CORRELATING THE PREVALENCE OF C174G POLYMORPHISM WITH IL-6, TNF-α AND Hs-CRP IN AN ELDERLY BLACK SOUTH AFRICAN POPULATION

by

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March, 2019

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DEDICATION

To my mother Alga, and husband Johann ~ you are my strength

ABSTRACT

Cardiovascular diseases (CVDs) are the leading cause of death worldwide, and the prevalence thereof is on the rise in developing countries due to the demographic transition and urbanization. The inflammatory process, atherosclerosis, is at the root of the majority of CVDs and is caused by unresolved inflammation. Various cardiovascular risk factors such as hyperglycaemia, dyslipidaemia, hypertension, smoking and aging stimulate the development of atherosclerosis through triggering inflammation. Being in a state of chronic low-grade inflammation therefor places an individual at higher risk of developing CVD, with inflammation playing a cause and effect role. The aim of this study was to investigate the inflammatory status of an elderly black South African population by analysis of inflammatory markers HS-CRP, TNF-α and IL-6, as well as the genetic polymorphism C174G associated with increased serum levels of IL-6 in some populations. The research was conducted in the field of Biomedical Sciences as a quantitative, cross-sectional, analytical observational design. The study was ethically approved and involved collection of 84 blood samples from volunteers in a purposively selected population as part of a larger collaborative study. Serum was used to analyse HS-CRP, TNF-α and IL-6 and DNA was extracted from whole blood for analysis of the C174G polymorphism. The median serum HS-CRP of 6.44mg/L (IQR = 2.82 - 9.86mg/L) fell within the highest risk (>5mg/L) of CVD and 75% of participants were at high (3.01-5mg/L) or very high (>5mg/L) risk. The median TNF-α of 0.00pg/mL was within the normal range and only 2.6% of participants had high serum TNF-α levels. The median serum IL-6 level was 1.92pg/mL and was also within the normal range with only 2.6% of participants who had high serum IL-6 levels. For the C174G polymorphism analysis, 98.6% had the GG, 1.4% the GC genotype and no participants had the CC genotype. The median serum IL-6 level of the homozygous GG group was 6.51mg/L, higher than the 4.13mg/L serum IL-6 of the heterozygous GC group. The difference in IL-6 should be considered with caution as only one participant had the C allele. A highly significant (p=0.001) correlation was found between HS-CRP and IL-6, as well as between IL-6 and TNF-α (p = 0.048). The elderly black Sharpeville community is in an increased inflammatory state which puts them at risk of CVD. The prevalence of the C allele in the C174G polymorphism is low in this population. Further research could be conducted as intervention studies to decrease the inflammatory state of the population and influence health policy changes to improve prevention of CVD.

Keywords: Inflammation, HS-CRP. IL-6, TNF-α, C174G, cardiovascular disease and elderly

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LIST OF ABBREVIATIONS

µL	Microlitre
µM	Micromolar
1q23.2	Position 23.2 on long arm of chromosome 1
6p21	Position 21 on short arm of chromosome 6
7p15.3	Position 15.3 on short arm of chromosome 7
A	Adenine
ADP	Adenosine diphosphate
AGE	Advanced glycation end-product
AIDS	Acquired Immune Deficiency Syndrome
Akt	Serine/threonine kinase Protein kinase B
AP-1	Activator protein-1
Apo A-I	Apolipoprotein A-I
Apo B	Apolipoprotein B
APRIL	A proliferation inducing ligand
Arid5a	AT-rich interactive domain-containing protein 5A
ASP-112	Aspartic acid-112
ATP	Adenosine triphosphate
BAFF	B-cell activating factor
BMI	Body mass index
BP	Blood pressure
bp	Base pairs
C	Cytosine
C/EBPβ	CCAAT/enhancer-binding protein beta
C/EBPδ	CCAAT/enhancer-binding protein delta
C174G	Cytosine-174-Guanine
CAM	Cell adhesion molecule
cAMP	cyclic AMP
CCAAT	Cytidine-Cytidine-Adenosine-Adenosine-Thymidine
CD	Cluster of differentiation
CHS	Cardiovascular Health Study
CIOMS	Council for International Organizations of Medical Sciences
CRP	C-reactive protein
CSIR	Council for Scientific and Industrial Research

CSL	Centre of sustainable livelihoods
CT	Computed tomography
CV	Coefficient variations
CVD	Cardiovascular disease
CVR	Cardiovascular risk
DD	Death domain
ddNTP	Dideoxynucleotide
DHA	Docosahexaenoic acid
DNA	Deoxyribose nucleic acid
dNTPs	Deoxynucleotide triphosphates
dsDNA	Double stranded deoxyribose nucleic acid
e.g.	Exempli gratia
EC	Endothelial cells
ED	Endothelial dysfunction
EDA-A1	Ectodysplasin A-A1
EDA-A2	Ectodysplasin A-A2
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
EPA	Eicosapentaenoic acid
ERK	Extracellular signal-regulated kinase
et al.	Et alia
EURIKA	European Study on Cardiovascular Risk Prevention and Management in Usual Daily Practice
FADD	Fas-associated protein with death domain
FasL	Fibroblast-associated ligand
Fcγ	Fc portion of immunoglobulin gamma
FIX	Factor nine
FIXa	Factor nine activated
FRET	Fluorescence resonance energy transfer
FRS	Framingham risk score
FVa	Factor V activated
FVII	Factor seven
FVIIa	Factor seven activated

FX	Factor ten
FXa	Factor ten activated
FXIII	Factor thirteen
G	Guanine
GITRL	Glucocorticoid-induced
Glu-81	Glutamic acid-81
GP	Glycoprotein
HbA_{1c}	Haemoglobin A_{1c}
HDL	High-density lipoprotein
HIV	Human Immunodeficiency Virus
HK	High molecular weight kininogen
HPCSA	Health Professionals Council of South Africa
HS-CRP	High sensitivity C-reactive protein
IBL	Immuno-Biological Laboratories Co. Ltd
ICAM-1	Intracellular adhesion molecule-1
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IFN-γ	Interferon-γ
Ig	Immunoglobulins
IKK	IκK kinase
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-17	Interleukin-17
IL-18	Interleukin-18
IL-1RA	Interleukin-1 receptor antagonist
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
IL-3	Interleukin-3
IL-6	Interleukin-6
IL-6R	Inteleukin-6 receptors
INF-β2	interferon-β2
IQR	Interquartile range
IκBα	Nuclear factor-κBα inhibitor
JAK	Janus kinase
JAK/STAT3	Janus kinase/signal transducer and activator of transcription 3

JHS	Jackson Heart Study
JNK	c-Jun NH2-terminal kinase
kDa	Kilodalton
kg/m^2	Kilogram per square meter
LDL	Low-density lipoprotein
LOX	Lipoxygenases
LTB_4	Leukotriene B_4
LT-β	Lymphotoxin β
MALDI-TOF	Matrix-assisted laser-desorption time-of-flight
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MCP-1	Monocyte chemoattractant protein-1
mCRP	Monomeric C-reactive protein
MCSF	Macrophage colony-stimulating factor
MEK	MAPK/ERK kinase
MEKK1	MAP/ERK kinase kinase 1
mg/L	Milligram per litre
$MgCl_2$	Magnesium chloride
MI	Myocardial infarction
MIDUS	Midlife in the United States
MKK3	MAP kinase kinase 3
MKK7	MAP kinase kinase 7
$ml/min/1.73m^2$	Millilitre per minute per 1.73 square metre
MLP	Medical Laboratory Professionals
mM	Millimolar
mmHg	Millimetre of mercury
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NCD	Non-communicable Disease
NEMO	Nuclear factor-κB essential modifier
NFκB	Nuclear factor kappa B
ng/µl	Nanogram per millilitre
NIBSC	National Institute for Biological Standards and Control

NK	Natural killer
N	Nitrogen
nm	Nanometre
nM	Nanomolar
NO	Nitric oxide
O	Oxygen
OPG	Osteoclast differentiating factor
OX40L	OX40 ligand
p	Probability
p38MAPK	P38 mitogen-activated protein kinase
PAF	Platelet-activating factor
PAI-1	Plasminogen activator inhibitor-1
PCR	Polymerase chain reaction
pCRP	Pentameric C-reactive protein
pg/mL	Picogram per millilitre
PGE_2	Prostaglandin E_2
PGI_2	Prostaglandin I_2
Phe-66	Phenylalanine-66
PI3K	Phosphatidylinositol-3-kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
POWIRS	Profiles of Obese Women with Insulin Resistance Syndrome
PPK	Plasma prekallikrein
PURE	Prospective Urban and Rural Epidemiology
qPCR	Real-time polymerase chain reaction
RANKL	Receptor activator of NF-κB ligand
Ras/MAPK	Ras/mitogen-activated protein kinases
RIP	Receptor interacting protein
RNA	Ribose nucleic acid
ROS	Reactive oxygen species
RRS	Reynolds risk score
SABPA	Sympathetic Activity and Ambulatory Blood Pressure in Africans
SAfrEIC	Sex, Age and Ethnicity on Insulin sensitivity and Cardiovascular function
SAMRC	South African Medical Research Council

SANAS	South African National Accreditation System
SAP	Shrimp Alkaline Phosphatase
SCORE	Systemic coronary risk evaluation
sIL-6R	IL-6 in complex with soluble IL-6 receptor
SMC	Smooth muscle cell
SNP	Single nucleotide polymorphism
SPM	Special pro-resolving mediators
ssDNA	Single stranded deoxyribose nucleic acid
SST	Serum separator tube
STAT3	Signal transducer and activator of transcription-3
Stats SA	Statistics South Africa
T	Thymine
T2DM	Type 2 diabetes mellitus
TAK1	TGF-β-activated kinase 1
TF	Tissue factor
Th1	T-helper 1
TH17	T helper 17
Th2	T-helper 2
TNF- β	Tumour necrosis factor- β
TNFR1	Tumour necrosis factor receptor 1
TNFR2	Tumour necrosis factor receptor 2
TNFSF	Tumour necrosis factor superfamily
TNF-α	Tumour necrosis factor alpha
TRADD	TNFR-associated death domain
TRAF2	TNFR-associated factor 2
TRAIL	Tumour necrosis factor related apoptosis-inducing ligand
TRAILR1	Tumour necrosis factor related apoptosis-inducing receptor 1
TRAILR2	Tumour necrosis factor related apoptosis-inducing receptor 2
TRAILR3	Tumour necrosis factor related apoptosis-inducing receptor 3
TRAILR4	Tumour necrosis factor related apoptosis-inducing receptor 4
Treg	Regulatory T
TWEAK	Tumour necrosis factor related weak inducer of apoptosis
TWEAKR	Tumour necrosis factor related weak inducer of apoptosis receptor
TXA2	Thromboxane A2

Tyr-175	Tyrosine-175
U/µL	Units per microlitre
UN	United Nations
VCAM-1	Vascular adhesion molecule-1
VEGI	Vascular endothelial cell-growth inhibitor
VLDL	Very low-density lipoprotein
VSMC	Vascular smooth muscle cell
VUT	Vaal University of Technology
VWF	Von Willebrand Factor
WHO	World Health Organization

LIST OF SYMBOLS

-	To or minus
%	Percentage
R	Rand
≥	Greater than or equal to
α	Greek small letter alpha
γ	Greek small letter gamma
κ	Greek small letter kappa
/	per
β	Greek capital letter beta
δ	Greek small letter delta
Å	The ångström or angstrom is a unit of length equal to one ten-billionth of a metre or 0.1 nanometre
>	Greater than
<	Less than
+	Plus or positive
^{o}C	Degrees Celsius
=	Equal

LIST OF ANNEXURES

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CHAPTER 1
PROBLEM AND SETTINGS

1.1 Introduction

Cardiovascular disease (CVD) may occur as a result of the normal physiology of aging and specific diseases or risk factors that cause damage to the cardiovascular system (Heron 2016; Benjamin et al. 2017; Tesauro et al. 2017). Normal aging may bring about a state of chronic low-grade inflammation, which may cause atherosclerosis, and ultimately result in cardiovascular disease (Franceschi et al. 2000a; Douglas & Channon 2014; Frank & Caceres 2015). Inflammatory biomarkers are measured to evaluate the inflammatory status of an individual. These inflammatory biomarkers are involved in or produced as a result of inflammation (Calder et al. 2013).

This study examined the inflammatory biomarkers, tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and high sensitivity C-reactive protein (HS-CRP), which have been shown to have an association with an increased risk for CVD (Biasillo et al. 2010; Hartupee & Mann 2013; Ofstad et al. 2013; Yu et al. 2016). The inflammatory response is influenced by ethnic differences, genetic variation, body fat and its distribution, socio-economic status and lifestyle factors. Therefore it is important to assess the contributing factors to establish the cardiovascular risk (CVR) profiles in different populations (Evans & Goedecke 2011; Yusuf et al. 2014; Fiatal & Adany 2017).

The biologically important single nucleotide polymorphism in the promoter region of the IL-6 gene, located on the short arm of chromosome 7 (7p15.3), rs1800795 C174G (Curti et al. 2011; Ataie-Kachoie et al. 2014), was also be investigated. The substitution from guanine (G) to cytosine (C) at the position 174 of the IL-6 gene, has a negative regulatory effect on gene expression (Fishman et al. 1998; Popko et al. 2008). However, previous studies have found varying results of IL-6 serum levels associated with the G174C polymorphism in different populations (Popko et al. 2010a; Giannitrapani et al. 2013; Spoto et al. 2015; Soysal et al. 2016). Limited data is available on this phenomenon in South Africa.

1.2 Geriatric Profile

The World Health Organization (WHO) stated that determinants for the definition for old age globally include: a person's chronological age, the age at which one receives pension benefits and one's loss of ability to perform physical activities (World Health Organization 2015). Based on these determinants, various definitions have been developed for the elderly. The United Nations (UN) generally refer to people of 60 years and older as elderly which is adapted in this study (United Nations 2011, 2015a).

1.2.1 Global geriatric profile

The elderly population has grown substantially from 378 million people in 1980 to 759 million in 2010. It was reported that there were 901 million elderly people in 2015. Of the total world's elderly population more than half reside in Asia, totalling about 508 million (56.4%). Europe followed Asia, accounting for 176.5 million (19.6%) elderlies; thereafter North America with 74.6 million (8.3%) elderlies, Latin America and the Caribbean followed with 70.9 million (7.9%) elderly people. Africa accounted for 64.4 million (7.2%) and Oceania for 6.5 million (0.7%) of the global elderly population. The global elderly population consisted of 54% women and 46% men in 2015 (United Nations 2015a). The elderly population is expected to increase from 901 million in 2015 to 2.1 billion by 2050, of which 80% will live in low-income or middle-income countries. The elderly population is growing at a higher rate in developing countries, compared to developed countries (United Nations 2015a). This can be attributed to reduced fertility, increased longevity and a transition of the leading causes of death from communicable to non-communicable diseases (Prince et al. 2015; United Nations 2015a).

1.2.2 South African geriatric profile

In 1996, 7.1% of the South African population constituted of elderly persons and that increased to 8.0% in 2015. Statistics South Africa (Stats SA) also reported that the proportion of black South Africans elderly is 6.1%, which is far less than the 20.1% elderly in the white population. There was also a 5.6% increase in the prevalence of elderly headed households from 14,6% in 1996 to 20.2% in 2011 (Statistics South Africa 2014, 2015). Multi-generational households are common in South Africa, often with an elderly caring for orphaned grandchildren. In some households three generations rely on the pension income of an elderly due to lack of resources (Oldewage-Theron et al. 2008; van der Pas et al. 2015).

1.2.3 Geriatric profile in Sharpeville

The elderly in Sharpeville attending the Centre of the Aged have been studied since 2004. This study population lives in poverty and experiences malnutrition. Though the majority of the elderly live in brick houses with access to safe water, electricity, sanitary services and waste removal services, the majority live with families averaging households of 4.9 members. They depend on a state pension, and have a household income of R501-R1000 per month of which less than R200 per week is spent for the household's food expenses (Grobler 2015). It was found that 29.8% of the sample population were overweight and 47.1% were obese. This sample population was reported to have increased CVR and 75% had low grade inflammation as indicated by increased levels of HS-CRP (Oldewage-Theron et al. 2015).

1.2.4 Health related problems in the elderly

Changes in physical appearance such as grey hair and wrinkles are visible manifestations of aging. However, the most significant changes are unseen and are within the human body. Aging can be defined as a time-dependant decline in function which affects most organisms; this progressive deterioration is the primary risk factor for human pathologies. Cellular and molecular alterations involved in aging include: impaired intracellular communication, stem cell exhaustion, mitochondrial dysfunction, loss of proteostasis, epigenetic alterations, telomere deterioration, genomic instability and deregulated nutrient sensing (Lopez-Otin et al. 2013).

These alterations result in various pathologies, associated with normal aging. It is common for elderly to experience nutrition-related health problems. Anaemia can be caused by vitamin B12, folate or iron deficiency. Deficiency of vitamin C, vitamin E, flavonoids, unsaturated fatty acids, vitamin B12 and folate can result in impaired cognitive function. Immune senescence or malnutrition may contribute to impaired immune dysfunction, placing the elderly at risk of infection. Osteoporosis may be caused by genetic or environmental factors including a lack of physical activity, calcium and vitamin D deficiency, medication or disease. Over-nutrition can lead to obesity, hypertension and chronic disease of lifestyle, which in turn can result in CVD (Navaratnarajah & Jackson 2013; Hsu et al. 2014; Grobler 2015; Prince et al. 2015).

Vascular endothelial dysfunction is a result of the aging process, causing vasospasms, thrombosis, macrophage infiltration and inflammation, and ultimately atherosclerosis. The presence of endothelial dysfunction in elderly is also associated with coronary artery disease,

erectile dysfunction, renal dysfunction and retinopathy (Berk et al. 2013; Prince et al. 2015; Badimon et al. 2016; Tesauro et al. 2017)

Hormonal homeostasis is affected resulting in the loss of the circadian rhythm (Jones & Boelaert 2015). Poor sleep or sleep deprivation have been linked to elevations of inflammatory markers (Motivala 2011). Visual impairment and cataracts, hearing impairment, urinary complaints and dementia are also health problems commonly seen in elderly people (Thakur et al. 2013; Prince et al. 2015).

1.3 Prevalence of Cardiovascular Disease

Non-communicable diseases (NCD) contribute to 70% of deaths worldwide and CVD is the leading cause of death in the global elderly population (World Health Organization 2016b). Epidemiological transition, demographic transition, urbanization and globalisation contributes to an expected rise in the incidence of CVD in South Africa (Maredza et al. 2011; Chatterji et al. 2015).

1.3.1 Global prevalence of cardiovascular disease

Cardiovascular disease is a growing global epidemic which resulted in 17 689 000 (31.3%) of deaths in 2015 worldwide. Ischaemic heart disease represents the leading cause of death globally and accounted for 15.5% of all deaths. Stroke accounted for 11.1% deaths, hypertensive heart disease led to 1.7% of deaths and 0.5% of deaths were a result of rheumatic heart disease (World Health Organization 2016b). In the Global health estimates study of 2015, WHO also reported that the WHO European Region and WHO Western Pacific region had the highest incidence of deaths attributed to CVD, 45.6% and 39.4%, respectively. The WHO Eastern Mediterranean Region attributed 32.2% of deaths to CVD. The WHO Region of Americas followed with a total of 28.1% of deaths as a result of CVD. In the South-East Asia Region CVD contributed to 27.7% of deaths. CVD accounted for 12.7% of deaths in African.

