 3.5 to 4.5 g/l and therefore double risk of CVD. According to the fibrinogen studies collaboration (2007), it was concluded that an increase of 1 g/l in plasma fibrinogen doubles the risk of CVD. A total of 29% of the same sample population had fibrinogen levels of >4.6 g/l and therefore four-time higher risk for CVD.
- The vitamin K intake of the sample population (94%) were low, as compared to the adequate intake of vitamin K.

- The correlation between dietary intake of vitamin K and homeostatic markers (PAI-1, FVII and fibrinogen status in this sample population was confirmed. A strong negative correlation was established between vitamin K and FVII.
- The correlation between the dominant allele G(Q) and FVII was statistically significant with the value of 0.010, which was within the was within the correlation significant level of 0.05
- The association between the genetic polymorphism R353Q and homeostatic markers was confirmed in this sample population. The R353Q genetic polymorphism was detected in 6.6% of subject's having high levels of FVII, 7.9% having high levels of fibrinogen and 10.5% of low PAI-1 levels. The allele G(Q) was detected mostly in 52.6% of subject's having increased levels of FVII, 52.3% of elevated fibrinogen levels and 46% of low PAI-1 levels. The R allele was only detected in 1% of the sample population having a normal level of FVII, fibrinogen and PAI-1. The insufficient samples of PAI-1 levels (25%) was inferred as insignificant.

6.5 SIGNIFICANCE

This study have provided information on the prevalence of the genetic polymorphism R353Q and its correlation with homeostatic markers in the Black South African elderly community of the Vaal Region.

6.6 CONCLUSION

This population is at a higher risk of developing CVD with the AG (RQ) genotype detected and with the increased levels of FVII and fibrinogen as well as a decreased level in PAI-1, has been confirmed as the CVR factor. More so, other homeostatic markers such as fibrinogen and PAI-1 levels have been found in this study to be associated with R353Q polymorphism. Thus, confirming the findings of previous studies which stated that, any changes in some coagulation factors such FVII, fibrinogen and PAI-1 (fibrinolytic system) have been strongly confirmed as a risk factor for development of CVD. Moreover, with R353Q genetic polymorphism which reduces the levels of FVIl activities which have been confirmed a risk factor for the development of CVD (Kaushansky *et al.,* 2010; Mo *et al.,* 2011; Lammertyn *et al.*,2014; Azzam *et al.,* 2017; Basu *et al.,* 2017; Tofler *et al.,* 2017). Vitamin K deficiency has been identified as an independent risk factor for CVD (Vermeer, 2012; Brunom 2016; Palermo *et al.,* 2017; Riphagen *et al.,* 2017) and this deficiency was confirmed in the majority of the subjects of the sample population.

6.7 RECOMMENDATION

6.7.1 Future studies

Other homeostatic marker or coagulation factors such as factor II, V, VIII, XI, X, and Von Willebrand factor should be investigated as CVR factors together with the genetic and non-genetic markers which are associated with the development of CVD. The genetic polymorphism A455G of the β-fibrinogen gene and 4G/5G of the PAI-1 gene, has been established to be associated with the development of CVD (Papageorgiou *et al.,* 2013; Martiskainen *et al.,* 2014; Sevimli *et al.,* 2014; Srivastava, 2016). Thus, it is suggested that these polymorphisms should be investigated as a CVR factor. Other studies on the R353Q polymorphism should be investigated in South Africa, since this study was the first to be conducted in an elderly population of South Africa. According to El-Mitwalli et al. (2014), Azzam et al. (2017), Huang et al. (2018), carriers of the dominant allele Q (G) of the genetic polymorphism R353Q are associated to low levels of FVII plasma levels, which have a protective mechanism against MI compared to the co-dominant allele R. This association has been proven to be uncertain by previous studies, including this study whereby 52.6% of the sample population who are carriers of the Q allele had higher levels of FVII and 1% of R allele carrier had normal FVII levels.

Fibrinolytic activity globally has been associated as a risk factor for CVD. It was recommended by other prospective studies to further investigate if there is an association between low level of PAI-1 and increased fibrinolysis (Lowe & Rumley 2014).