The risk for CVD is lower in developing countries and is the highest in developed countries. Developed countries have a greater burden of risk factors, such as smoking, and consequently a higher prevalence of CVD. The same is true for rural and urban communities within the same country, where the latter have a higher risk for CVD. Though developed countries and urban populations have a higher CVR burden, treatment and preventative medication is more accessible, reducing the incidence of fatalities. Developing countries and rural areas often lack

health care due to low budgets in which case CVD often results in major cardiovascular events and fatalities (Celermajer et al. 2012; Yusuf et al. 2014). The increase in the incidence and prevalence of CVD in the developing world is attributed to demographic change, industrialization and urbanization (Gersh et al. 2010; Maredza et al. 2011; Owolabi et al. 2014).

1.3.2 Africa

The leading cause of death in the African region in 2015 was found to be infectious and parasitic diseases which caused 31.2% of deaths. A total of 33.5% of deaths were attributed to NCD of which CVD was responsible for 12.7% of deaths. The rising epidemic of CVD cannot be ignored with urbanization and lifestyle changes associated with economic growth as drivers of the disease. While ischaemic heart disease represents the leading cause of death in high income countries, stroke is the leading CVD causing death in the African region, totalling 4.9% of deaths (World Health Organization 2016b). This reiterates the importance of epidemiological transition, demographic transition and unique genomic patterns in risk determination and disease (Owolabi et al. 2014). Stroke was followed by ischaemic heart disease which caused 4.8% of deaths in Africa and other circulatory diseases caused 1.3% of deaths. Hypertensive heart disease resulted in 1.1% of deaths, cardiomyopathy, endocarditis and myocarditis in 0.5% of deaths, and 0.3% of deaths were attributed to rheumatic heart disease (World Health Organization 2016b).

1.3.3 South Africa

South Africa is in a phase of demographic transition, urbanisation and globalisation, resulting in a higher burden of CVR factors in the population (Owolabi et al. 2014). Variations in genetic and ethnic factors cause some populations to be more susceptible to NCD than others (Evans & Goedecke 2011). Infectious and parasitic infections were the leading cause of death in the South African population in 2015 causing 34% of deaths, of which 26% is attributed to Human Immunodeficiency Virus (HIV) or Acquired Immune Deficiency Syndrome (AIDS). CVD was responsible for 18% of deaths in South Africa and represents the leading cause of death in the elderly population of South Africa which amounted to 34% of deaths. Further breakdown of CVDs showed that ischaemic heart disease caused 14.2% and stroke 12.3% of deaths. It was followed with 3.8% of deaths attributed to hypertensive heart disease, 1.9% to other circulatory diseases, 1.5% to cardiomyopathy, endocarditis and myocarditis, and 0.4% of deaths resulted from rheumatic heart disease (World Health Organization 2016b).

1.3.4 Sharpeville

The elderly population of Sharpeville have an increased risk for CVD. Previous studies found that the majority of females and almost 40% of males were overweight or obese (Oldewage-Theron et al. 2015). In another study the population showed that hypertension, obesity and increased total serum cholesterol as CVR factors (Oldewage-Theron et al. 2008). Grobler (2015) confirmed the prevalence of hyperhomocysteinaemia, hyperfibrinogenaemia, hypertension, obesity, hyperinflammatory state, dyslipidaemia and metabolic syndrome in this population all of which are CVD risk factors.

1.4 Background to the Study

This study was carried out in conjunction with the Centre of Sustainable Livelihoods (CSL) at the Vaal University of Technology (VUT). It forms part of the multi-disciplinary Sharpeville Integrated Nutrition Programme which is managed by Prof. W.H. Oldewage-Theron. This programme is offered to volunteers attending a day care centre in Sharpeville, Gauteng, South Africa, and was established in 2004. The day care centre provides the elderly of Sharpeville with food, skills training and offer recreational activities. Cardiovascular risk of the elderly in Sharpeville is assessed as part of this programme, under the leadership of Dr. C.J. Grobler This study was approved by the ethics committee of the University of the Witwatersrand, Johannesburg (M070126) (annexure B), and VUT (20140827-1ms) (annexure C).

Sharpeville is situated approximately 70 kilometres south of Johannesburg in the Vaal triangle or Vaal region, a highly industrialised and polluted area (Oldewage-Theron et al. 2008). It was determined that 46.1% of the Sharpeville population of 37599 live in poverty, with 4.2% of the population being 60 years and older (Statistics South Africa 2011). This elderly community has been reported to have a high risk for cardiovascular disease (Oldewage-Theron et al. 2008; Grobler 2015).

1.5 Significance of the Study

The study provided insight into the role of inflammatory biomarkers, specifically HS-CRP, TNF-α and IL-6 and regulating genetic marker (C174G) in CVR in the elderly population of Sharpeville. The study thus contributes to a better understanding, prediction, and early detection of CVD in elderly black South Africans.

As a pro-inflammatory cytokine IL-6 contributes to inflammation, which is a cardiovascular risk. A meta-analysis was conducted, based on whether the polymorphism C174G causes increased levels of IL-6. It was reported that the polymorphism does not have the same outcome in different populations, and suggested that this polymorphism in relation to IL-6 levels be further investigated in various populations of different descent (Kumar et al. 2015). A literature review performed to determine the role of the C174G polymorphism on chronic liver disease and hepatocellular carcinoma found that this polymorphism resulted in increased levels of circulating IL-6 (Giannitrapani et al. 2013). A review reported that studies performed in Swedish, Canadian and American populations found an association with the prevalence of the C allele and obesity. Visceral fat tissue secretes about two to three times more IL-6 than subcutaneous tissue, linking it to increased levels of circulation IL-6. Other studies found that the G allele was linked to increased IL-6 levels (Curti et al. 2011). Another study conducted on females aged 15-35 found a correlation between the C allele and increased circulating IL-6 levels (Soysal et al. 2016). It was reported that the CC allele had significantly lower circulating levels of IL-6 than the GC genotypes in normal patients. The same study also found that there was no significant difference in the circulating levels of IL-6 in obese people based on their genotype, though the obese group had increased levels on IL-6 in the same population (Popko et al. 2010a). It is therefore suggested that the C174G polymorphism that has been linked to increased CVR, requires further studies to determine its effect in various populations (Curti et al. 2011).

Current data suggests that the C174G polymorphism has varying effects on the circulating levels of IL-6 in different populations. Studying the prevalence of the C174G polymorphism in different populations will bring insight to its role in CVD (Meenagh et al. 2002; Phulukdaree et al. 2013; Joffe et al. 2014). This created the need for this study, as there was limited information thereof in South Africa up to date, and even more so in elderly black South Africans.

1.6 Aims and Objectives

1.6.1 Aim

The aim of this study was to evaluate the prevalence of the C174G polymorphism, in correlation with the inflammatory biomarkers, HS-CRP, TNF-α and IL-6 as CVR markers.

1.6.2 Objectives

The objectives of this study were:

1) To measure the serum levels of HS-CRP.
2) To evaluate the serum levels of TNF-α.
3) To evaluate the serum concentration of IL-6.
4) To determine the prevalence of the C174G polymorphism.
5) To correlate the C174G polymorphism prevalence and serum IL-6 levels.
6) To correlate the serum HS-CRP with IL-6 and TNF-α levels.

1.7 Relevance of the Study

The health status of the elderly change over time and is influenced by several factors. There is a drastic increase in prevalence of CVD disease in developing countries resulting from an increase in the CVR factor burden created by urbanization and demographic change in these elderly populations (Celermajer et al. 2012; Owolabi et al. 2014). Other than the normal physiology of aging, these CVR factors, such as smoking, unhealthy diet and inactivity, cause inflammation (Franceschi et al. 2000a; Yu et al. 2016). Inflammation causes endothelial damage which contributes to the progression of atherosclerosis and ultimately results in CVD (Bell et al. 2012; Taleb 2016).

The health profiles of elderly living in high-income countries have been studied significantly more than that of low-income and middle-income countries. Very little data is available about the health profiles of elderly in developing countries leaving an urgent need for research in this population and its influence by urbanization and demographic change (Chatterji et al. 2015). Unfortunately, the treatment and prevention of NCD is less studied than the devastating burden of communicable diseases in South Africa (Maredza et al. 2011). Each population has unique genetic and ethnic factors, resulting in varying susceptibility to disease (Gersh et al. 2010). Consequently, population based CVR profiling is invaluable to indicate disease trends and enable the most effective allocation of limited resources for disease prevention and treatment (Mensah 2013; van der Ende et al. 2017)

1.8 Outline of Dissertation

Chapter 1 outlines the background of the study and gives a description of the problem. The significance of the study is introduced, together with the objectives and limitations thereof.

To provide better understanding of the study and all its components, a comprehensive literature review is covered in **Chapter 2**.

Chapter 3 is a theoretical analysis of the body of principles and procedures applied in this study to collect data.

Results obtained in this study are set out in **Chapter 4**, followed by a discussion thereof.

A conclusion is drawn in **Chapter 5** of the outcome of the study and the set objectives. Recommendations are made based on the findings to further improve knowledge and understanding of the subject.

A **Reference** list according to the VUT modified Harvard method stipulates all sources utilized during this study.

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

Cardiovascular diseases (CVDs) refers to a group of diseases affecting the heart and vascular system and are the leading cause of deaths worldwide (World Health Organization 2011, 2016b). Multiple biological pathways have been implicated in the pathogenesis of CVDs. Atherosclerosis is the underlying cause of the majority of CVDs. It results in plaque formation in the coronary arteries and decreased blood supply to the heart. The plaque can also rupture causing myocardial infarction or stroke (Sakakura et al. 2013; Newby 2016).

Inflammation is a fundamental factor in the development of atherosclerosis, more particularly the failure of inflammatory resolution (Ross 1999; Viola & Soehnlein 2015). Elderly people are in a chronic state of inflammation as a result of normal aging, placing them at risk of developing atherosclerosis (Badimon et al. 2016). Other CVR factors may also contribute by damaging the endothelial wall, triggering inflammation, resulting in atherosclerosis and ultimately causing CVD (Grundy 2012; Liao & Solomon 2013). This chapter will discuss these concepts in more detail.

2.2 Cardiovascular Disease

Various causes of CVD have been reported and investigated indicating that the underlying cause of the majority of CVDs is atherosclerosis. The danger of plaque formation during atherosclerosis does not only lie in the stenosis and narrowing of arteries but also in the thrombogenic potential thereof (Otsuka et al. 2016). Atherosclerosis can lead to ischaemic heart disease or coronary artery disease, cerebrovascular disease or stroke, diseases of the aorta and diseases of the arteries or hypertension (Mudau et al. 2012). . The elderly population is at a greater risk of developing CVD caused by atherosclerosis. Physiological changes of the vascular endothelium due to aging creates a proatherogenic environment, which can be exaggerated by the addition of other risk factors such as smoking, hyperlipidaemia and hyperglycaemia (Tesauro et al. 2017).

Other causes of CVD include congenital heart disease, rheumatic heart disease and cardiomyopathies. Congenital heart disease is a disease caused by a malformation of the heart which is present at birth. Rheumatic fever from Streptococcal infection can damage heart

muscle and valves, resulting in rheumatic heart disease. Other CVDs such as cardiomyopathies, heart valve disease and cardiac arrhythmias do not often cause heart attacks or stroke (World Health Organization 2011; Benjamin et al. 2017).

2.3 Atherosclerosis

Atherosclerosis is a chronic inflammatory disease of large and medium arteries. It is generally understood as plaque formation in response to stimuli with subsequent rupture, erosion or calcification of the plaque surface. Cardiovascular risk factors are atherogenic stimuli, triggering the inflammatory response through endothelial injury and ultimately leading to plaque formation and atherosclerosis as seen in Figure 1 (Ross 1999; Park & Park 2015). Irrespective of other CVR factors such as smoking, hyperlipidaemia and diabetes, elderly persons are at risk of developing atherosclerosis due to the physiological effects of aging. Advanced age not only causes structural changes of the vascular system contributing to endothelial dysfunction , but also results in chronic low-grade inflammation (Jackson & Wenger 2011; Badimon et al. 2016).

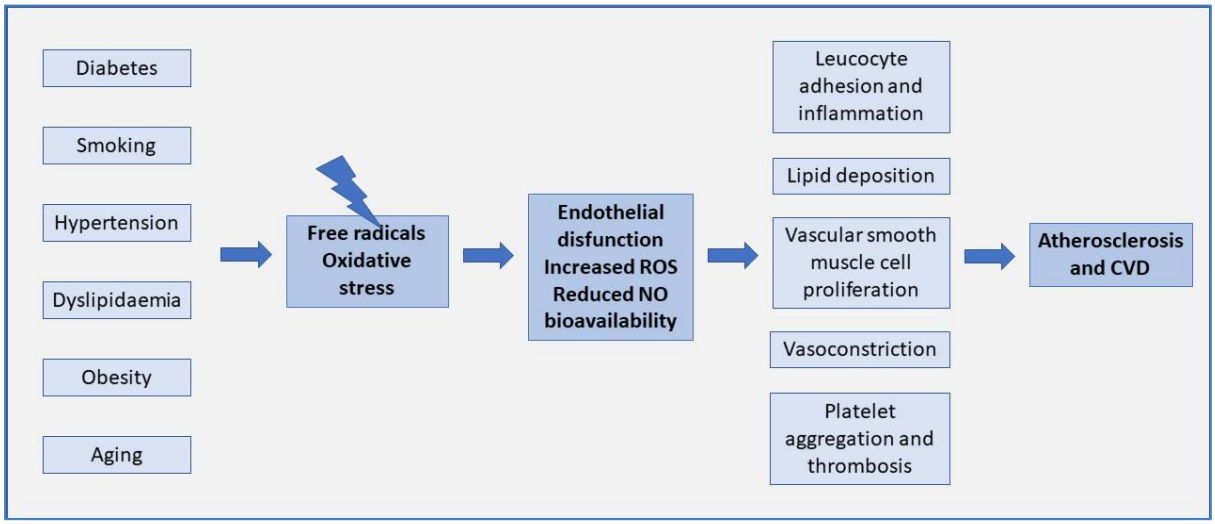


Figure 1: Linking CVR factors to atherosclerosis**.** Adapted from Park and Park (2015)

Atherosclerosis is characterized by endothelial dysfunction, vascular inflammation and accumulation of modified lipids, inflammatory cells and cellular debris in the plaques within the vascular wall (Viola & Soehnlein 2015). Plaque formation usually occurs at vascular curvatures and bifurcations, where there are non-laminar flow and shear stress. At these sites of non-laminar flow and shear stress, cells have reduced protective nitric oxide (NO) bio-availability and increased superoxide production (Forstermann et al. 2017). In normal vasculature, endothelial cells (ECs) produce endothelial NO synthase (eNOS), which act on L-arginine to

produce NO (Hirase & Node 2012). Aging results in a decline in the endothelium-derived NO, resulting in enhanced reactivity to vasoconstrictors (Badimon et al. 2016).

Endothelial dysfunction occurs as a result of damage to the endothelial layer. Injury may occur due to non-laminar flow shear stress and/or chemical or metabolic stress from CVR factors such as diabetes mellitus, hypertension, high serum cholesterol and the effects of cigarette smoking, among others. Dysfunctional ECs acquire a pro-inflammatory phenotype, which is the major pathophysiological link between exposure to CVR factors and the development of atherosclerosis. Nuclear factor kappa B (NFκB) in dysfunctional ECs activates gene transcription for pro-inflammatory cytokines (Tesauro et al. 2017). The increased production of reactive oxygen species (ROS) during endothelial dysfunction, especially superoxide, reacts with NO. Normal ECs produce NO for signalling vasodilation, prohibition of vascular smooth muscle cells (VSMCs) proliferation, inhibition of platelet activation and aggregation, and inhibition of inflammatory cell adhesion and migration. Reduced bio-availability of NO in endothelial dysfunction thus results in vasoconstriction, inflammation, coagulation and increased production of superoxide (Hirase & Node 2012; Douglas & Channon 2014).

In the elderly, the non-enzymatic effects of advanced glycation end-products results in more permanent cross-linkages between collagen fibres. Collagen thus becomes more resistant to breakdown and consequently there is a decreased rate of cell turnover. The increased collagen in the arterial walls make them more rigid. Together with reduced production of NO, it causes the elderly to have impaired endothelial dependant vasodilation (Jackson & Wenger 2011; Tesauro et al. 2017). Reduced production of NO in elderly is due to a decrease in the activity of eNOS in aged ECs resultant from decreased regulatory proteins, and an increased expression of arginase II which degrades L-arginine (Tesauro et al. 2017).

Smoking, hypertension, diabetes, obesity and hypercholesterolaemia cause free-radicals, and consequently oxidative stress as indicated in Figure 1 (Wang et al. 2012). During oxidative stress, elevated ROS, particularly superoxide, scavenges available NO, forming peroxynitrite which is cytotoxic and subsequently damages deoxyribonucleic acid (DNA), proteins and lipids (Tesauro et al. 2017). Peroxynitrite and other oxidants, such as hydrogen peroxide have been found to uncouple eNOS, responsible for the synthesis of NO, contributing to reduced availability of NO. Increased oxidant production results in increased VSMC proliferation which thickens the vascular wall, endothelial apoptosis and increased expression of matrix-degrading proteinases, matrix metalloproteinases. Oxidative stress also aggravates endothelial dysfunction by magnifying vascular inflammation, and conversely inflammatory cells release superoxide (Mudau et al. 2012; Heusch et al. 2014).

Proteoglycans are among the extracellular matrix molecules produced by the VSMCs. The exposure of proteoglycans by ECs enable the retention of low-density lipoproteins (LDL), cholesterols and phospholipids in the vascular intima. These lipids are oxidized by ROS and enzymes released from inflammatory cells such as myeloperoxidase, lipoxygenase and nitric oxide synthases (Wang et al. 2012; Heusch et al. 2014). After initiating of the fatty streak formation tissues produce cytokines in response to oxidized LDL which attract circulating monocytes to the site of endothelial dysfunction. Oxidized LDL causes epithelial damage and necrosis due to its cytotoxic nature and can also induces monocyte adhesion to epithelial cells (Viola & Soehnlein 2015).

The production of cell adhesion molecule (CAM) is amplified as a result of tissue damaged caused by oxidized LDL, increasing the adhesion of leukocytes and platelets to the endothelial surface. Monocyte chemoattractant protein-1 (MCP-1) is responsible for firm initial endothelial attachment via activation of leukocyte integrin, and together with vascular adhesion molecule-1 (VCAM-1) monocytes migrate into tissues. Monocytes then differentiate into macrophages as a result of the action by macrophage colony-stimulating factor (MCSF) (Wang et al. 2012). These macrophages produce pro-inflammatory cytokines and express scavenger receptors and take up the oxidized LDL via endocytosis for degradation by lysosomes. Oxidized LDL builds up in the macrophages as a result of impaired degradation due to its oxidized nature. As a result, macrophages become foam cells that further exacerbate the inflammatory response. Interferon-γ (IFN-γ) promotes the formation of foam cells and development of plaque (Wang et al. 2012; Krychtiuk et al. 2013).

Although monocytes and macrophages are the major cellular role players in atherosclerosis, lymphocytes also contribute. Cytokines and adhesion molecules expressed during vascular inflammatory reactions recruit T cells to atherosclerotic lesions. T-helper 1 (Th1) cells in turn secrete inflammatory pro-atherogenic cytokines, IFN-γ, TNF-α, interleukin-1 (IL-1) and interleukin-18 (IL-18) (Douglas & Channon 2014; Taleb 2016). The cytokines, TNF-α and IL-1, in turn stimulate the production of adhesion molecules; VCAM-1 and intracellular adhesion molecule-1 (ICAM-1), MCP-1 and IL-6. The action of IL-18 stimulates VSMCs to proliferate. IL-6 promotes hepatic production of C-reactive protein (CRP). Macrophage apoptosis is induced by TNF-α and IFN-γ secreted by activated T cells, forming cellular debris aggregates of the necrotic core (Mudau et al. 2012; Wang et al. 2012). In atherosclerosis, the clearance of apoptotic cells is defective, a process called efferocytosis, thus aggravating the necrotic core formation and inflammation (Viola & Soehnlein 2015). Atherosclerotic lesions have small quantities of T helper 2 (Th2) cells which secrete interleukin-4 (IL-4) and interleukin-10 (IL-10)

and antagonize Th1, and scanty regulatory T (Treg) cells which secrete anti-inflammatory IL-10 and transforming growth factor-β (Hovland et al. 2015; Taleb 2016).

Some atherosclerotic plaques accumulate in VSMCs in response to cytokines and growth factors. The VSMCs migrate from the media layer, proliferate and synthesize the extracellular matrix proteins, collagen and elastin. A strong fibrous cap is formed, isolating the thrombogenic necrotic core from circulation and providing stability (Douglas & Channon 2014). The migration of VSMCs to the arterial intima with formation of fibrous lesions result in arterial stenosis and impaired blood flow. The arterial loss of elasticity together with the intimal thickening which results, ultimately results in hypertension (Heusch et al. 2014).

When a plaque ruptures the exposed collagen and tissue factor (TF) activates platelets and the coagulation cascade, as with vessel injury. The resultant thrombus formation in the vascular system is termed atherothrombosis (Krychtiuk et al. 2013; Otsuka et al. 2016). Platelets bind to the subendothelial matrix proteins, glycoprotein (GP) Ia/IIa at sites with low shear stress. At vascular sites with high shear stress, the vessel wall is coated with von Willebrand factor (VWF) multimers. Collagen-bound VWF encourages platelets to adhere to the surface via the GPIb-XI-V complex, and initiates platelet rolling. The decreased speed at which platelets flow allows stronger adherence of VWF and activated GPIIb/IIIa as well as GPVI and integrin α1/β2 with collagen. Activated platelets spread out on the vascular wall and release granules. Adenosine diphosphate (ADP) and thromboxane A2 (TXA2) released from platelets further enhance platelet activation and formation of a platelet plug (Badimon & Vilahur 2014; Mastenbroek et al. 2015; Olie et al. 2018). The activated platelets also express P-selectin, recruiting monocytes and neutrophils into the expanding thrombus (Krychtiuk et al. 2013).

The exposed TF from foam cells and VSMCs initiate the proteolytic coagulation cascade. During the coagulation cascade, the transmembrane receptor TF forms a complex with factor VII activated (FVIIa). The TF-FVIIa complex triggers the conversion of factor IX (FIX) to factor IX activated (FIXa) and factor X (FX) conversion to factor X activated (FXa). Prothrombin is converted to thrombin by the prothrombinase, which is a complex of FXa and co-factor FV activated (FVa). The resultant thrombin cleaves fibrinogen to fibrin and activates the transglutaminase factor XIII (FXIII), enhancing fibrin cross-linking and stabilising the haemostatic clot (Badimon & Vilahur 2014; Mastenbroek et al. 2015; Ten Cate et al. 2017; Olie et al. 2018). The inflammatory process is usually self-limiting, resolving by means of anti-inflammatory mediators. During resolution of inflammation, inflammatory cell recruitment is terminated. Inflammatory cells are removed from the site by phagocytes and macrophages

are reprogrammed for anti-inflammatory processes through efferocytosis (Lim et al. 2017). Lastly, tissue regenerative processes are triggered. This is not the case in atherosclerosis since inflammation does not resolve due to hampered efferocytosis. Failed resolution of the inflammatory process translates to apoptotic cells not being cleared, inflammatory cell recruitment not being terminated, macrophages not being reprogrammed for anti-inflammatory processes and no tissue regeneration taking place (Viola & Soehnlein 2015). As a result, the plaque continues growing, expanding the diameter of the vessel so that the lumen is not affected or narrowing the vascular lumen (Wang et al. 2012).

The development of plaque during atherosclerosis may result in the narrowing of the arteries, ultimately occluding the arteries and causing myocardial infarction or cerebrovascular disease (Douglas & Channon 2014). Atherosclerotic plaque is classified using the Modified American Heart Association classification of atherosclerosis based on morphological description as indicated in Table 1 (Bhanvadia et al. 2013; Badimon & Vilahur 2014). Coronary thrombosis arises from 3 different morphological entities: rupture, erosion and calcified nodules. Plaque ruptures develop in a lesion with a necrotic core accounting for >30% of the plaque area which is covered by a thin fibrous cap and contains numerous macrophages and T-lymphocytes. Physical contact between the necrotic core and circulating blood results in the formation of a platelet rich luminal thrombus. Plaque rupture most commonly cause sudden coronary death from thrombi. Erosions are characterized by a luminal thrombus overlying a peptidoglycan-rich matrix with mostly VSMCs and very few inflammatory cells. Calcified nodules are characterized by calcified plates which penetrate the lumen and disrupt overlying collagen and endothelium (Sakakura et al. 2013; Otsuka et al. 2016).

Table 1: Modified American Heart Association classification of atherosclerosis based on morphological description. Adapted from (Bhanvadia et al. 2013; Badimon & Vilahur 2014)

	Description	Thrombosis
Non-atherosclerotic lesions		
Intimal thickening	Normal accumulation of Smooth muscle cells (SMCs) in the intima in the absence of lipid or macrophage foam cells Layers of smooth muscle cells and extracellular matrix	Absent
Intimal xanthoma, or "fatty streak"	Luminal accumulation of foam cells without a necrotic core or fibrous cap.	Absent
Progressive atherosclerotic lesions		
Pathological intimal thickening	Layers of SMCs in a proteoglycan-collagen matrix with areas of extracellular lipid accumulation without necrosis Variable accumulation of macrophages outside the lipid pool and presence of micro-calcification	Absent
Erosion	Luminal thrombosis; plaque same as above	Thrombus mostly mural and infrequently occlusive
Fibrous cap atheroma	Well-formed acellular necrotic core containing free cholesterol and covered by a thick fibrous cap consisting of smooth muscle cells in a proteoglycan-collagen matrix (type I and type III)	Absent
Erosion	Luminal thrombosis; plaque same as above; no communication of thrombus with necrotic core	Thrombus mostly mural and infrequently occlusive
Thin fibrous cap atheroma	A thin fibrous cap infiltrated by macrophages and lymphocytes with rare SMCs and an underlying necrotic core	Absent; may contain intraplaque haemorrhage/fibrin
Plaque rupture	Fibroatheroma with cap disruption; luminal thrombus communicates with the underlying necrotic core	Thrombus usually occlusive

Calcified nodule	Eruptive nodular calcification with underlying fibrocalcific plaque	Thrombus usually nonocclusive
Fibrocalcific plaque	Collagen-rich plaque with significant stenosis usually contains large areas of calcification with few inflammatory cells; a necrotic core may be present.	Absent

2.4 Cardiovascular Risk

Since CVD is the leading non-communicable disease, it is of paramount importance to determine the risk profiles of various populations in order to design qualifying intervention plans to decrease mortalities caused by this disease (World Health Organization 2016b). Currently, different risk prediction formulae are used to determine a patient's risk in the clinical environment, such as the Framingham Risk Score (FRS), Reynolds Risk Score (RRS) and the Systematic Coronary Risk Evaluation (SCORE). Each of these utilize different risk factors as indicated in table 2 (Liao & Solomon 2013).

The FRS algorithm is most commonly used in the United States and has been modified for used in population in other countries. The use of FRS should be validated and recalibrated in the target population. Variables included in risk determination using the FRS are age, gender, diabetes status, smoking status, total cholesterol, high density lipoprotein (HDL) cholesterol and blood pressure measurements. These determinants are easily accessible in clinical settings (Batsis & Lopez-Jimenez 2010; Cook & Ridker 2014). These risk prediction systems could also be utilized to predict patients who have an increased risk for prolonged hospital stay and death post-acute coronary syndrome (Loudon et al. 2016).

The European society of cardiology recommend using the SCORE algorithm, in which Europe has been classified as a low risk country. The SCORE algorithm includes age, gender, smoking status, systolic blood pressure, and serum total cholesterol (Jorstad et al. 2015). Determination of CVR using the SCORE algorithm involves reading of a chart designed for low-risk countries as seen in annexure D and high-risk countries as seen in annexure E (Piepoli et al. 2016a) . However, using this chart only could result in elderly using unnecessary medication since their age alone would place them at risk as seen in Figure 2. For this reason, the SCORE system is being modified to incorporate HDL (Perk et al. 2012), emphasizing the importance to integrate new CVR markers into risk assessment strategies.

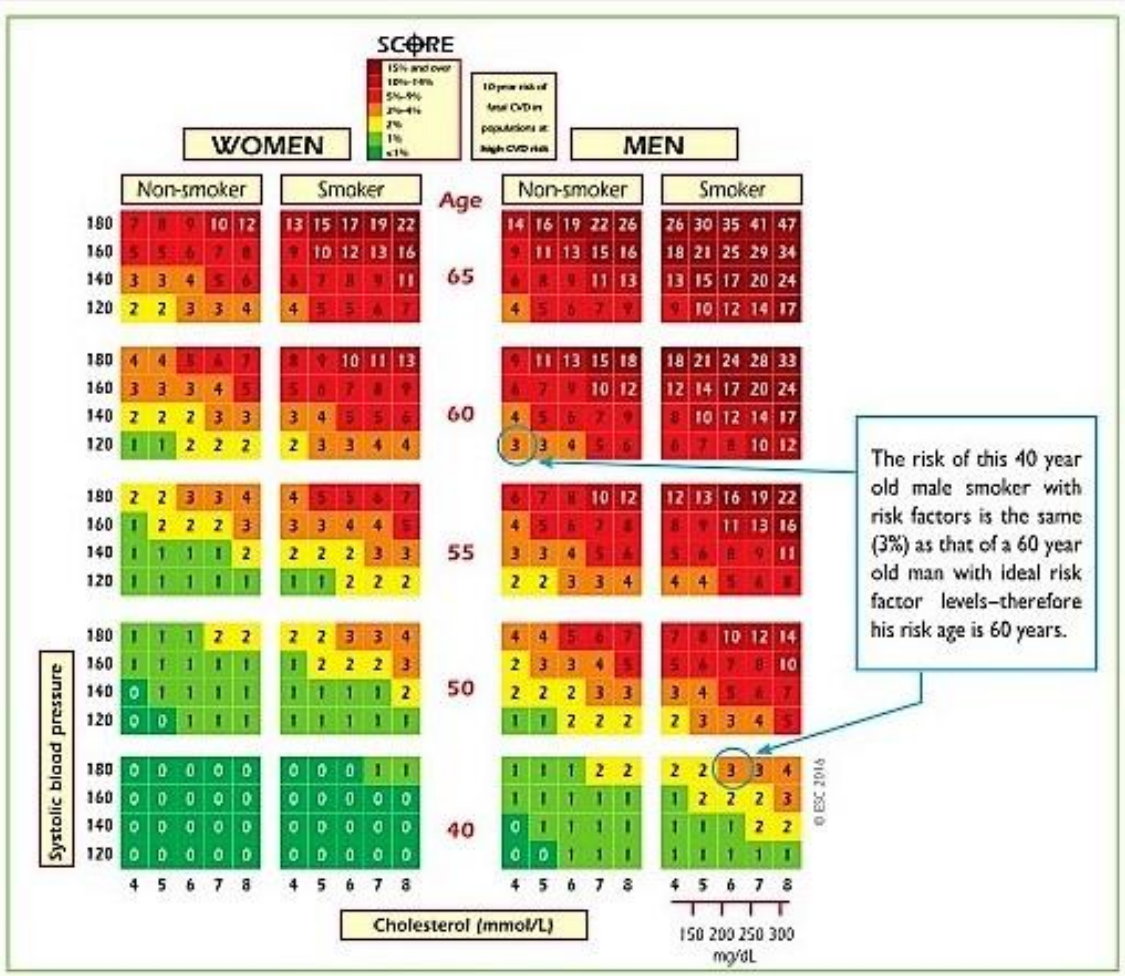


Figure 2: SCORE chart illustrating the effect of age on risk classification (Piepoli et al. 2016b).

The Reynolds Risk Score was developed in 2007 (Ridker et al. 2007). As with FRS, this global risk algorithm makes use of the variables age, gender, smoking status, total cholesterol, HDL cholesterol, blood pressure and diabetes status. The RRS uses haemoglobin A_{1c} (HbA_{1c}) for diabetes evaluation, and additionally includes evaluation of HS-CRP and family history of myocardial infarction (MI) (Paynter et al. 2014; Willeit et al. 2014).

Table 2: Comparison of clinical variables used for FRS, RRS and SCORE

	FRS	RRS	SCORE
Age	√	√	√
Gender	√	√	√
Smoking status	√	√	√
Total cholesterol	√	√	√
HDL cholesterol	√	√	
Diabetes status	√	√ (HbA$_{1c}$)	
Blood pressure	√	√	√
Treatment for hypertension	√		
Family history of MI		√	
HS-CRP		√	
High/Low risk country			√

There are various risk modifiers not currently used in these common risk determination algorithms. These include socio-economic status, body mass index (BMI) or central obesity status, and computed tomography (CT) coronary calcium score (Piepoli et al. 2016b).

Various risk factors have been identified to cause CVD, as illustrated in Figure 3. These risk factors are involved in the pathophysiology and development of CVD. Risk factors are classified into modifiable and non-modifiable risk factors. Modifiable risk factors include tobacco smoking, dyslipidaemia, hypertension, diabetes mellitus and obesity or lack of exercise. Non-modifiable risk factors are advancing age, gender and genetics. Treatment and prevention of CVD is currently obtained by targeting modifiable risk factors (Douglas & Channon 2014).

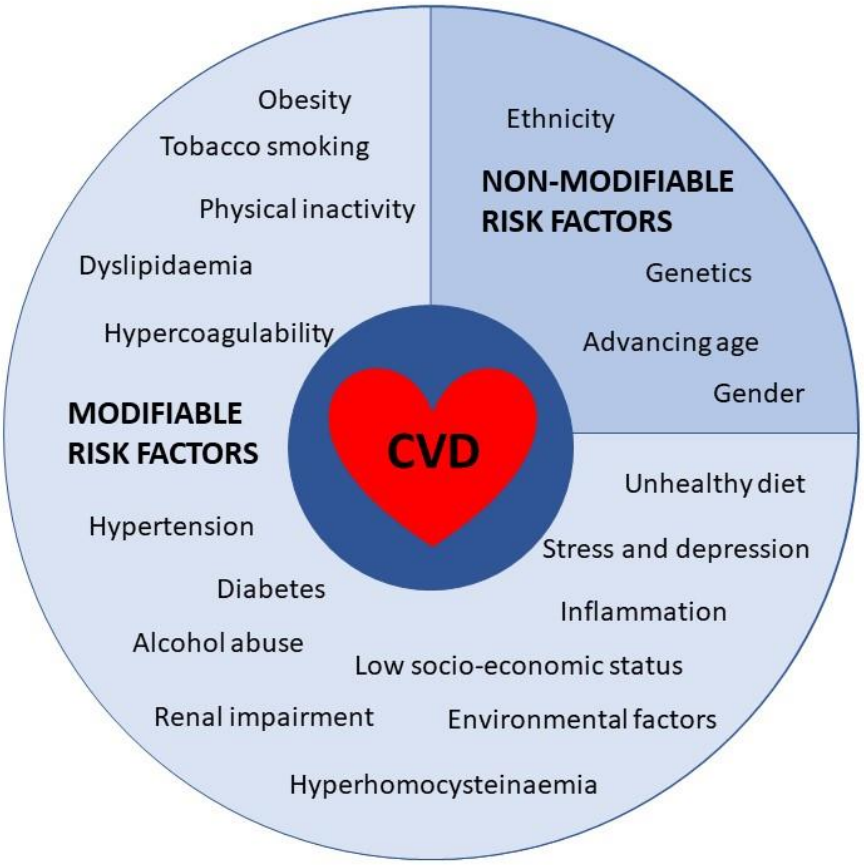


Figure 3: Cardiovascular risk factors. (Douglas & Channon 2014; Piepoli et al. 2016b)

2.4.1 Modifiable cardiovascular risk factors

2.4.1.1 Tobacco smoking

Though the global prevalence of smoking has decreased, the total number of smokers has increased with the growing global population. Cigarette smoking and second-hand smoking are responsible for 6.3 million deaths each year, of which one third is CVD related (Morris et al. 2015). Smoking is not only harmful to the smoker but also to the non-smoker exposed to second-hand smoke (Writing Group et al. 2016). Arterial walls are exposed to free radicals from cigarette smoke, resulting in oxidative stress. Smoking also activates inflammatory cells, which in turn release ROS (Park & Park 2015). Hypercoagulability, impaired fibrinolysis and increased blood viscosity resulting from smoking can cause atherothrombosis (Vella & Petrie 2015).

2.4.1.2 Dyslipidaemia

To determine an individual's lipid status, a lipid profile is requested by the physician. Total cholesterol, LDL cholesterol, HDL cholesterol and triglyceride serum values are measured in a lipid profile. Circulating LDL cholesterol is deposited in arterial walls causing atherosclerosis. Oxidized LDL cholesterol which is taken up by macrophages form foam cells. These foam cells contribute to plaque formation. HDL cholesterol removes LDL cholesterol from arterial walls, protecting against CVD (Mudau et al. 2012; Douglas & Channon 2014). Increased LDL cholesterol levels and decreased HDL cholesterol levels have been found to be associated with CVD (Douglas & Channon 2014; Nordestgaard & Varbo 2014; Park & Park 2015). Though low levels of HDL cholesterol have been associated with increased CVR, high levels of HDL cholesterol have not proven to decrease CVR (Piepoli et al. 2016b).

2.4.1.3 Obesity and physical inactivity

Obesity is classified as individuals with a BMI of ≥30 kilogram per square meter (kg/m^2), and is strongly associated with other CVR factors such as hypertension, hyperglycaemia and insulin resistance, systemic inflammation, prothrombotic state, albuminuria and dyslipidaemia (World Health Organization 2011; Stepien et al. 2014). Excess calories are stored as fat, resulting in adipocyte hypertrophy, greater triglyceride storage, macrophage infiltration and apoptosis, and ultimately inflammation (Vella & Petrie 2015). Under normal conditions adipocytes synthesize lipids and store and secrete anti-inflammatory molecules. Obesity results in the hypertrophic adipocytes secreting pro-inflammatory molecules, ultimately resulting in increased production of CRP. In visceral and epicardial obesity, adipocytes also contain higher ROS than subcutaneous adipose tissue, causing oxidative stress (Agra et al. 2014). Adiponectin is an anti-inflammatory expressed by adipocytes under normal conditions, and lower levels are found in obesity. It is a high molecular weight polymeric protein which has been indicated to sensitize insulin (Vella & Petrie 2015).

A sedentary lifestyle increases the risk for cardiovascular disease and exercise decreases cardiovascular risk. In order to gain the cardioprotective benefits from exercise, national and international guidelines suggest 150 minutes of moderate intensity exercise per week or 75 minutes of high intensity exercise per week, or a combination of the two (Bouchard et al. 2015; Despres 2016). Physical activity utilizes calories to prevent fat storage, improves endothelial function and vasodilation which prevents hypertension, improves insulin sensitivity and contributes to satisfactory glycaemic control (Park & Park 2015).