Vitamin K supplements should be implemented in this sample population suffering from Vitamin K deficiency. These vitamin K supplements have been proven as a preventive intervention for vitamin K insufficiency (Vermeer, 2012; Riphagen *et al.,* 2017).

6.7.2 Community

The early pattern trend of managing the disease needs to be communicated to this elderly community as this community living in a poor, low socio-economic status, have been identified by previous studies to be at higher risk of CVD (homeostatic imbalance) in association with CDL and environmental conditions, influencing the health fundamental changes.

This community should be educated with the information obtained from these studies about preventative health, nutrition, life style and family history in relation to genetic diseases (because they are inherited). This sampled population should consume more of leafy greens and other vegetables as these contain large compounds of vitamin K1 and K2, which are very essential nutritional components.

6.7.3 Policy makers

To reduce or minimize the risk of CVD, certain policies should be recommended and implemented to understand other associated risk factors, which could be managed more effectively before the manifestation of the disease pattern. Nutritional awareness (in relation to vitamins deficiencies), physical activities and screening tests leading to CVD should be implemented. More so, CVR factors (modifiable and non-modifiable) awareness campaigns should be implemented in the community.

6.7.4 Scientific capacity building

The genetic polymorphism R353Q of the FVII gene has been established in South Africa in the elderly community of Sharpeville as a CVR factor, with the majority having the dominant G (Q) allele having a protective mechanism against myocardial infarction. This G (Q) allele was also dominant in individuals with low levels of PAI-1 as compared to individuals of higher levels of FVII and fibrinogen, which have been established to be strongly associated as a risk factor for CVD. This is the first study to detect the genetic polymorphism R353Q of the FVII gene in a South African elderly population. The findings of this study will be published in order to supplement current information about the polymorphism associated with CVD.

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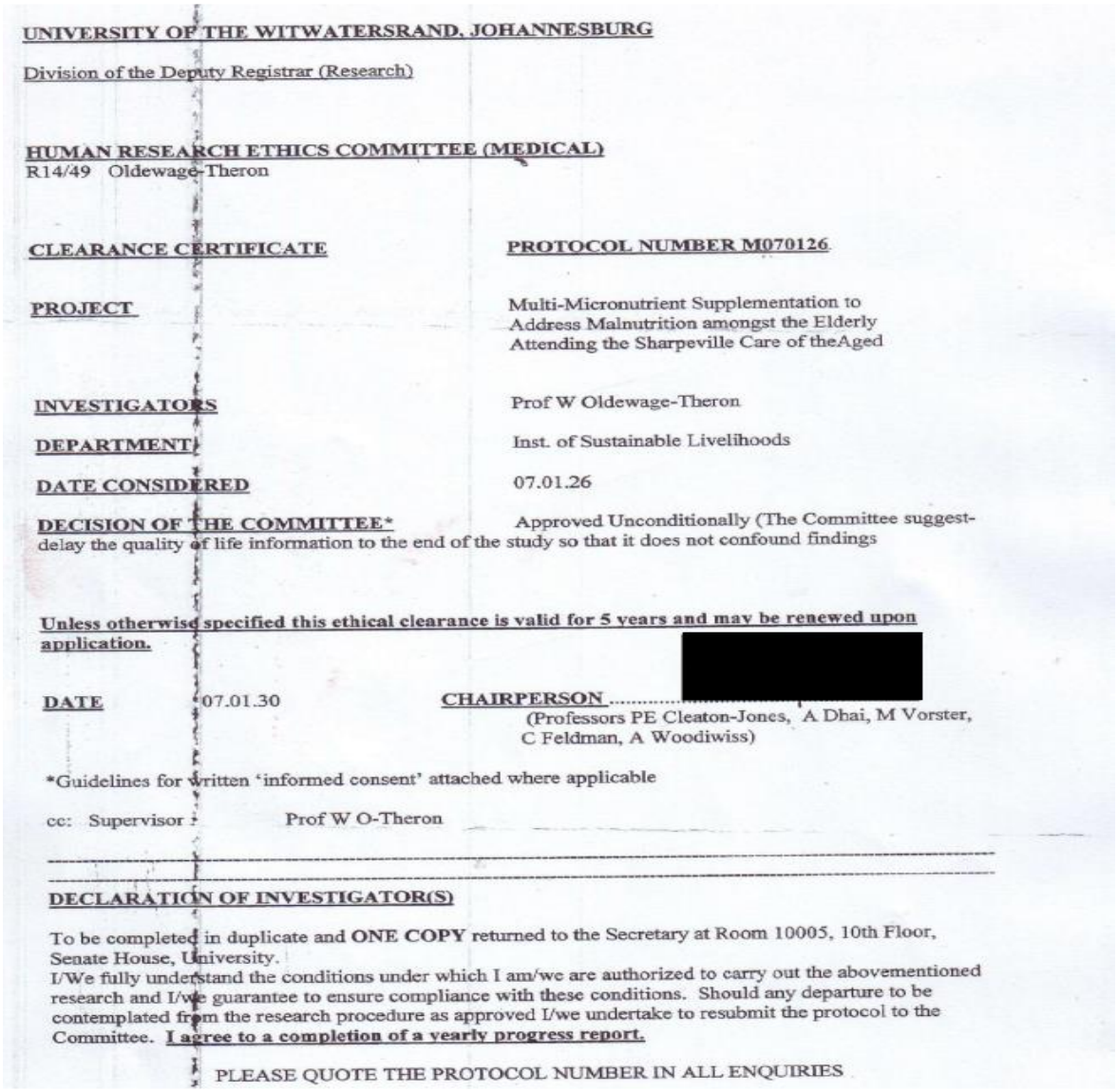
LIST OF ANNEXURES