2.4.1.4 Hypertension

Hypertension alters shear stress, causing dysfunction of ECs by increasing endothelial cell permeability and endothelial cell activation resulting in increased inflammatory cell recruitment (Douglas & Channon 2014). Increased production of endothelial derived vasoconstrictors, ROS and decreased NO have been associated with hypertension, which may result in atherosclerosis (Mudau et al. 2012; Park & Park 2015).

Normal blood pressure (BP) range is a systolic pressure of 120-129 millimetre of mercury (mmHg) and diastolic pressure of 80-84. High normal BP range is 130-139mmHg systolic and 85-90mmHg. A BP of 140mmHg systolic and above and/or 90mmHg diastolic and above is classified as hypertension (Perk et al. 2012). An untreated systolic BP of 140mmHg and above or diastolic BP of 90mmHg and above, as well as individuals using therapy for hypertension have a 2-3 fold increased rick for CVD (Landsberg et al. 2013).

2.4.1.5 Diabetes mellitus

Diabetes is defined by the WHO as “a serious, chronic disease that occurs either when the pancreas does not produce enough insulin (a hormone that regulates blood glucose), or when the body cannot effectively use the insulin it produces”. The resultant hyperglycaemia causes damage to the cardiovascular system, nervous system, kidneys and eyes (World Health Organization 2016a). An individual with diabetes is at greater risk for CVD, the CVR increases as glucose levels increase. Diabetic individuals experience increased oxidative stress and eNOS uncoupling due to hyperglycaemia. This results in decreased NO production and increased synthesis of vasoconstrictive substances, ultimately causing ED. Increased superoxide anions produced also promote VSMC proliferation and amplifies the inflammatory response. These macrovascular complications cause are the major causes of mortality in diabetic patients (Mudau et al. 2012; Maschirow et al. 2015).

Non-enzymatic glycation of proteins and lipids takes place during hyperglycaemia with the production of advanced glycation end-products (AGEs). These AGEs that accumulate in the arterial wall cause damage to the endothelium and basement membrane structure and cause a decrease in NO activity which results in ED. Intensification of the inflammatory response, increased vascular permeability and oxidative stress occur as a result of AGEs binding to specific surface receptors of monocytes, macrophages and VSMCs (Park & Park 2015).

2.4.1.6 Alcohol abuse

The complex effects of alcohol can result in both increased and decreased risk of CVD, depending on the dosage and frequency of consumption. Within hours of consumption, moderate and high alcohol intake is associated with increased heart rate, electromechanical delay and impaired fibrinolysis. The increased risk is continued even after 24 hours with high average alcohol intake. Moderate alcohol intake results in increased risk within 24 hours after consumption and decreased risk thereafter due to improvement in blood pressure and coagulation (Mostofsky et al. 2016). Moderate average alcohol consumption has a protective effect, demonstrated by decrease in fibrinogen, and increase in adiponectin and HDL cholesterol (Brien et al. 2011). The polyphenols in wine and beer have antioxidant, anti-inflammatory, hypotensive activities and prevent platelet aggregation (Chiva-Blanch et al. 2013).

Drinking patterns and not only the average consumption is vital in determining CVR. Even with moderate average alcohol consumption, occasional heavy drinking can eliminate the beneficial effects of moderate drinking and increase CVR. In conclusion, higher levels of average alcohol consumption and binge drinking are linked to an increased risk for developing CVD (Roerecke & Rehm 2014).

2.4.1.7 Unhealthy diet

Nutrition plays an invaluable role in CVR. A poor diet, consisting of large amounts of saturated fats, trans-fat and cholesterol, and low intake of fruits, vegetables and fish has been associated with CVD. Excess calories in the body are converted to triglycerides and stored as fat throughout the body which may result in increased BMI, obesity and ultimately increased CVR (World Health Organization 2011). A healthy diet is one which incorporates all food groups in appropriate amounts. It is suggested that a healthy diet is high in vegetables, fruit, whole grains, low-fat or non-fat dairy, seafood, legumes and nuts. A moderate amount of alcohol, red meat and processed meat may form part of a balanced healthy diet. Sugar, sweetened foods and refined grains should be consumed in low amounts (Yu et al. 2016; Benjamin et al. 2017).

Vitamin D deficiency has been demonstrated to be associated with increased CVR. Vitamin D receptors have been found in all cells involved in atherosclerosis. The role of vitamin D in preventing atherosclerosis includes stimulation of NO production and eNOS activation (Norman & Powell 2014; Park & Park 2015). Deficiencies of folate, vitamin B6 or vitamin B12

may result in hyperhomocysteinaemia, which is an independent CVR factor (Ganguly & Alam 2015). The consumption of antioxidants plays a beneficial role in the prevention of endothelial damage from ROS. There are numerous antioxidants, each with a unique structure and function. This creates a need for further studies into anti-oxidants as a preventative measure or its use for treatment (Mangge et al. 2014; Siti et al. 2015).

2.4.1.8 Social and environmental factors

Low socio-economic status including poverty, low educational level, environmental degradation and poor housing, have been associated with CVR (Piepoli et al. 2016b). Rapid unplanned urbanization results in limited healthy food choices, limited environment for physical activity and high rates of exposure to air pollutants, including tobacco smoke. This has a negative impact on general health, including increased risk for CVD (World Health Organization 2011; Dutta & Ray 2012). Exposure to air pollution triggers an inflammatory response in the vasculature system contributing to atherosclerosis (Hajat et al. 2015). Psychological factors such as isolation from other people, personal stress, clinical depression and anxiety have all been linked to risk for CVD (Piepoli et al. 2016b).

2.4.1.9 Inflammation

Inflammation is the common underlying mechanism of endothelial dysfunction and atherosclerosis (Ross et al. 1977; Ross 1999; Douglas & Channon 2014). Though it may occur alone, it is usually a result of another modifiable or non-modifiable risk factor. A normal endothelium produces NO to control vascular inflammation; however, a dysfunction endothelium produces ROS which exaggerates vascular inflammation. Oxidative stress resulting from various other risk factors could amplify vascular inflammation signalling pathways resulting in further superoxide production by inflammatory cells (Mudau et al. 2012). Unlike acute inflammatory events which are self-limiting and resolve once the cause of inflammation is removed, atherosclerosis does not progress to the resolution phase (Viola & Soehnlein 2015).

2.4.1.10 Haemostasis

Several studies have shown an association between abnormal haemostasis and CVR through investigations of biomarkers of coagulation and inflammation. Fibrinogen is a clotting factor as well as inflammatory marker demonstrated to be an independent CVR marker. Controversial results have been reported regarding the association of factor VII with CVR. Increased circulating levels of von Willebrand: factor VIII complex have been linked to CVR (Lowe & Rumley 2014).

Atherosclerotic plaque may rupture as a result of inflammatory activation of macrophages, increased apoptosis within the plaque and thinning of the cap. Upon plaque rupture, collagen and tissue factor is exposed to the circulating blood which triggers platelet and coagulation activation. This ultimately results in atherothrombosis, which could partially or completely occlude the arteries (Krychtiuk et al. 2013).

2.4.1.11 Albuminuria and reduced glomerular filtration rate

There is a strong association between CVD and renal impairment with an estimated glomerular filtration rate (eGFR) of less than 60 millilitre per minute per 1.73 square metre (ml/min/1.73m^2). Microalbuminuria is linked to an almost doubled risk for CVD in diabetic and non-diabetic patients (Vella & Petrie 2015). Hypertension, dyslipidaemia and hyperglycaemia are often observed in patients with kidney disease, resulting in albuminuria and reduced eGFR. Pro-inflammatory substances and promotors of calcification are also a feature of kidney disease, causing endothelial injury and adding to the risk of CVD (Piepoli et al. 2016b).

2.4.1.12 Hyperhomocysteinaemia

Homocysteine is an amino acid and intermediate product in the synthesis of amino acids methionine and cysteine. Hyperhomocysteinaemia may arise from genetic abnormalities of the enzymes involved in homocysteine metabolism or nutrition deficiencies of folate, vitamin B6 or vitamin B12. Hyperhomocysteinaemia contributes to CVD through endothelial damage, reduction in arterial flexibility, VSMC growth, increased LDL cholesterol oxidation and uptake into vascular wall and increased platelet adhesion (De Farias Leal et al. 2013; Ganguly & Alam 2015). Increased levels of homocysteine have been reported to result in increased CVR (Perk et al. 2012).

2.4.2 Non-modifiable cardiovascular risk factors

2.4.2.1 Advancing age

An association between aging and CVD have been established. Aging may result in an increased prevalence of CVR factors and there is usually a longer exposure to these factors. There are also structural and functional changes of the cardiovascular system that occur during the normal physiology of aging. These changes include decreased vascular wall elasticity, changes in coagulation and haemostasis, ED, pro-inflammatory state and impaired regeneration as demonstrated in Figure 4 (Badimon et al. 2016).

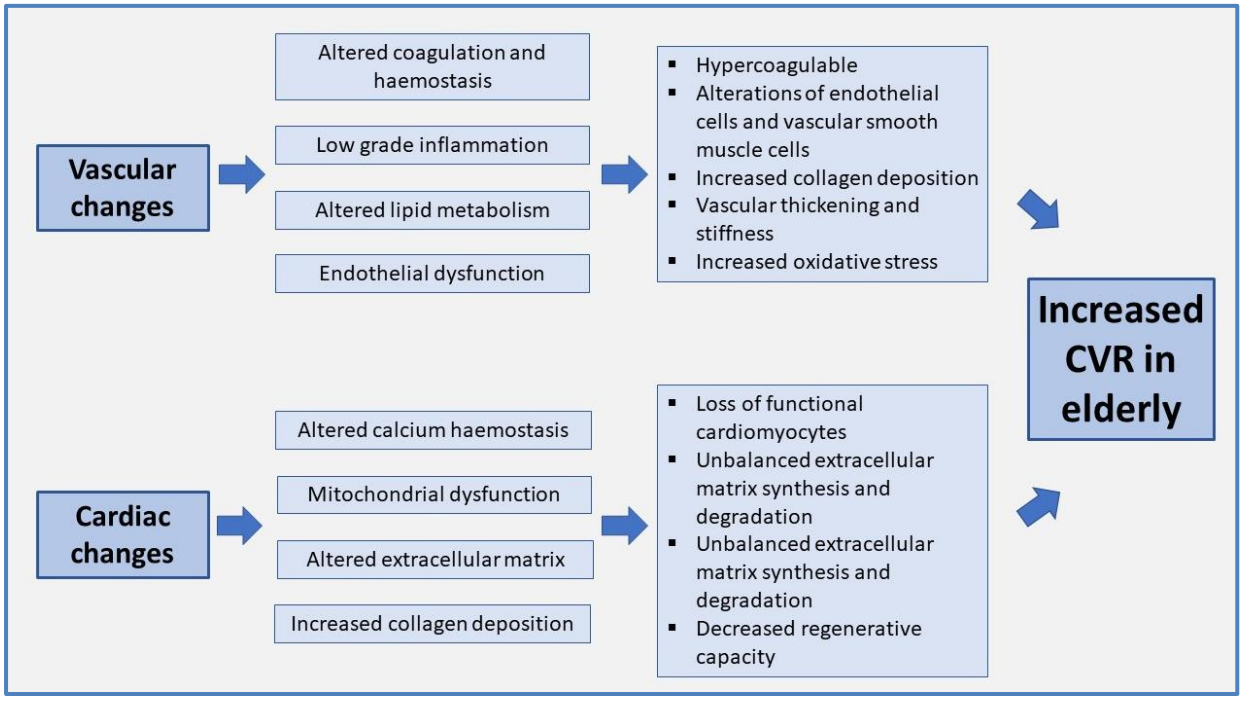


Figure 4: Effects of age-related vascular and cardiac changes. Adapted from Badimon et al. (2016)

Decreased vascular wall elasticity is caused by phenotypic alterations of ECs and VSMCs, collagen deposits and vascular wall thickening. This arterial wall stiffening results in arterial hypertension (Badimon et al. 2016). Together with increased deposition of collagen, collagen fibres have more permanent cross linkages resultant from AGE cross links which are more resistant to routine breakdown. With advancing age, the upregulation of elastase results in reduced elastin and ultimately impaired elasticity of arteries (Jackson & Wenger 2011).

Coagulation and haemostasis changes in elderly place them in a hypercoagulable state. Several studies have demonstrated the elderly to have increased coagulation factors such as

fibrinogen, factors VII, VIII, IX, X and XII, von Willebrand factor, high molecular weight kininogen and prekallikrein. Plasminogen activator inhibitor-1 (PAI-1) has been indicated to play an imported role in fibrinolysis of the elderly (Badimon et al. 2016).

Aging is associated with endothelial dysfunction as a result of a decline in 3 major endothelium-derived vasodilators, NO, prostacyclin and endothelium derived hyperpolarizing factor, resulting in a decreased endothelium-dependant vasodilation and increased reactivity to vasoconstrictors (Tesauro et al. 2017). An increase in endothelium-derived vasoconstrictors such as cyclooxygenase-derived prostanoids and endothelin-1, also a powerful vascular growth factor, has also been demonstrated to be associated with aging (Park & Park 2015; Badimon et al. 2016). Increase in specific metalloproteinases and angiotensin associated with aging also contributes to ED. Alterations of the renin-angiotensin-aldosterone system can lead to sodium reabsorption, resulting in blood pressure fluctuations (Jackson & Wenger 2011).

The elderly population has been reported to be in a pro-inflammatory state, with increased circulation levels of CRP and IL-6. This not only results in an exaggerated immune response, but also affects fibrinolysis (Badimon et al. 2016). Increased expression of IL-1 and TNF-α is also observed in the chronic low-grade inflammatory state of the elderly (Capuron et al. 2011). The increased production of inflammatory cytokines with advancing age is termed inflamm-aging (Franceschi et al. 2000a; Graja & Schulz 2015).

Age-related cardiovascular remodelling demonstrated unbalanced extracellular matrix synthesis and degradation with an increase in collagen in elderly cardiac structures. Abnormal left-ventricular diastolic function occurs as a result of excessive collagen deposition and cross linking which thickens the ventricular wall (Gupta et al. 2015). Aging is also associated with impaired calcium recycling and reduced calcium sensitivity of myofilament proteins, causing reduced cardiomyocyte relaxation. Age-related mitochondrial dysfunction due to decreased adenosine triphosphate (ATP) production hinders cardiac function. Damage to mitochondrial DNA and redox-sensitive mitochondrial proteins in aging result in the excessive formation of mitochondrial ROS, consequently causing further mitochondrial damage and ROS production (Badimon et al. 2016). Age-related decline in eGFR results from reduced glomerular surface area. As the glomerular basement membrane becomes increasingly permeable, microalbuminuria and proteinuria is observed (Navaratnarajah & Jackson 2013)

2.4.2.2 Gender, genetics and ethnicity

Studies have reported a higher prevalence of CVD in males compared to females (Perk et al., 2012, Vella and Petrie, 2014). This is also true for pre-menopausal women as oestrogen has a cardioprotective effect of lowering blood pressure. As in the case of gender, genetics plays an undeniable role in CVD. Numerous CVR factors have been shown to demonstrate strong evidence of hereditability (Perk et al. 2012). An individual with a close relative diagnosed with CVD before the age of 60 has an almost a doubled risk of CVD (Vella & Petrie 2015).

Certain genetic polymorphisms lead to an individual being more susceptible to developing CVD upon exposure to social, behavioural and metabolic risk factors. In a study reviewing the literature on the application of single nucleotide polymorphisms (SNPs) in genetic susceptibility to CVD, several SNPs were identified to correlate with risk factors. The majority of studies investigating genetic variants associated with CVD in the past 10 years have been conducted in populations of different ancestry and ethnicity (Fiatal & Adany 2017). Relating to inflammation, C174G polymorphism has been associated with increased levels of serum IL-6 in various populations. Consequently, the inflammatory state increases the risk of CVD (Giannitrapani et al. 2013; Nadeem et al. 2013; Ramirez Garcia 2017).

2.4.3 Biochemical cardiovascular risk markers

The effects of CVR factors and ultimately their ability to result in CVD are experienced differently in populations which is a consequence of unique genetic and ethnic factors. With CVD as the leading cause of non-communicable disease, the need for population based CVR profiling is essential for effective risk determination, disease prevention and treatment (van der Ende et al. 2017). CVR markers have been identified which allows measuring and classification of the effects of the CVR factors. Evaluation of risk factors are done through collection of data on demographic and behavioural factors, physical biometric measurements and biochemical measurements (Mensah 2013). The identification of independent markers of CVR in various populations are of paramount importance to simplify risk stratification in low-income regions, with restricted resources for CVD detection and prevention (Celermajer et al. 2012). Various biomarkers are being investigated as CVR markers, as outlined in Table 3.

Table 3: Biomarkers of interest in cardiovascular disease risk stratification

Biomarker	Description
Inflammation	
CRP (HS-CRP)	Inflammatory acute phase protein, demonstrated as an independent risk marker for CVD (Batsis & Lopez-Jimenez 2010; Emerging Risk Factors Collaboration et al. 2012).
IL-6	Pro-inflammatory cytokine and major stimulator of hepatic acute phase response and CRP synthesis, associated with increased CVR (Fan et al. 2011; Bell et al. 2012).
TNF-α	Pro-inflammatory cytokines, associated with increased risk for CVD in some populations (Cui et al. 2012; Rodriguez-Hernandez et al. 2013).
IL-1	Increased circulating levels of pro-inflammatory cytokine IL-1 is associated with CVD (Tsimikas et al. 2014).
IL-10	IL-10 is an anti-inflammatory cytokine, promoting inflammatory resolution and protects against endothelial dysfunction (Kinzenbaw et al. 2013).
Dyslipidaemia	
Total cholesterol	Increased total cholesterol (consisting of LDL cholesterol, HDL cholesterol and triglycerides) indicates increased CVR (Liao & Solomon 2013).
LDL	LDL cholesterol plays a crucial role in plaque formation, increased LDL levels are associated in CVR (Liao & Solomon 2013; Peer et al. 2014).
HDL	Low HDL cholesterol levels have been indicated to increase CVR (Liao & Solomon 2013; Peer et al. 2014).
Lipoprotein (a)	Increased levels of lipoprotein (a) is associated with CVR (Batsis & Lopez-Jimenez 2010; Willeit et al. 2014).
Apolipoprotein B	Apolipoprotein A (Apo B) is the structural protein carrying very low-density lipoprotein (VLDL), demonstrated as a measure of atherogenic potential and CVR (Mangalmurti & Davidson 2011).
Apolipoprotein A-I	Apolipoprotein A-I (Apo A-I) is a structural component of HDL, which is atheroprotective (Mangalmurti & Davidson 2011).