ANNEXURE A



UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Oldewage-Theron

CLEARANCE CERTIFICATE **PROTOCOL NUMBER M070126**

PROJECT Multi-Micronutrient Supplementation to Address Malnutrition amongst the Elderly Attending the Sharpeville Care of theAged

INVESTIGATORS Prof W Oldewage-Theron

DEPARTMENT Inst. of Sustainable Livelihoods

DATE CONSIDERED 07.01.26

DECISION OF THE COMMITTEE* Approved Unconditionally (The Committee suggest-delay the quality of life information to the end of the study so that it does not confound findings

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 07.01.30 **CHAIRPERSON** .....
(Professors PE Cleaton-Jones, A Dhai, M Vorster, C Feldman, A Woodiwiss)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof W O-Theron

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

ANNEXURE B

VAAL UNIVERSITY OF TECHNOLOGY

RESEARCH & INNOVATION

ETHICAL CLEARANCE CERTIFICATE

Applicant:	Mrs. Christa Grobler
Project:	To establish the effect of vitamin B6 supplementation on the cardiovascular risk (CVR) and to determine the prevalence of genetic polymorphisms in correlation with CVR markers, in a black elderly community in Sharpeville.
Institution:	Vaal University of Technology
Date Approved:	21st August 2014
Ethical Clearance Number:	20140827-1ms
Approved: Yes/No	Approved

Approved by: BJ Du Soe Date: 21/8/14

Dr. B.J. Johnson
Chairperson: Research & Innovation Ethics Committee

Private Bag X021 – Vanderbijlpark – 1900
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ANNEXURE C

Informed Consent Form

Persons to Contact in the Event of Any Problems or Queries:

Mrs. C.J. Grobler	Prof. W.H. Oldewage-Theron
0169509210	0169305085

Statement of Agreement to Participate in the Research Study:

I,……subject's full name,

ID number……., have read this document in its entirety and understand its contents. Where I have had any questions or queries, these have been explained to me by ……to my satisfaction. Furthermore, I fully understand that I may withdraw from this study at any stage without any adverse consequences and my future health care will not be compromised. I, therefore, voluntarily agree to participate in this study.

Subject's name …….. **ReSubject's signature**……..

Date:……..…….....

Researcher's name ……..... Researcher's signature……..……

Date:……..…….....

Witness name ……..…….. Witness signature……..……..

Date:……..…….....

Principle investigator's name……Principle investigator's signature……

Date:……..…….....

ANNEXURE D

FIELDWORKER CONTROL SHEET

Subject ID number:.....Date: .....