Table 3: continued

Metabolic syndrome	
Glucose	Increased glucose levels could indicate pre-diabetes, diabetes or metabolic syndrome, which is associated with increased CVR (Grundy 2012).
Insulin	Insulin resistance and increased circulating levels of insulin have been connected to the development of CVD (Yoon et al. 2014).
HbA1c	Used to diagnose diabetes, increased levels indicate hyperglycaemia and therefore it is associated with CVR (Grundy 2012).
Adiponectin	Previous studies have demonstrated that adiponectin has anti-atherogenic and anti-inflammatory properties which can prevent atherosclerosis. Decreased levels of adiponectin is therefore linked to increased CVR (Agra et al. 2014; Vella & Petrie 2015; González et al. 2017).
Oxidative stress	
Myeloperoxidase (MPO)	Cationic protein, mainly found in azurophilic granules of neutrophils, induces the formation of oxidants causing tissue damage. Through LDL oxidization and NO reductions contributes to atherosclerosis. Several studies associating increased MPO with CVR (Biasillo et al. 2010).
Coagulation	
Fibrinogen	Coagulation factor which increases in response to inflammatory stimuli, elevated levels of fibrinogen is associated with CVR (Bell et al. 2012).
Factor VII	Several studies have associated increased factor VII activity with CVR, as it results in a pro-thrombotic state (Azzam et al. 2017). Whilst other studies have reported contradicting results (Lowe & Rumley 2014).
Factor VIII: von Willebrand factor complex	An association has been demonstrated between increased circulating levels of Factor VIII: von Willebrand complex and an increased risk of developing CVD (Lowe & Rumley 2014).
D-Dimer	Increased levels of D-Dimer, a fibrin breakdown product and marker of activated coagulation, have been associated with the development of atherothrombosis (Lowe & Rumley 2014).

Table 3: continued

Renal function	
Creatinine	Creatinine is measured to assess renal function and is increased in renal damage or failure. Chronic kidney disease and renal failure are associated with an increased CVR (Piepoli et al. 2016b).
Microalbuminuria	Microalbuminuria indicates endothelial dysfunction and is therefore associated with increased risk to develop CVD (Vella & Petrie 2015).
Other	
Vitamin D	Controls inflammatory and immune responses (anti-inflammatory effect), as well as calcium and phosphorus metabolism (Norman & Powell 2014). Decreased levels of vitamin D have been associated with increased CVR (Arnson et al. 2013).
Homocysteine	Homocysteine is an intermediate amino acid, in the normal synthesis of amino acids methionine and cysteine (Ganguly & Alam 2015). It has been demonstrated as an independent CVR factor through previous investigations (Batsis & Lopez-Jimenez 2010; De Farias Leal et al. 2013).
Vitamin B12	Vitamin B12 is required as a coenzyme in the re-methylation process of homocysteine, decreased levels of vitamin B12 will consequently result in hyperhomocysteinaemia and increased CVR (De Farias Leal et al. 2013).
Folate	The metabolism of homocysteine also involves folate as a coenzyme. Decreased levels of folate is therefore associated with CVR (De Farias Leal et al. 2013).
ICAM-1	Increased levels of circulating endothelial cell adhesion molecule, ICAM-1, is associated with endothelial activation and atherosclerosis, therefore increased CVR (Lopez-Mejias et al. 2016).
VCAM-1	The development of CVD and increased endothelial cell adhesion molecule, VCAM-1, have been reported to be directly associated (Lopez-Mejias et al. 2016).

2.5 Inflammation

Inflammation is a component of innate, natural, native or non-specific immunity. Body barriers, epithelial cell-derived chemicals and normal flora provide a first line of defence, protecting against infection and tissue injury. The inflammatory response is the second line of response, activated to respond to infection and tissue injury (Huether & McCance 2012). Inflammation is a complex nonspecific response to tissue or cellular injury, characterized by increased blood flow, capillary dilation, leukocyte infiltration and localized production of host chemical mediators. This leads to the manifestation of the five typical signs of inflammation such as heat, redness, swelling, pain and loss of function (Medzhitov 2010; Calder et al. 2013; Motwani et al. 2017). The purpose of the inflammatory response is to minimize the effects of injury or infection through diluting, destroying and removing damaged or necrotic tissue and pathogens, generating new tissue and facilitating healing (Minihane et al. 2015; Porth 2015).

Inflammation can be classified as acute or chronic, with the main differentiating factors being duration and intensity. Acute inflammation is the first reaction to defend the body against harm, whether is it septic, such as infectious agents, or sterile, such as cell damage resultant from irradiation. The rapid onset of acute inflammation is followed by short lived exudation of fluid and plasma proteins and the immigration of leukocytes to the infected or injured site. The self-limiting process of acute inflammation lasts minutes to days, resolving upon removal of the causal agent (Calder et al. 2013; Porth 2015).

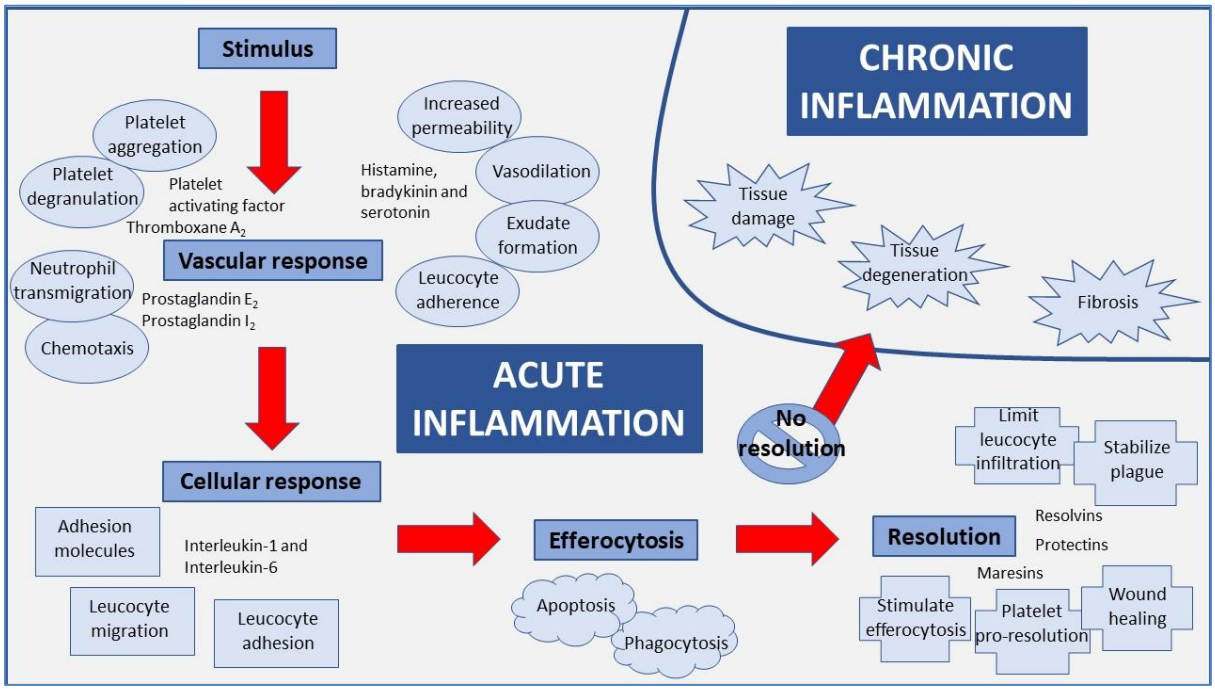


Figure 5: Acute and chronic inflammation adapted from (Buckley et al. 2014)

Chronic inflammation is longer lasting, can be days to years, and is usually of lower intensity than acute inflammation. Whereas acute inflammation facilitates tissue repair, chronic inflammation tends to result in tissue degradation (Franceschi & Campisi 2014). Chronic inflammation is associated with the proliferation of blood vessels, tissue necrosis and fibrosis. Though unresolved acute inflammation is often the cause of chronic inflammation and both may also occur simultaneously (Porth 2015).

2.5.1 The inflammatory response

Acute inflammation is initiated upon exposure to inflammatory triggers, such as microbial products, tissue and cellular damage and metabolic stress. The resultant systemic manifestations and increased serum proteins is termed acute phase response. Acute inflammation has two stages, a vascular stage followed by the cellular stage (Porth 2015).

The vascular stage is the initial response to injury starting with vasoconstriction of the arterioles which only last a few seconds (Kokkas 2010). During increased microvascular permeability protein-rich fluid moves from the vasculature into the extravascular spaces forming an exudate. The exudate dilutes the pathogen or harmful agent in the tissues. The loss of proteins from the vasculature to tissues spaces aids in the movement of fluid into tissue spaces against the osmotic pressure gradient. Swelling, pain and loss of function manifests

as a consequence of increased interstitial fluid. The fluid loss results in blood becoming more viscous, with more concentrated blood cells and clotting factors (Huether & McCance 2012; Buckley et al. 2014). The sluggish blood flow allows clotting and limits the spread of infectious or harmful agents from the injury site. Histamine, bradykinin and serotonin bind to epithelial receptors and cause retraction of capillaries lining ECs. Histamine also increases vascular permeability through rapid constriction of smooth muscle cells, dilation of postcapillary venules, and increased adherence of leukocytes to the endothelium. This leads to separation of intercellular junctions, increasing vascular permeability and allow vascular leakage. NO also mediates endothelium dependant vasodilation.(Porth 2015; Shayganni et al. 2016; Shakya & Gupta 2017).

Phospholipases releases the arachidonic acids in the phospholipids of endothelial cell membranes (Huether & McCance 2012). This leads to the synthesis of prostaglandins through the cyclo-oxygenases pathway and leukotrienes through the lipoxygenase pathway. Leukotrienes induce smooth muscle contraction, constrict pulmonary airways and increase microvascular permeability. Prostaglandins induce vasodilation and bronchoconstriction, decreases IL-1 and IL-6 and enhances tissue remodelling. Prostaglandin E_2 (PGE_2) has a role as an inflammatory antagonist, increasing production of anti-inflammatory IL-10 and decreasing TNF-α. It also acts together with PGI_2 to allow transmigration of neutrophils across ECs of postcapillaries along the gradient of the chemoattractant leukotriene B_4 (Ashina et al. 2015; Chiurchiu & Maccarrone 2016; Serhan 2017). Prostaglandin thromboxane A_2 promotes vasoconstriction, bronchoconstriction and platelet aggregation (Chiurchiù & Maccarrone 2016). The membrane phospholipid of nearly all the inflammatory cells give rise to platelet-activating factor (PAF). PAF induces platelet aggregation and platelet degranulation which amplifies serotonin release and thereby increasing vascular permeability. It also aids in leukocyte adhesion, chemotaxis and leukocyte degranulation, as well as stimulate the synthesis of prostaglandins (Gros et al. 2014; Porth 2015).

There are three patterns of changes in the vascular endothelium, based on the severity of the injury. Minor injury results in the immediate transient response, lasting 15-30 minutes. Direct damage to the endothelium will result in the longer lasting immediate sustained response, involving arterioles, capillaries and venules. Injuries from radiation, such as sunburn, trigger the delayed response which involves the arterioles and capillaries. Increased vascular permeability is established after a delay of 2-12 hours, and may last for days (Porth 2015).

The cellular stage involves changes in the ECs that line the vasculature, leukocyte margination and adhesion, transmigration, chemotaxis and activation and phagocytosis. Leukocyte

margination is the process of leukocyte accumulation as a result of slowed blood flow. Mast cells and macrophages adjacent to the blood vessels release chemical mediators (Krychtiuk et al. 2013; Porth 2015). Inflammatory cytokines TNF-α and IL-1 are released, causing the ECs of the vascular lining to express adhesion molecules and result in tethering (Mittal et al. 2014; Porth 2015). During tethering the adhesion molecules, E-selectins (associated with ECs) and P-selectins (associated with platelets) bind to carbohydrates on the leukocytes to slow them down; this is known as leukocyte rolling. After rolling along the endothelial lining, leukocytes stop when they adhere strongly to the intercellular endothelium adhesion molecules. Endothelial cell separation allows leukocytes to transmigrate through the vessel wall into tissue spaces, where phagocytosis will take place (Silva-Herdade et al. 2016). The circulating interleukin-1 Beta (IL-1β) not only enhances further production of itself, but also of IL-6. Consequently, IL-6 stimulates hepatic production of CRP. Inflammatory cells involved in local inflammation can also produce CRP at concentrations too low to cause systemic effects (Ridker 2016).

The final phase of the cellular stage is phagocytosis by neutrophils, monocytes and macrophages. Specific receptors on phagocytes recognise and bind to the target cell triggering engulfment. Target cells are recognized directly by phagocytes, or indirectly through opsonization. During opsonization the target cells are coated with lectins, antibodies and/or complement. The cytoplasm of phagocytes extends to surround and ultimately engulf the opsonized particle, forming a phagosome. In the cytoplasm the phagosome fuses with the lysosome, forming a phagolysosome. The enzymes from the lysosome kill and digest the particle. Lysozymes, proteases and defensins are responsible for intracellular killing. These require oxygen and the enzyme reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and nitric oxide synthase (Porth 2015; Lim et al. 2017).

Phagocytosis plays a vital role in the resolution of inflammation, finally removing the cause of inflammation. Neutrophils that have been involved in the removal of pathogens or pro-inflammatory stimuli are subsequently engulfed by macrophages for leukocyte clearance. If these neutrophils are not removed, the molecules released form the apoptotic cells would prolong the inflammatory response (Gordon 2016). Phagocytosis of apoptotic cells, as in the case of atherosclerosis, is termed efferocytosis (Lim et al. 2017).

It is important to note the difference between anti-inflammation and pro-resolution. Anti-inflammatory relates to processes which inhibits the factors that promote inflammation and it does not remove the cause of inflammation. Using anti-inflammation as a pathway for treatment may cause the patient to be immunocompromised (Fredman & Tabas 2017;

Motwani et al. 2017). Buckley et al. (2014) defined resolution of inflammation as the rate at which neutrophils are cleared from the site of tissue injury, until there is an absence of neutrophils. They explained that the process involves removal of the pro-inflammatory stimuli and resultant proinflammatory signals, normalizing chemokine levels and neutrophil apoptosis followed by efferocytosis.

Specialized pro-resolving mediators (SPMs) are the regulators of inflammatory resolution. These lipid derived SPMs include resolvins, lipoxins, protectins and maresins. The SPMs are synthesized at the site of injury upon activation of the inflammatory response through action of lipoxygenases (LOX). Proteins and peptides, nucleotides and gases also play a role in resolution of inflammation (Fredman & Tabas 2017). Resolvin E1 and protein Annexin A1 with its bioactive peptide Ac2-26 decreases leukocyte infiltration at the site of inflammation. Maresin 1 promotes the pro-resolving actions of platelets. Resolvin D1 stabilizes plaque by increasing fibrous cap thickness and decreases necrosis and oxidative stress through an increased rate of efferocytosis (Buckley et al. 2014; Fredman & Spite 2017; Heinz et al. 2017).

Unresolved acute inflammation develops into chronic inflammation. Unlike the reparative process of acute inflammation, chronic inflammation causes tissue damage and degeneration (Franceschi & Campisi 2014; Fredman & Spite 2017). Chronic low-grade sterile inflammation is characterised by a slight increase in inflammatory markers. Though it does not present clinical signs, it has proven to negatively affect metabolic pathways and normal physiology (Mraz & Haluzik 2014).

2.5.2 Inflammatory mediators

Mediators of the acute inflammatory response are plasma-derived and cell-derived. Cell-derived mediators include preformed and newly synthesized mediators as a result of the inflammatory response as seen in Figure 6. These mediators are responsible for initiation, intensification and resolution of inflammation.

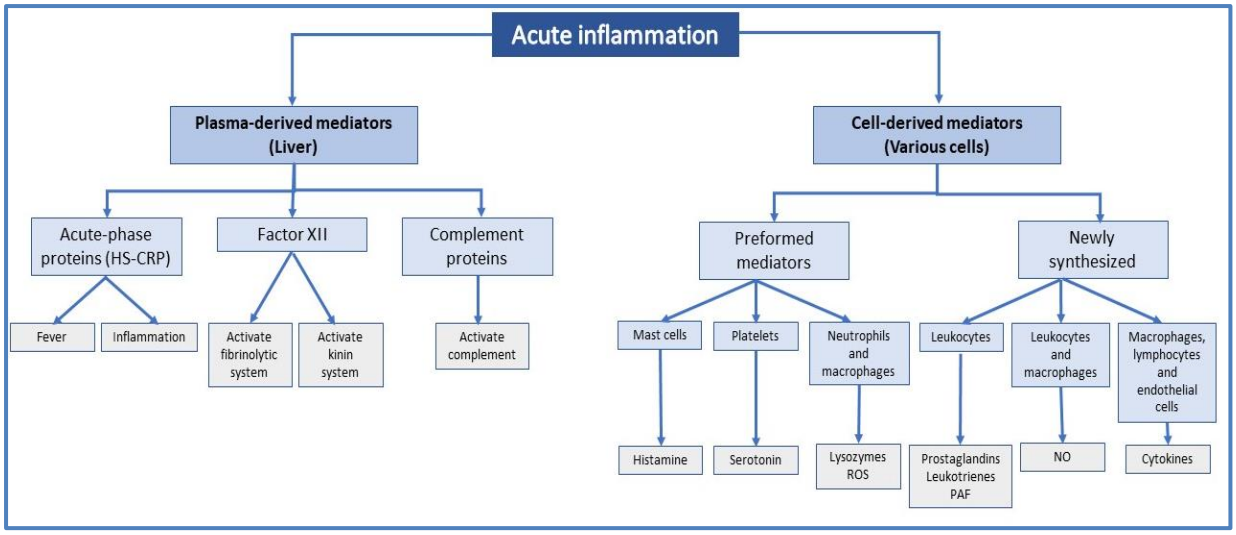


Figure 6: Mediators of acute inflammation. Adapted from Porth (2015)

2.5.2.1 Cytokines

a) Interleukin-1

Interleukin-1 is a proinflammatory mediator of acute and chronic inflammation that activates the endothelium during inflammation and increases TNF-α, CAM and IL-6 production. Membrane bound interleukin-1 alpha (IL-1α) and circulating IL-1β both bind to type 1 IL-1 receptor (Ridker 2016). The production of IL-1 is regulated by interleukin-1 receptor antagonist (IL-1RA). The precursor IL-1α is 31 kilodalton (kDa) and is biologically active when uncleaved. Once cleaved by proteases elastase, granzyme B and mast cell chymase the activity of IL-1α drastically increases. Precursor IL-1β is 31kDa in size and requires proteolytic activity of caspase-1. After cleavage into 17 kDa active IL-1β, it can bind to IL-1 receptor (Garlanda et al. 2013; Afonina et al. 2015).

b) Interleukin-6

Proinflammatory cytokine, IL-6 is produced by the liver. In turn, IL-6 stimulates the production of CRP (Ridker 2016). IL-6 is discussed in more detail in 2.8.

c) Interleukin-10

Interleukin-10 is an anti-inflammatory cytokine. IL-10 decreases the intensity of inflammation by decreasing oxidative stress, matrix metalloprotease and necrosis. It promotes inflammatory

resolution by increasing the fibrous cap and efferocytosis (Kinzenbaw et al. 2013; Fredman & Tabas 2017).

d) Tumour necrosis factor-α

The pro-inflammatory cytokine TNF-α is expressed by macrophages, lymphocytes, mast cells, ECs, fibroblasts and neurons (Slebioda & Kmiec 2014). TNF-α will be discussed in more detail where.

2.5.2.2 High Sensitivity C-Reactive Protein

The acute phase protein HS-CRP is produced by the liver in response to tissue injury and inflammation (Braig et al. 2017). This non-specific inflammatory marker will be discussed in further detail in 2.6.

2.5.2.3 Complement

The complement system is part of the innate immunity and consists of a cascade of proteins which are activated to cause cell lysis (Merle et al. 2015b). Complement proteins C1-C9 are always present in an inactive state in plasma. The complement cascade can be activated via the classical pathway, alternative pathway and lectin pathway. Antibodies bound to antigen activate complement via the classical pathway as part of the humoral immune system. The alternative pathway is activated by exposure to microbial cell surface antigens in the absence of antibodies. Lectin-bound microbial mannose activates the lectin pathway which in turn activates the classical complement pathway (Merle et al. 2015a; Porth 2015).