Stations	Activity	Baseline	Follow-up
Station 1: **Check/control**	Handing out of file		
Station 2: **Blood**	• Blood pressure		
Station 3: **Dietary intake data**	• 24-hour recall • FFQ • Food insecurity		
Station 4: **Anthropometry**	• Height • Weight		
Station 5: **Health questionnaire**	Health questionnaire		
Station 1: **Check/control**	Control that all fieldwork is complete		

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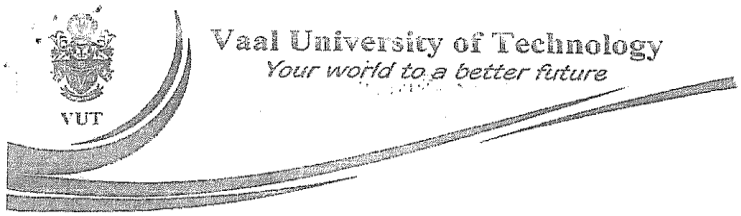
ANNEXURE E



SHARPEVILLE INTERGRATED NUTRITION PROJECT
HEALTH QUESTIONNAIRE

1.

Subject ID		Age	
Gender	Male. Female ☐		

HEALTH QUESTIONNAIRE:

2. **Are you or any of your family member suffering from the following?**

	YES	NO
1. Any skin disease?		
2. Any affection of the skeleton and/or joints?		
3. Any affection of the eyes, ears, nose or teeth?		
4. Any affection of the heart or circulatory system?		
5. Any affection of the chest or respiratory system?		
6. Any affection of the digestive system?		
7. Any affection of the urinary system and/or genital organs?		
8. Any nervous affection or mental abnormality?		
9. Any headaches		
10. Any other illness?		

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3. Have you experienced any of the following?

	YES	NO
Weight loss during the past month?		
A recent change in appetite?		
Tiredness		
Problems with the following:		
* chewing?		
* swallowing?		
* nausea?		
* diarrhoea?		
* vomiting?		
* constipation?		
Follow a special diet?		
If yes, specify…….		
Allergic to any foods?		
If yes, specify		

4.Do you smoke at this moment?

	Tick the correct block
5.1. Yes	
5.2. No (Never smoked)	
5.3. No (Stopped)	

5.Does your spouse or partner smoke at this moment?

	Tick the correct block
1. Yes	
2. No	
3. Not applicable	

6.Do you make use of snuff at this moment?

	YES	NO
1. Yes		
2. No (Never used)		
3. No (Stopped)		

7.Do you use alcohol on a regular basis?

	YES	NO
1. Yes		
2. No		
3. Not applicable		

8.

	YES	NO
Have you undergone any operations during the past five years?		
GIVE TYPE OF THE OPERATION		

9.Do you use chronic medication?

	YES	NO
3. If yes, what for/why?		

10. Do you take any supplements?

	YES	NO

11. If yes in previous question.

Specify the type	Vitamins, specify…… …… ……	Minerals, specify…… …… ……	Multivitamin	Other, specify…… …… …… …… ……

12.Which health facility is commonly used you?

	Tick the correct block
1. Private Doctor	
2. Clinic	
3. Hospital	
4. Traditional Healer	
5. Other (please state)	

13.How do you travel to the health facility?

	Tick the correct block
1. On foot	
2. Taxi	
3. Bus	
4. Own transport	
5. Other (please state)	

Thank you very much for your co-operation. We appreciate the time.

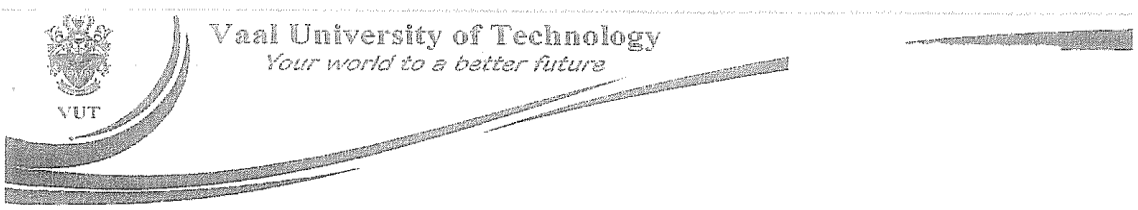
Mrs. C.J. Grobler

0169509210

Prof. W.H. Oldewage-Theron

0169509792

ANNEXURE F



24-HOUR RECALL

Subject ID number : _____ Gender: Male/Female: _____

Interviewer: _____ Date: _____ / _____ / 2014

Tick what the day was yesterday:

Monday	Tuesday	Wednesday	Thursday	Friday	Saturday

Would you describe the food that you ate yesterday as typical of your habitual food intake?