Activation of either of these pathways results in the cleavage of complement protein C3 to C3b and C3a proteins. Complement protein C3a is a chemoattractant which recruits' neutrophil whereas C3b opsonizes the microbe for phagocytosis. Complement protein C5 is cleaved by the C3b enzyme complex. The resultant C5a fragments recruits more neutrophils and activates the vascular phase of acute inflammation. The fragment C5b remains bound to the microbe and initiates the membrane attachment phase of the complement cascade as illustrated in Figure 7. Activated complement proteins C6, C7, C8 and C9 form a pore in the microbe allowing fluid to enter and lyses it (Hovland et al. 2015; Porth 2015).

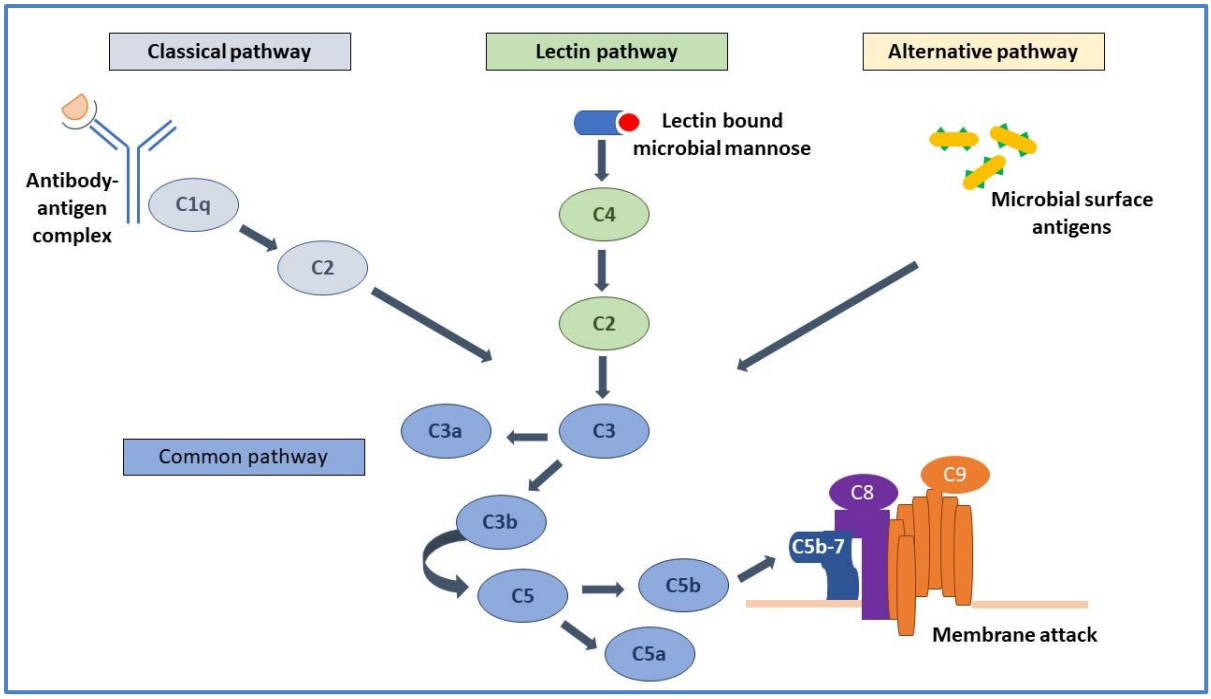


Figure 7: Complement system. Adapted from Hovland et al. (2015); Merle et al. (2015a)

2.5.2.4 Factor XII (Hageman factor)

Coagulation factor XII is also known as the Hageman factor. It is an 80kDa single chain protease, consisting of a heavy and light chain linked by a disulphide bond. Upon contact with anion surfaces, it is activated into a two-chain form. Once activated, Hageman factor initiates the intrinsic coagulation pathway, converts plasma prekallikrein to kallikrein and releases bradykinin from high molecular weight kininogen (Lin et al. 2017; Didiasova et al. 2018).

2.5.2.5 Bradykinin

The vasoactive peptide bradykinin is an end-product of the intrinsic coagulation system and is produced via the kinin system. The main function of this peptide is its hypotensive action. Factor VII (FVII) is activated when it binds with kaolin, FVIIa then activates plasma prekallikrein (PPK). In turn PPK cleaves bradykinin from high molecular weight kininogen (HK). The process of bradykinin formation is linked to the coagulation system through factor XI (Hofman et al. 2016; Anton et al. 2018).

2.5.2.6 Serotonin

Serotonin is a vasoactive amine with the chemical composition 5-hydroxytryptamine. Tryptophan is hydroxylated by tryptophan hydroxylase and further decarboxylated by aromatic acid decarboxylase to form serotonin. There are two pools of serotonin; 5% of the total body serotonin is synthesized in the brain and serves as a neurotransmitter (Tonello et al. 2015). The remaining 95% is produced by cells of peripheral organs, such as enterochromaffin cells of the gut, pancreatic β cells, adipocytes and osteoclasts. Peripheral serotonin regulates pancreatic β cell, liver and adipocyte metabolism. It is also involved in neutrophil and macrocyte recruitment, increased vascular permeability and cytokine production during inflammation (Gros et al. 2014; El-Merahbi et al. 2015).

2.5.2.7 Histamine

Histamine is a vasoactive amine derived from histidine and known for its role in acute allergic inflammation. It is released upon degranulation of platelets, basophils and mainly mast cells. Histamine causes vasodilation and increased vascular permeability (Hofman et al. 2016). Histamine binds to numerous target cells via the surface receptors H1 and H2 (Ashina et al. 2015; Panula et al. 2015).

2.5.2.8 Nitric oxide

Nitric oxide is a critical signalling antioxidant molecule produced by leukocytes, macrophages and ECs. NO plays a role in blood flow and pressure, platelet inhibition and inflammation. This anti-inflammatory role is achieved through relaxation of VSMCs, inhibition of VSMC proliferation, inhibition of platelet activation and aggregation, and inhibition of cell adhesion and migration (Douglas & Channon 2014; Shrivastava et al. 2015a). NO is produced by eNOS activity on L-arginine converting it to citrulline, in response to chemical and mechanical stimuli. The enzyme eNOS is mainly expressed by ECs upon exposure to shear stress, bradykinin and acetylcholine (Park & Park 2015; Forstermann et al. 2017). Oxidative stress and ROS uncouple eNOS, which consequently becomes a ROS producer (Park & Park 2015).

2.5.2.9 Reactive oxygen species (ROS)

Reactive oxygen species is defined as oxygen containing reactive molecules such as superoxide, hydrogen peroxide, peroxynitrite and the hydroxyl radical. These metabolites are capable of oxidizing other molecules. The formation of ROS results from normal physiological metabolism and signalling, as well as pathological oxidative processes (Mittal et al. 2014; Brown & Griendling 2015; He & Zuo 2015). The partial reduction of oxygen during aerobic mitochondrial metabolism forms ROS as a by-product. The formation of ROS is also initiated by cytokines, xenobiotics and microbial invasion, during which it acts as a signalling molecule. ROS is abnormally increased during conditions resulting in oxidative stress such as diabetes, atherosclerosis and aging. In these cases, ROS causes damage to proteins, nucleic acids and lipids. ROS can be produced intracellularly in platelets, which play an important role in platelet activation and adherence (Qiao et al. 2018). During the inflammatory response, NADPH oxidases and myeloperoxidases in ECs and phagocytes produce ROS (Mittal et al. 2014).

2.5.2.10 Prostaglandins and leukotrienes

Prostaglandins are produced from the release of arachidonic unsaturated fatty acids from cell membrane phospholipids. When phospholipases release arachidonic acids, prostaglandins are formed via the cyclooxygenase metabolic pathway. The metabolism of arachidonic acids via the lipoxygenase pathway results in the formation of pro-inflammatory leukotrienes. The PGE_2 and PGI_2 are responsible for inducing diapedesis and leukotriene B_4 (LTB_4) and amplifies neutrophil recruitment (Buckley et al. 2014; Porth 2015).Its synthesis is dependent on the amount of available arachidonic acids, NO and ROS intermediates (Viola & Soehnlein 2015).

2.5.2.11 Platelet activating factor (PAF)

The pro-inflammatory and anti-inflammatory PAF is an ether phospholipid, with the most active form being alkyl-PAF (1-alkyl-2-acetyl-sn-glycero-3-phosphocholine) (Chaithra et al. 2018). PAF induces platelet aggregation and degranulation, which in turn stimulates serotonin release and ultimately increases vascular permeability. The action of PAF also results in leukocyte adhesion, chemotaxis, leukocyte degranulation and prostaglandin production. PAF requires platelet activating factor receptor on cell membranes to initiate its action via the G-protein coupled receptors (Gros et al. 2014; Porth 2015; Jacob et al. 2017).

2.5.2.12 Adhesion molecules

a) Selectins

Selectins are membrane glycoproteins with carbohydrate recognition domains, expressed on ECs and leucocytes (Mittal et al. 2014). The transmembrane, Calcium 2+ dependant selectins, E-selectin, L-selectin and P-selectin form the selectin family. L-selectin is expressed by leucocytes, E-selectin is expressed by activated ECs and P-selectin is expressed by both activated platelet α-granules and activated ECs. Selectins are responsible for leucocyte rolling, during which leucocyte ligands reversibly bind to selectins (McEver 2015; Silva-Herdade et al. 2016).

b) Integrins

The integrin family consists of almost 30 transmembrane receptor proteins that promote cell-to-cell and cell-to-extracellular matrix interaction. They are heterodimers of α- and β-subunits which are non-covalently bound. Blood cell integrins require activation before they can bind with ligands. Selectin-mediated rolling is slowed down even further through short-lived bonds between leucocyte integrins and their ligands such as ICAM and VCAM (Nourshargh & Alon 2014; McEver 2015). Adherence of the β_2-integrins on leucocytes with ICAM-1 on ECs facilitate transendothelial migration of leucocytes (Mittal et al. 2014).

2.5.2.13 Specialized pro-resolving mediators (SPMs)

Specialized pro-resolving mediators are derived from poly unsaturated fatty acids, which enhance the resolution of inflammation through efferocytosis. Lipoxins are derived from arachidonic acids and E-resolvins are derived from eicosapentaenoic acid (EPA). Maresins, protectins and D-resolvins are derived from docoshexaenoic acid (DHA) (Buckley et al. 2014; Fredman & Spite 2017).

Other than the common function of promoting efferocytosis, SMPs have various other functions which contribute to inflammatory resolution. Resolvins act on neutrophils, macrophages and epithelial cells to reduce neutrophil adhesion, ROS generation, pro-inflammatory cytokine production, leucocyte transmigration and organ fibrosis. Resolvins also increase neutrophil apoptosis, phagocytosis, microbial killing IL-10 production and tissue regeneration (Chiang & Serhan 2017; Fredman & Spite 2017; Heinz et al. 2017). Lipoxins act on neutrophils, monocytes and macrophages to increase phagocytosis, nonphlogistic

monocyte recruitment and IL-10 production. Neutrophil adhesion and tissue damage are also reduced through the action of lipoxins (Buckley et al. 2014; Serhan 2017).

Maresins regulate neutrophil infiltration, reduce pain and increase tissue regeneration and wound healing through action on neutrophils, monocytes and macrophages (Serhan 2014). Protectins are primarily neuroprotective, targeting T cells and microglial cells. However, protectins also regulate T-cell and neutrophil infiltration, reduce TNF-α production, reduce renal fibrosis and reduce retinal pigmentation (Buckley et al. 2014; Chiang & Serhan 2017; Serhan 2017).

2.5.3 Inflammation and aging

The link between inflammation and aging has been studied for many years with new concepts evolving as research developed. The network theory of aging was proposed in 1989. It generally suggested that aging is indirectly controlled by a network of cellular and molecular defence mechanisms (Franceschi 1989). This theory evolved from the evolution of aging theory which proposed that mortality results from reduced error regulation in somatic cells and new data on cellular and biological aging (Kirkwood 1977; Franceschi et al. 2000b). In summary, the theory proposed that cells cope with stressors through various mechanisms, such as DNA repair, antioxidant defence, and enzyme action among others. A fault in any of these integrated mechanisms will lead to defective homeostasis. Further studies on human immune-senescence and the new model of healthy centenarians led to the development of the remodelling theory of aging in 1995. Some immune components decrease, some increase and some remain the same during aging, thus concluding that the body is continuously adapting to deterioration (Franceschi & Cossarizza 1995; Franceschi et al. 1995; Franceschi et al. 2000a).

The “inflamm-aging/ inflammaging” phenomenon was introduced in 2000 and is considered an extension of the network theory of aging and remodelling theory of aging. Inflammaging refers to the chronic, low-grade inflammatory state as a result of physiological changes associated with aging (Franceschi et al. 2000a; Franceschi & Campisi 2014). Current research is focused on genetic mutations linked to inflammaging, biomarkers of inflammaging and the impact of diet and exercise on prevention of inflammaging (Frank & Caceres 2015; Das 2017).

2.5.4 Inflammation and cardiovascular disease

The role of inflammation in atherosclerosis and destabilization of plaque has been widely accepted (Taleb 2016; Fredman & Tabas 2017). Atherosclerosis results from chronic inflammation, triggered by vessel injury and endothelial dysfunction as discussed in 2.3 (Minihane et al. 2015; Shrivastava et al. 2015b; Fredman & Spite 2017).

2.6 High Sensitivity C-Reactive Protein

With inflammation playing a key role in the development of atherosclerosis, destabilization of plague and rupture of the plague, inflammatory biomarkers have become a focal point of investigation. Studies of these inflammatory biomarkers aim to improve risk determination and treatment stratification of CVD. Of these inflammatory biomarkers, CRP has been the most commonly studied in association with CVD. CRP is a non-specific inflammatory marker (Shrivastava et al. 2015b).

CRP was discovered by Tillet and Francis in the 1930s, in a study on patients with acute *Streptococcus pneumonia* infection. The name CRP originated from its ability as a protein to react with and precipitate phosphorylcholine residues of the C polysaccharide derived from the teichoic acid in the cell wall of *Streptococcus pneumoniae.* It was also found to be able to precipitate calcium ions (Tillett 1930; Salazar et al. 2014).

In the 1940s, Avery and McCarthy described CRP as an acute phase reactant which was increased in the serum of patients with various inflammatory conditions. Ridker et al. (1997) indicated the presence of an association between increased levels of CRP and CVD. With this revitalized interest of CRP associated with CVD in the 1990s, the immunoassay for HS-CRP was developed, which allowed the analysis of CRP levels with greater sensitivity. This led to the discovery that even slight increases in CRP, within the ranges which were previously thought to be normal, were associated with the risk of developing CVD (Shrivastava et al. 2015b).

2.6.1 Physical characteristics

C-reactive protein is a member of the pentraxin family of calcium dependant, ligand-binding plasma proteins. Pentraxins characteristically have 5 identical non-glycosylated globular subunits or protomers. These are noncovalently associated, forming a symmetric cyclic pattern around a central pore which gives these proteins their pentameric, discoidal, and flattened doughnut configuration (Salazar et al. 2014). Each subunit of the pentameric CRP (pCRP) molecule weighs approximately 23 kDa (Braig et al. 2017).

Each protomer is made up of 2 anti-parallel β-pleated sheets and has the flattened beta-jellyroll lectin fold. The binding face, B face or recognition face of each protomer has a phosphocholine binding site, which is concave. This ligand-binding site contains two calcium ions adjacent to the hydrophobic pocket which are 4 Å apart, bound by protein carboxylate and amide side chains. The residues phenylalanine-66 (Phe-66) and glutamic acid-81 (Glu-81) are responsible for binding phosphocholine. The positively charged nitrogen of phosphocholine head interacts with Glu-81, while the methyl groups on the tail hydrophobically interact with Phe-66 on the opposite side of the pocket (Pepys & Hirschfield 2003; Black et al. 2004; Du Clos 2013; Shrivastava et al. 2015b). These characteristics allow the B face to bind to damaged or apoptotic cells and bacterial cell walls (Braig et al. 2017).

The effector face or A face carries a single α-helix and has the ability to initiate fluid-phase pathways of host defence and cell-mediated immunity pathways. The globular recognition domain of complement, C1q binds at this site and activates the classical complement pathway up to level C3 convertase. This site also serves as receptors for the Fc portion of immunoglobulin gamma (Fcγ). The residues aspartic acid-112 (Asp-112) and tyrosine-175 (Tyr-175) along the groove leading from the centre of the protomer to the central pore are vital for binding C1q. (Black et al. 2004; Di Napoli et al. 2011; Chandrashekara 2014; Thiele et al. 2015; Braig et al. 2017).

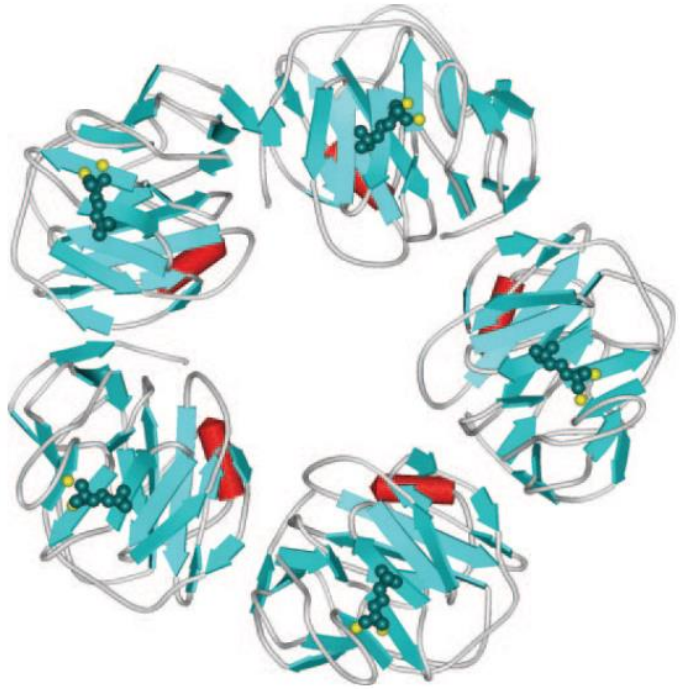


Figure 8: Structure of c-reactive protein (Black et al. 2004)

C-reactive protein is made up of 206 amino acids and is present in pentameric or monomeric form. The freely soluble pCRP as illustrated in Figure 8, has pro- and anti-inflammatory properties and is easily quantifiable in plasma. pCRP can undergo spontaneous and irreversible conformation to monomeric CRP (mCRP), which is found in associated with particles or in tissues such as atheromas. The structure changes from a β-sheet to a less soluble α-helix and no longer binds to phosphocholine. Aggregates of mCRP form matrix-like lattices in tissues. (Di Napoli et al. 2011; Shrivastava et al. 2015b; Braig et al. 2017). The biologically active mCRP is not freely soluble, it can be detected in plasma as a complex with lipid containing particles and has powerful pro-inflammatory properties (Trial et al. 2016).