Yes	No

If not, why? _____

I bought some food	My visitor brought me some food	Other reasons (pls. specify)

I want to find out about everything you ate or drank yesterday, including food you bought. Please tell me everything you ate from the time you woke up to the time you went to sleep. I will also ask you where you ate the food and how much you ate.

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ANNEXURE G

FFQ LIST OF FOODS AND FOOD GROUP DIVERSITY : VUT NUTRITION SUPPORT STUDY

Subject number:.....

Date:.....Fieldworker:.....

Gender:.....Age:.....

PLEASE INDICATE ONLY THE FOOD YOU ATE DURING THE PAST SEVEN (7) DAYS BY A (X or √)

GROUP 1: Flesh foods (meat, poultry, fish) diversity	Yes
Chicken	
Beef, including steak, stew	
Pork	
Fish, fresh or frozen (hake, haddock, etc)	
Fish (tinned pilchards/tuna)	
Lekgotlwane (finely chopped, cooked meat)	
Mutton, including chops	
Chicken runners and heads, livers	
Goat (meat)	
Mogodu and malana	
Dried meat (biltong)	
Viennas / polony /.Russians	
Sausage (wors)	
Other:	
Other:	

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Group 2: Eggs diversity	
Eggs (boiled, fried, poached, scrambled, omelette)	
Group 3: Diary products diversity	
Evaporated milk (Ideal milk)	
Maas/ inkomasi	
Powdered milk	
Milk, full cream, 2% or low fat (pasteurized or unpasteurized)	
Cheese	
Custard / Ultramel	
Ice cream	
Yoghurt / Yogisip	
Other:	
Other:	
Group 4: Cereals, roots and tubers diversity	**Yes**
Rice	
Maize meal porridge / Pap	
Macaroni/pasta/spaghetti	
Maize rice (mielierys)	
Samp (stampmielies)	
Bread / Buns / bread rolls (white / brown / wholewheat)	
Dumpling / "Vetkoek"	
Scones	
Rusks	
Biscuits	
Mabela (soft porridge)	
Corn flakes / wheat bix / rice krispies / other breakfast cereals	
Oats	
Mageu	
Potatoes	
Sweet potatoes	
Umqombothi / Traditional beer	
Other:	

Group 5: Legumes and nuts	
Sugar or other dried beans	
Peas (dried)	
Baked beans, tinned	
Peanut butter / peanuts	
Other nuts (pistachio, brazil, walnut, pecan, etc)	
Soya	
Other:	
Other:	
Group 6: Vitamin A rich fruits and vegetables diversity	
Pumpkin	
Carrots	
Wild leafy vegetables (morogo), fresh and dried	
Spinach	
Butternut	
Apricots (Applelkoos)	
Peach (yellow cling)	
Mango	
Other:	
Other:	
Group 7: Other fruits (and juices) diversity	Yes
Deciduous fruits	
Apple	
Peaches	
Pear	
Grapes (black/green)	
Plum	

Sub – tropical fruit	Yes
Lemon	
Orange	
Naartjie	
Banana	
Pineapple	
Avocado	
Blueberry	
Cherry	
Kiwi fruit	
Raspberry	
Watermelon	
Wild watermelon(tsamma)	
Guava	
Other:	
Other:	
Juices	
Juice (100% pure juice e.g. Ceres/Liquifruit)	
Group 8: Other vegetables diversity	
Onions	
Cabbage	
Beetroot	
Rhubarb	
Turnips (raap)	
Gem-squash (lemoenpampoen)	
Tomatoes	
Green beans (fresh)	
Peas (fresh – green)	
Cauliflower	
Chili (red/green)	
Lettuce	
Mushroom	

Baby marrow	
Green pepper	
Sweet-corn (baby)	
Corn-on-the-cob(white)	
Garlic	
Other:	
Other:	
Group 9: oils and Fats diversity	
Butter	
Sunflower oil	
Margarine	
Lard	
Salad oil	
Other:	
Other:	

Thank you.