2.6.2 Biochemical metabolism

The majority of CRP found in circulation is synthesized in the liver during the transcriptional phase, regulated by proinflammatory cytokines in response to tissue injury and inflammation. The main regulator of CRP synthesis is IL-6, which upregulates the transcription factors Cytidine-Cytidine-Adenosine-Adenosine-Thymidine (CCAAT)/ enhancer-binding protein β (C/EBPβ) and CCAAT enhancer-binding protein δ (C/EBPδ). IL-1β and TNF also increase the rate of CRP transcription (Salazar et al. 2014; Thiele et al. 2015). The CRP gene is located on long arm of chromosome 1, position 23.2 (1q23.2) (Floyd-Smith et al. 1986). CRP has also been found to be expressed in adipocytes in response to proinflammatory mediators. Visceral

adipose tissue is the underlying inflammatory component of obesity, promoting inflammation by releasing IL-1β and IL-6 which contribute to CRP production (Sadashiv et al. 2015).
Other sites where messenger ribonucleic acid (mRNA) of CRP have been found include neurons, epithelial cells of the lungs, epithelial cells of renal cortical tubules, Kupffer cells, lymphocytes and both macrophages and smooth muscle cells of atherosclerotic lesions. These extrahepatic sites of CRP production are thought to be the sources of sustained low levels of CRP, which are implicated in CVR (Chandrashekara 2014; Salazar et al. 2014).

The acute phase protein CRP is produced as a pentamer in the liver and secreted into circulation. Exposure to conditions that separate the subunits of the pCRP, results in the formation of mCRP. Bioactive lipids encourage the conformational rearrangement to mCRP, which cannot reassemble to pCRP. The two structurally and functionally different forms of CRP have different epitopes for antibody recognition (Thiele et al. 2015; Trial et al. 2016).

Small soluble bioactive peptides resultant from CRP degradation constrain the pro-inflammatory and tissue destructive abilities of neutrophils. CRP residues have different functions; CRP residue 201-206 (CRP-III), 83-90 (CRP-IV) and 77-82 (CRP-V) from the intact protein act additively to inhibit superoxide production from activated neutrophils at 50µM. CRP-III and CRP-V have been indicated to prevent neutrophil chemotaxis. CRP residue 174-185 and CRP-III activates neutrophils to shed the IL-6 receptors, reduces neutrophil attachment and decreases L-selectin expression (Chandrashekara 2014).

Serum CRP levels increase significantly 6-8 hours after the initial stimulation and peaks at 24-48 hours. The concentration of CRP in the serum is determined by the rate of production, it can increase by 1000-fold or more during injury or inflammation. The half-life of CRP is approximately 19 hours (Chandrashekara 2014; Shrivastava et al. 2015b).

2.6.3 Physiology

The major function of CRP is to protect the body against foreign pathogens through promoting agglutination, activating the classical complement pathway, causing bacterial capsular swelling, and promoting the phagocytosis and precipitation of polycationic and polyanionic molecules (Shrivastava et al. 2015b). In this response to infectious organisms, CRP plays a role in innate immune. CRP is also involved in inflammation caused by sterile tissue damage (Trial et al. 2016).

C-reactive protein binds with the highest affinity to the ligand phosphocholine. It also binds to autologous ligands such as native and modified plasma lipoproteins, damaged cell membranes, various phospholipids, small ribonucleic particles and apoptotic cells. Extrinsic ligands to which CRP binds include elements of bacteria, fungi, parasites and plants such as glycan, phospholipids, as well as capsular and somatic components (Shrivastava et al. 2015b). CRP also binds to macromolecular ligands such as phosphor-ethanolamine, chromatin, histones, fibronectin, small nuclear ribonucleoproteins, laminin and polycations (Chandrashekara 2014).

The role of CRP:

Activation of complement

The innate immunity is amplified and facilitated by CRP through activation of the classical complement pathway through binding of C1q (Shrivastava et al. 2015b). Though CRP plays a crucial role in the acute phase response and acts as a regulator of the innate immunity, it has been associated with various chronic inflammatory conditions (Salazar et al. 2014).

Immune cell recruitment and activation

C-reactive protein promotes adhesion molecule expression in ECs, which attract monocytes to the vessel injury site. Macrophages are activated by CRP to secrete the pro-coagulant tissue factor (Shrivastava et al. 2015b). Alveolar macrophages and blood mononuclear cells are stimulated by CRP to produce IL-1α, IL-1β, TNF-α and IL-6. ECs are triggered to express adhesion molecules, chemokines and cytokines (Chandrashekara 2014).

Fibrinolysis

Plasminogen activator inhibitor-1 is a protease inhibitor which regulates fibrinolysis. CRP promotes PAI-1 expression and activity resulting in decreased fibrinolysis and ultimately, atherosclerosis (Shrivastava et al. 2015b).

Activation of metalloproteases

The release of matrix metalloprotease-1 is promoted by CRP (Chandrashekara 2014). Metalloproteases decrease the thickness of plaque fibrous cap, increasing the probability of plaque rupture (Tesauro et al. 2017).

Nitric oxide synthesis

The expression of NO synthase in EC is inhibited by CRP. Decreased NO results in decreased anti-atherogenic reactions which decrease platelet aggregation, vasoconstriction and VSMC proliferation (Shrivastava et al. 2015b).

Lipoprotein interaction

C-reactive protein binds to oxidized phospholipids and lipids play a vital role in the dissociation of pCRP to mCRP (Trial et al. 2016). It also mediates the uptake of LDL into macrophages (Chandrashekara 2014).

2.6.4 Methods of detecting serum HS-CRP

Assays for HS-CRP make the use thereof for clinical diagnosis, management and treatment of CVD simple and relatively inexpensive (Salazar et al. 2014). These assays have become widely available commercially and are reliable and reproducible. The similarity of results from fresh, frozen, or stored plasma demonstrates that CRP is a stable and robust clinical marker (Shrivastava et al. 2015b). These assays measure soluble pCRP (Trial et al. 2016). CRP can be detected with direct immunoturbidimetric or immunonephelometric assays. However, the detection limit for these assays are not low enough to accurately measure concentrations of CRP less than 0.3 milligram per litre (mg/L), known as HS-CRP. For the measurement of HS-CRP concentrations, particle-enhanced assays or sandwich immunoassays are used (Rifai et al. 2018).

2.6.4.1 Enzyme linked immunosorbent assay (ELISA)

a) Principle

An antibody is adsorbed onto a solid phase such as a microtiter well. If the sample that is added contains the corresponding antigen, such as CRP, it will bind to the solid phase antibody. The mixture is washed, leaving only the complexes on the solid phase. An antibody (also to CRP) labelled with an enzyme is then added, upon binding it forms a “sandwich-complex” of antibody-antigen-antibody. The mixture is washed once again to remove unbound antibodies. An enzyme substrate is added to react with the enzyme-labelled antibody producing a coloured product. The intensity of the coloured product is measured through

colourimetry and is proportional to the concentration of the analyte in the sample. Standards are used to draw a curve and the concentration of the samples are read from the curve (Nimse et al. 2016; Rifai et al. 2018).

b) Advantages

Highly sensitive, allow detection of target antigen or antibody at low concentrations (Albrecht et al. 2008; Gan & Patel 2013).

c) Disadvantages

Expensive and only well-equipped laboratories have ELISA automation. It has a long turn-around time. The method requires trained and competent technologists to prevent false positive results due to prolonged colorimetric reaction by enzymes if the method is not adhered to. In order to detect an antigen, an antibody to the target antigen has to be available for the solid phase and vice versa. Nonspecific antigen or antibodies binding to the plate may lead to false high or positive results (Albrecht et al. 2008; Gan & Patel 2013).

2.6.4.2 Immunonephelometry

a) Principle

The principle of nephelometry is based on the measurement of light scattered at a fixed angle. The reagent contains polystyrene particles coated with monoclonal anti-human CRP antibodies. When samples containing CRP are mixed with the reagent the antibody coated particles and CRP bind to form aggregates of polystyrene particles. A beam of light passed through the sample will scatter as a result of these aggregates. The intensity of scattered light is directly proportional to the concentration of CRP in the analysed sample. A standard curve is drawn using known standards, and the concentration of the samples are read from the curve (Ziv-Baran et al. 2017).

b) Advantages

In general nephelometric methods are more sensitive than turbidimetry, detecting lower concentrations of the target protein (Burtis & Bruns 2015).

c) Disadvantages

Immunonephelometric assays are performed on specialized instruments (Ariyurek 2012).

2.6.4.3 Immunoturbidimetry

a) Principle

When latex particles coated with anti-human CRP antibodies are mixed with a sample containing CRP, latex aggregates will form. The latex aggregates increase the turbidity of the sample. A beam of light passes through the sample and a detector measures the absorbance of light by the sample. Standards are measured and used to draw a curve from which samples are read to determine the concentration (Ziv-Baran et al. 2017).

b) Advantages

The addition of latex particles lowers the detection limit of the assay (Burtis & Bruns 2015). Immunoturbidimetric assays are more readily available on general chemistry analysers than immunonephelometric assays. The availability of automated immunoturbidimetric assays reduces turn-around time and allows high volume testing (Ariyurek 2012).

c) Disadvantages

The method is less sensitive than nephelometric assays if it is not particle-enhanced (Burtis & Bruns 2015).

2.6.5 Increased serum HS-CRP

C-reactive protein has been widely accepted and used as a diagnostic marker for inflammation. When detecting levels below 5 mg/L it is termed HS-CRP (Ziv-Baran et al. 2017). Increased levels of CRP indicate the presence of inflammation but does not specify the cause of inflammation (Salazar et al. 2014; Ziv-Baran et al. 2017). Elevated levels of CRP are consequently associated with various inflammatory conditions such as atherosclerosis, prediabetes, type II diabetes mellitus, obesity (BMI), hypertension, hyperlipidaemia, metabolic syndrome, infections, smoking, coffee consumption, oral contraceptive use, infections in new-borns (CRP does not cross the placenta), poor prognosis in some cancers, physical stress, systemic inflammatory conditions such as rheumatic disease, and to a lesser extent single

nucleotide polymorphisms in CRP genes (Aguiar et al. 2013; Chandrashekara 2014; Halcox et al. 2014; Phosat et al. 2017).

2.6.6 HS-CRP as a cardiovascular risk marker

CRP undeniably contributes to CVD through its involvement in atherosclerosis formation. CRP plays a role in atherosclerosis formation through various mechanisms such as activation of the complement cascade, mediation of lipid uptake by macrophages, increased secretion of pro-inflammatory molecules and expression of tissue factor in monocytes, promotion of endothelial dysfunction and hindering the production of NO (Shrivastava et al. 2015b).
Elevated levels of CRP have been associated with numerous non-communicable diseases including insulin resistance, hypertension and metabolic syndrome. However, its role in CVD has been of most interest. Several studies have demonstrated HS-CRP is an independent marker of CVR in both sexes, across a wide age range, in diverse ethnic backgrounds and various clinical settings. An increasing number of studies suggest that elevated levels of HS-CRP, in addition to risk determination of asymptomatic patients, also predict a poor prognosis in patients with symptomatic CVD (Greenland et al. 2010). Recent studies have indicated that CRP levels respond to the management or elimination of CVR factors, such as exercising and losing weight, controlling diabetes and cessation of smoking. Therefor CRP can be useful in determining treatment compliance (Chandrashekara 2014; Salazar et al. 2014; Shrivastava et al. 2015b; Ziv-Baran et al. 2017).

2.7 Tumour Necrosis Factor Alpha

During research on haemorrhagic necrosis of tumour cells by gram-negative bacteria Carswell et al. (1975) discovered a substance is released by the host in response to the bacterial endotoxin which is responsible for causing necrosis of tumour cells, rather than the endotoxin itself. The substance was found to be selectively toxic to malignant cells and was consequently named tumour necrosis factor. This endorsed the research done by Algire et al. (1952) which concluded that the endotoxin indirectly kills tumour cells but disproved that tumour cell death is caused by hypotension and haemostasis as a result of endotoxin. Aggarwal et al. (1985) went on to clone, purify and describe the characteristics of TNF-α and TNF-β (initially lymphotoxin-α).

Tumour necrosis factor alpha forms part of the TNF superfamily, consisting of 19 ligands and 29 receptors as outlined in Table 4. Receptors can be further classified into receptors that do and do not have the intracellular death domain (DD). All members of the TNF superfamily play

a pro-inflammatory role, some also play a role in haematopoietic proliferation, differentiation and apoptosis. TNF-α has a molecular mass of 17 kDa and TNF-β is a protein of 25 kDa. Though these proteins share 50% similarity in amino acid make-up, they have been proven to be two different proteins with immunological reactions. Another differentiating characteristic is that TNF-α is mainly produced by macrophages and tumour necrosis factor- β (TNF-β) by lymphocytes. The rest of the tumour necrosis factor superfamily (TNFSF) were identified genetically first, unlike TNF-α and TNF-β were the proteins were isolated first (Kelker et al. 1985; Aggarwal et al. 2012).

2.7.1 Physical characteristics

The pleiotropic, pro-inflammatory cytokine TNF-α is made up of 233 amino acids (Wang et al. 2015). It is a glycoprotein with three identical polypeptide subunits, which combine after exposure to the surface of the cell membrane. Trimeric TNF-α is biologically active in its transmembrane or soluble form (Blandizzi et al. 2014; Olmos & Llado 2014). The transmembrane form of the newly synthesized TNF-α is 27 kDa in size and may be cleaved to soluble TNF-α of 17 kDa. This soluble form of TNF-α consists of two antiparallel β-pleated sheets with antiparallel β-strands that form a jelly-roll β-structure as seen in Figure 9 (Parameswaran & Patial 2010).

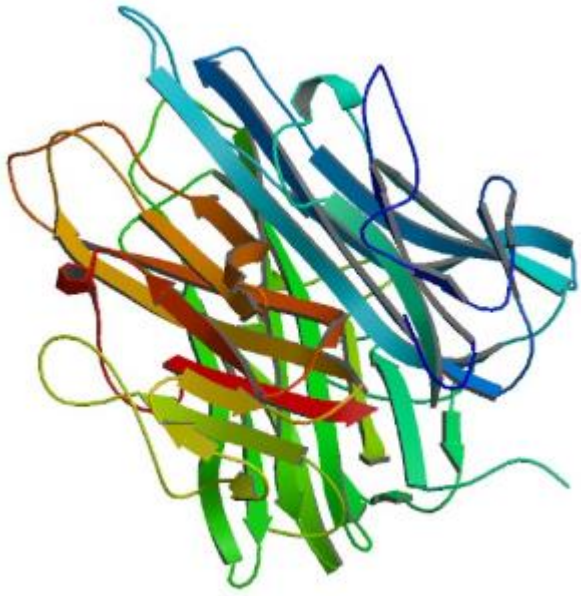


Figure 9: Structure of TNF-α (Eck & Sprang 1989)

Table 4: Tumour necrosis factor superfamily. Adopted from Aggarwal et al. (2012)

Ligand	Ligand name	Symbol	Expressed by	Receptor	Receptor name	Symbol	Expressed by	DD
TNF-β	Tumour necrosis factor β	TNFSF1	Natural killer (NK) cells T cells B cells	**TNFR1 (DR1)**	Tumour necrosis factor receptor 1	TNFRSF1A	Haematopoietic and immune cells	Yes
				TNFR2	Tumour necrosis factor receptor 2	TNFRSF1B	Immune and ECs	
TNF-α	Tumour necrosis factor α	TNFSF2	Macrophages NK cells T cells B cells	**TNFR1**	Tumour necrosis factor receptor 1	TNFRSF1A	Immune and ECs	Yes
				TNFR2	Tumour necrosis factor receptor 2	TNFRSF1B	Immune and ECs	
LT-β	Lymphotoxin β	TNFSF3	Activated $CD4^+$ T cells Dendritic cells NK cell	**LT-βR**		TNFRSF3	NK cells $CD4^+$ T cells $CD8^+$ T cells	

Table 4: continue

OX40L	OX40 ligand	TNFSF4	B cells T cells Dendritic cells ECs Smooth muscle cells	**OX40**		TNFRSF4	Activated $CD4^+$ T cells Neutrophils	
CD40L		TNFSF5	Activated $CD4^+$ T cells NK cells Mast cells Basophils Eosinophils	**CD40**		TNFRSF5	B cells Monocytes Dendritic cells Thymic epithelium Reed-Sternberg cells	
FasL	Fibroblast-associated ligand	TNFSF6	Activated splenocytes Thymocytes Nonlymphoid tissues NK cells	**Fas (DR2)**	Fibroblast-associated	TNFRSF6	Epithelial cells Hepatocytes Activated mature lymphocytes	Yes
				DcR3		TNFRSF6B	Lung cells Colon cells	

Table 4: continue

CD27L		TNFSF7	NK cells T cells B cells Mast cells Smooth muscle cells Thymic epithelial cells	**CD27**		TNFRSF7	Haematopoietic progenitor cell $CD4^-$ T cells $CD8^-$ T cells	
CD30L		TNFSF8	Activated T cells B cells Monocytes Granulocytes Medullary thymic epithelial cells	**CD30**		TNFRSF8	Reed-Sternberg cells	
4-1BBL		TNFSF9	B cells Macrophages Dendritic cells	**4-1BB**		TNFRSF9	T cells NK cells Mast cells Neutrophils	

Table 4: continue

TRAIL	Tumour necrosis factor related apoptosis-inducing ligand	TNFSF10	NK cells T cells	**TRAILR1 (DR4)**	Tumour necrosis factor related apoptosis-inducing receptor 1	TNFRSF10 A	Most normal and transformed cells	Yes
			Dendritic cells	**TRAILR2 (DR5)**	Tumour necrosis factor related apoptosis-inducing receptor 2	TNFRSF10 B	Most normal and transformed cells	Yes
				TRAILR3	Tumour necrosis factor related apoptosis-inducing receptor 3	TNFRSF10 C	Most normal and transformed cells	

Table 4: continue

				TRAILR4	Tumour necrosis factor related apoptosis-inducing receptor 4	TNFRSF10D	Most normal and transformed cells	
				OPG	Osteoclast differentiating factor	TNFRSF11B	Most normal and transformed cells	
RANKL	Receptor activator of NF-κB ligand	TNFSF11	T cells Thymic cells Lymph nodes	**RANK**	Receptor activator of NF-κB	TNFRSF11A	Osteoclasts Osteoblasts Activated T cells	
				OPG	Osteoclast differentiating factor	TNFRSF11B	Osteoclast precursors ECs	
TWEAK	Tumour necrosis factor related weak inducer of apoptosis	TNFSF12	Monocytes	**TWEAKR**	Tumour necrosis factor related weak inducer of apoptosis receptor	TNFRSF12A	ECs Fibroblasts	

Table 4: continue

APRIL	A proliferation inducing ligand	TNFSF13	Macrophages Lymphoid cells Tumour cells	**BCMA**		TNFRSF13 A/17	B cells Peripheral T cells Spleen Thymus Lymph nodes Liver Adrenals	
				TACI		TNFRSF13 B	B cells Activated T cells Peripheral T cells Spleen Thymus Small intestine	
BAFF	B-cell activating factor	TNFSF13 B	T cells Monocytes Macrophages Dendritic cells	**TACI**		TNFRSF13 B	B cells Activated T cells Peripheral T cells Spleen Thymus Small intestine	

Table 4: continue

				BAFFR		TNFRSF13C	B cells T cells Spleen Thymus Small intestine	
				BCMA		TNFRSF17	B cells T cells Spleen Lymph nodes	
LIGHT		TNFSF14	T cells Granulocytes Monocytes Dendritic cells	**LIGHTR**		TNFRSF14	T cells B cells Monocytes Lymphoid cells	
				LT-βr		TNFRSF3	Nonlymphoid haematopoietic cells Stromal cells	
VEGI	Vascular endothelial cell-growth inhibitor	TNFSF15	Epithelial cells	**DR3**		TNFRSF25	NK cells $CD4^+$ T cells $CD8^+$ T cells	Yes
			B cells Macrophages Dendritic cells	**DcR3**		TNFRSF6B	Activated T cells	

Table 4: continue

GITRL	Glucocorticoid-induced	TNFSF18	Human umbilical vein cells	**GITR**		TNFRSF18	$CD4^+CD25^+$ T cells	
EDA-A1	Ectodysplasin A-A1		Skin	**EDAR**			Ectodermal derivative	
EDA-A2	Ectodysplasin A-A2		Skin	**XEDAR**			Ectodermal derivative Embryonic hair follicles	
Not identified yet		TNFSF19		**TROY**		TNFRSF19	Embryo skin Epithelium Hair follicles Brain	
Not identified yet		TNFSF19L		**RELT**		TNFRSF19L	Lymphoid tissues Haematopoietic tissues	
Not identified yet		TNFSF21		**DR6**		TNFRSF21	T cells	Yes
Not identified yet		TNFSF16		**NGFR**		TNFRSF16	Neuronal axons Schwann cells Perineural cells	

2.7.2 Expression and signalling

Monocytes or macrophages are the main producers of TNF-α (Wang et al. 2015; Hu et al. 2017). TNF-α is also produced to a lesser degree in lymphocytes, natural killer cells, mast cells glomerular mesangial cells, astrocytes, fibroblasts and microglial cells of the brain, and Kupffer's cells of the liver (Slebioda & Kmiec 2014; Hu et al. 2017). Several transcription factors stimulate the expression of TNF-α, including interleukin-17 (IL-17), IL-1β, NFκB, nuclear factor activated T cells and microbial lipopolysaccharides (Jovanovic et al. 1998; Parameswaran & Patial 2010; Slebioda & Kmiec 2014). The TNF-α gene is found on the short arm of chromosome 6 at position 21 (6p21) and has a length of 1585 baser pairs (bp) (Wang et al. 2016). TNF-α is expressed as a transmembrane protein when newly synthesized. Transmembrane TNF-α is capable of reverse-signalling, during which the signal is transmitted from the receptor to the cell containing the transmembrane ligand mediating anti-inflammatory activities (Blandizzi et al. 2014). In order to release the soluble homotrimer TNF-α into circulation, the transmembrane ligand is cleaved by the proteolytic enzyme TACE, also known as ADAM17 (Watts et al. 1999; Kalliolias & Ivashkiv 2016). TNF-α binds to two receptors as indicated in Table 4, TNFR1 (p55, CD120a) or TNFR2 (p75, CD120b) (Tartaglia & Goeddel 1992). The death domain is present in the cytoplasmic tail of TNFR1 (Zheng et al. 1995).

Signalling via TNF-α results in the following, as seen in Figure 10:

Activation of NF-κB

To activate NF-κB, TNF-α binds to TNFR1 and recruits TNFR-associated death domain (TRADD), followed by TNFR-associated factor 2 (TRAF2) and receptor interacting protein (RIP) (Hsu et al. 1996; Aggarwal et al. 2012). TGF-β-activated kinase 1 (TAK1) is then recruited, followed by IκK kinase (IKK) complex. Two kinases, IKK1/IKKα and IKK2/IKKβ, and the regulatory protein, NF-κB essential modifier (NEMO) also known as IKKγ make up the IKK complex. The redox sensitive transcription factor NF-κB is bound to the nuclear factor-κBα inhibitor (IκBα) in its inactive form. The IKK complex initiates activation of NF-κB through action on the inhibitor. IκBα undergoes phosphorylation, addition of ubiquitin and degradation by 26S protease. NF-κB is then formed as hetero- or homodimers of proteins p50 and/or p65. Activation of NF-κB by TNF-α results in the regulation of proteins expressed in association with cell survival and proliferation (Tedgui & Mallat 2006; Aggarwal et al. 2012; Brenner et al. 2015).

Activator protein-1 (AP-1)

The transcription factor AP-1 consists of homodimers of protein Jun or heterodimers of Jun (c-Jun, JunB, JunD) and Fos (c-Fos, FosB, Fra-1 and Fra2) proteins (Tedgui & Mallat 2006). AP-1 can be activated by TNF-α through two pathways.

As in 2.8.1.a) above, TNF-α recruits TNFR1, followed by TRADD and TRAF2. Hereafter mitogen activated protein (MAP)/ Extracellular signal-regulated kinase (ERK) kinase kinase 1 (MEKK1) and MAP kinase kinase 7 (MKK7) is recruited. The c-Jun is phosphorylated by c-Jun NH2-terminal kinases (JNKs) which activates AP-1 which in turn activates cellular proliferation (Tedgui & Mallat 2006; Aggarwal et al. 2012). In the second pathway TNF-α recruits TRADD, TRAF2 and TAK1, followed by MAP kinase kinase 3 (MKK3). This results in activation of P38 mitogen-activated protein kinase (p38MAPK) and consequently AP-1 (Aggarwal et al. 2012).

Apoptosis

When TNF-α binds to TNFR1, protein TNFR-associated death domain (TRADD) is recruited through the DD region. Hereafter apoptosis can be achieved through two pathways. In one of the pathways TRADD then recruits Fas-associated protein with death domain (FADD) (Hsu et al. 1996). Caspase-8 and caspase-3 are consequently activated by FADD resulting in apoptosis. In the second pathway TNF-α can signal the release of ROS, cytochrome c and Bax which in turn activates caspase-9 and caspase-3, resulting in apoptosis (Aggarwal et al. 2012; Brenner et al. 2015).

Extracellular signal-regulated kinase

The activation of ERK is through the recruitment of TRADD, TRAF2, RIP, TAK1 and MKK3/6 (Aggarwal et al. 2012).

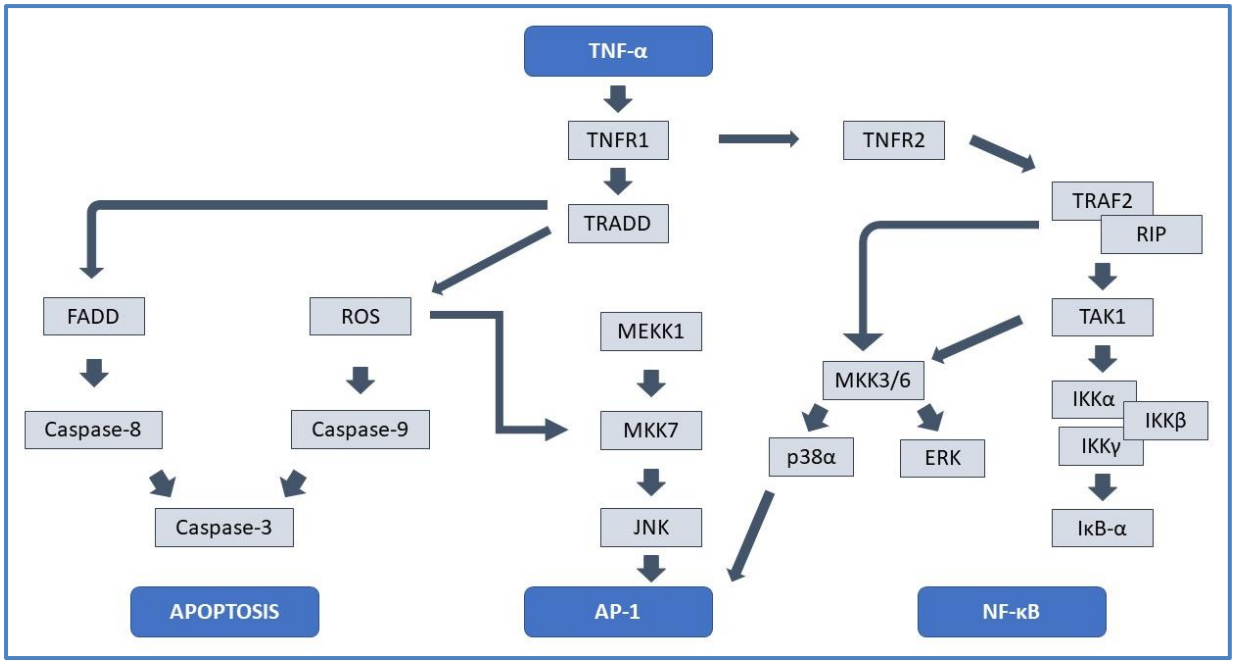


Figure 10: TNF-α signalling. Adapted from Tedgui and Mallat (2006); Aggarwal et al. (2012)

2.7.3 Physiology

The main function of TNF-α is the role it plays in mediating the inflammatory response. TNF-α activates the vascular endothelium and it also stimulates the release of chemokines and adhesion molecules proteion-1α, ICAM-1, VCAM-1 and E-selectin (Kalliolias & Ivashkiv 2016; Hu et al. 2017). Anti-inflammatory activity of TNF-α through reverse signalling inhibits T cell proliferation, inhibits pro-inflammatory cytokine release and mediates apoptosis (Blandizzi et al. 2014).

Through signalling as described in 2.8.1. TNF-α activates caspase-3, AP-1 and NF-κB which results in morphogenesis, differentiation and apoptosis. After priming of macrophages by IFN-γ, extracellular TNF-α can activate macrophages leading to enhanced TNFα-induced NFκB activation (Parameswaran & Patial 2010; Aggarwal et al. 2012; Blandizzi et al. 2014). Through the ERK signalling pathway, TNFα has been shown to enhance the survival of differentiated osteoclasts (Lee et al. 2001; Yu et al. 2018).

Lipid metabolism is altered by TNF-α, as it increases lipolysis in adipocytes, increases free fatty acids and decreases endothelial lipoprotein lipase activity (Pedersen 2017). Exposure of fatty acids to the vascular endothelial leads to endothelial dysfunction and the uptake of oxidized-LDL by macrophages (Chen et al. 2015; Ertunc & Hotamisligil 2016).

Platelets express receptors TNFR1 and TNFR2, when TNF-α binds to these receptors it activates platelets and causes coagulation (Page et al. 2018).

2.7.4 Methods of detecting serum TNF-α

The trusted method for TNF-α analysis is ELISA. An anti-human TNF-α antibody is adsorbed onto a solid phase such as a microtiter well. If the sample that is added contains the corresponding antigen, in this case human TNF-α, it will bind to the solid phase antibody. The mixture is washed, leaving only the complexes on the solid phase. A biotin-conjugated anti-human TNF-α antibody then binds to the TNF-α-antibody complex already formed on the microwell, forming a “sandwich-complex” of antibody-antigen-antibody. The mixture is washed once again to remove unbound antibodies. An enzyme substrate is added to react with the enzyme-labelled antibody producing a coloured product. The colour intensity of the product is measured using a colourimeter and is proportional to the concentration TNF-α in the sample. Standards are measured to draw a curve and the concentration of the samples are read from the curve (Immuno-Biological Laboratories International GmbH 2012b; Nimse et al. 2016; Rifai et al. 2018). As mentioned in 2.6.4.1.a., the ELISA method is highly sensitive, though it is expensive and requires a trained technologist.

2.7.5 Increased serum TNF-α

Although TNF-α was discovered due to its ability of causing tumour cell necrosis, its use as cancer treatment is limited by the side effect of TNF systemic toxicity. On the other hand, TNF-α is associated with cancer due to its ability to activate NF-κB, which enhances the expression of genes that support tumour cell proliferation, invasion, angiogenesis and metastasis and supress tumour cell apoptosis (Aggarwal et al. 2006; Kalliolias & Ivashkiv 2016). Interest in TNF-α has increased in recent years, implicating this pro-inflammatory cytokine in immunologic, cardiovascular, neurologic, pulmonary and metabolic disorders. Increased serum TNF-α levels have been associated with traditional CVR factors (Cui et al. 2012; Brenner et al. 2015). This protein acts on haematopoietic cells through activation of various mitogen-activated kinases, resulting in proliferation of these cells (Hu et al. 2017).

2.7.6 Increased serum TNF-α as a cardiovascular risk marker

Since inflammation mediates the formation of atherosclerosis and TNF-α is a role player of inflammation, TNF-α is involved in the formation of atherosclerosis. TNF-α recruits monocytes to the arterial sub-endothelial, affects lipid metabolism by reducing lipoprotein activity, activates the endothelium to secrete cytokines and enhances further production of TNF-α (Cui et al. 2012; Wang et al. 2015; Kalliolias & Ivashkiv 2016). In chronic inflammation these pro-inflammatory activities of TNF-α are continuously leading to damage of the vasculature, and ultimately resulting in atherosclerosis (Viola & Soehnlein 2015; Yurdagul et al. 2017). Several studies have found an association between increased serum TNF-α levels and CVD (Mendall et al. 1997; McKellar et al. 2009; Cui et al. 2012; Enayati et al. 2015; Pedersen 2017)

2.8 Interleukin-6

Interleukin-6 is a multifunctional pro-inflammatory cytokine, involved in inflammation as well as haematopoiesis. Numerous names have been allocated to IL-6 in the past, each reflecting the physiological activity of the cytokine discovered in the various previous studies (Simpson et al. 1997; Kishimoto & Tanaka 2014; Masjedi et al. 2018). It was initially known as interferon-β2 (INF-β2) as in a study during which Weissenbach et al. (1980) were able to clone its mRNA. The term 26K factor was used to describe it by Haegeman et al. (1986) and was initially only thought to be a similar protein. In this study, the 26K factor was found to have identical stimulatory conditions, molecular size, immunological properties and nucleotide-sequence, but lacked the antiviral activity of INF-β2. The weak antiviral activity raised the opportunity to further investigate the protein's functions, leading to the discovery of the protein's ability to promote the growth of B cell hybridomas and plasmacytomas (Van Damme 1987). It was termed B-cell stimulatory factor (Hirano et al. 1985), hybridoma growth factor and plasmacytoma growth factor (Van Snick et al. 1986). IL-6 was also termed hepatocyte stimulating factor as in studies conducted by Gauldie et al. (1987).

2.8.1 Physical characteristics

Interleukin-6 is a single-chain glycoprotein of 21-30 kDa molecular weight. The molecular weight of IL-6 varies based on the cells from which it was produced and post-translation modifications (May et al. 1988; Simpson et al. 1997; Ataie-Kachoie et al. 2014). It is made up of a bundle of 4 α-helices in an up-up-down-down arrangement, meaning that helices A and B in Figure 11 run in the same direction and helices C and D run in the same direction as seen in Figure 11. The helices are joined together by long loops. There are two shorter helices,

helix S1 and helix S2 found lying outside of the main 4 helix bundle (Somers et al. 1997; Veverka et al. 2012; Ataie-Kachoie et al. 2014). The cytokine IL-6 consists of 184 amino acids and has two potential N-glycosylation sites as well as four cysteine residues (Hirano et al. 1986). IL-6 undergoes modification after translation of the protein, in form of addition of nitrogen (N)- or oxygen (O)-linked glycans (Simpson et al. 1997; Lokau et al. 2017).

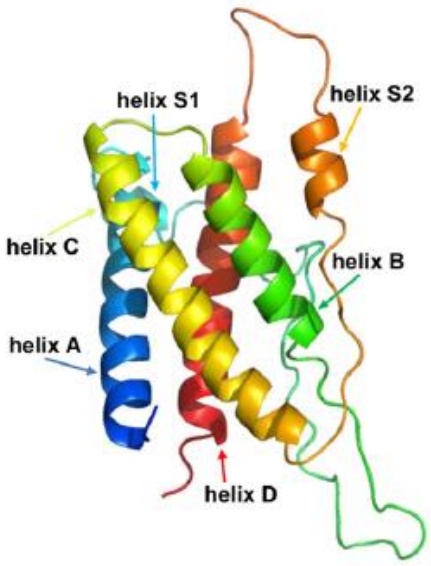


Figure 11: Structure of IL-6 (Veverka et al. 2012)

2.8.2 Expression and signalling

All epithelial and ECs are able to produce IL-6, but it is mainly produced by immunological and stromal cells. A cyclic AMP (cAMP) element, AP-1, C/EBPβ or NF-IL6 as it is also known, and NF-κB bind to the promotor region of the IL-6 gene to trigger IL-6 synthesis. The major trigger of IL-6 transcription and secretion is NF-κB. (Ataie-Kachoie et al. 2014; Lokau et al. 2017). IL-6 transcription can also be initiated by IL-1 and TNF-α (Zilberstein et al. 1986) bacterial lipopolysaccharides, viruses, and growth factors such as epidermal growth factor, platelet-derived growth factor and transforming growth factor-β The post-transcription expression of IL-6 is limited by RNase regnase-1 which destabilizes mRNA via the 3'untranslated region to inhibit translation. When IL-6 levels needs to be increased, IL-β and toll like receptors phosphorylates RNase regnase-1 to allow translation of IL-6 (Uehata & Akira 2013). Another post-transcription regulator of IL-6 is AT-rich interactive domain-containing protein 5A (Arid5a). In contrast to RNase regnase-1, Arid5a stabilizes IL-6 mRNA through binding to the 3′ untranslated region allowing IL-6 translation (Masuda et al. 2013).

The Human interleukin-6 gene has been reported to be located on 7p15-21 (Ferguson-Smith et al. 1988; Ma et al. 2016). Transcription of the IL-6 gene locus results in the synthesis of the

IL-6 precursor protein which is 212 amino acids in length. The precursor protein is cleaved to remove the 28 amino acid long signal peptide from the 184 amino acid long mature segment (Hirano et al. 1986; Ataie-Kachoie et al. 2014). After secretion of IL-6, it travels through the circulatory system to the liver where it binds to inteleukin-6 receptors (IL-6R) on hepatocytes. Neutrophils, T cells, B cells, megakaryocytes, monocytes, macrophages and dendritic cells also express IL-6R (Ataie-Kachoie et al. 2014). IL-6 further stimulates secretion of acute phase proteins such as CRP, α1-antitrypsin, α2-macroglobulin, fibrinogen, plasminogen and complement proteins C3, C4 and C1 inhibitor (Kishimoto 2006; Lokau et al. 2017).

A functioning IL-6R complex is a hexamer composed of two IL-6 polypeptide chains, two soluble or transmembrane 80 kDa IL-6 ligand-binding receptors and two 130 kDa signal transducers (gp130) (Hunter & Jones 2015). The transmembrane form of IL-6 and interleuikin-6 receptor-α (IL-6Rα) complex binds with gp130 after stimulation and starts intracellular signalling. This is the classical signalling pathway (Saito et al. 1992; Kishimoto 2006; Ataie-Kachoie et al. 2014). IL-6 in complex with soluble IL-6 receptor (sIL-6R) can bind with gp130 to further trigger cellular events via the transmembrane domain of gp130; this is termed trans-signalling (Jones et al. 2001; Ataie-Kachoie et al. 2014). Signalling through the classic pathway results in anti-inflammatory activities, and IL-6 trans-signalling results in pro-inflammatory activities (Masjedi et al. 2018).

There are three intracellular pathways in which activation can occur as indicated in Figure 12; the Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) pathway, the Ras/mitogen-activated protein kinases (Ras/MAPK) pathway and the phosphatidylinositol-3-kinase (PI3K)/Akt pathway (Askevold et al. 2014).

When IL-6 ligand binds to the IL-6R, it results in the homodimerization of gp130 (Masjedi et al. 2018). This activation of gp130 leads to the activation and phosphorylation of the intracytoplasmic Janus kinase (JAK). Tyrosine residues of the IL-6R are then phosphorylated by JAKs, leading to the phosphorylation of signal transducer and activator of transcription-3 (STAT3) which contain phosphotyrosine SH2 binding domains. The STAT3 dimers that are formed translocate to the nucleus, resulting in gene expression for cell growth, differentiation and survival (Heinrich et al. 2003; Ataie-Kachoie et al. 2014).

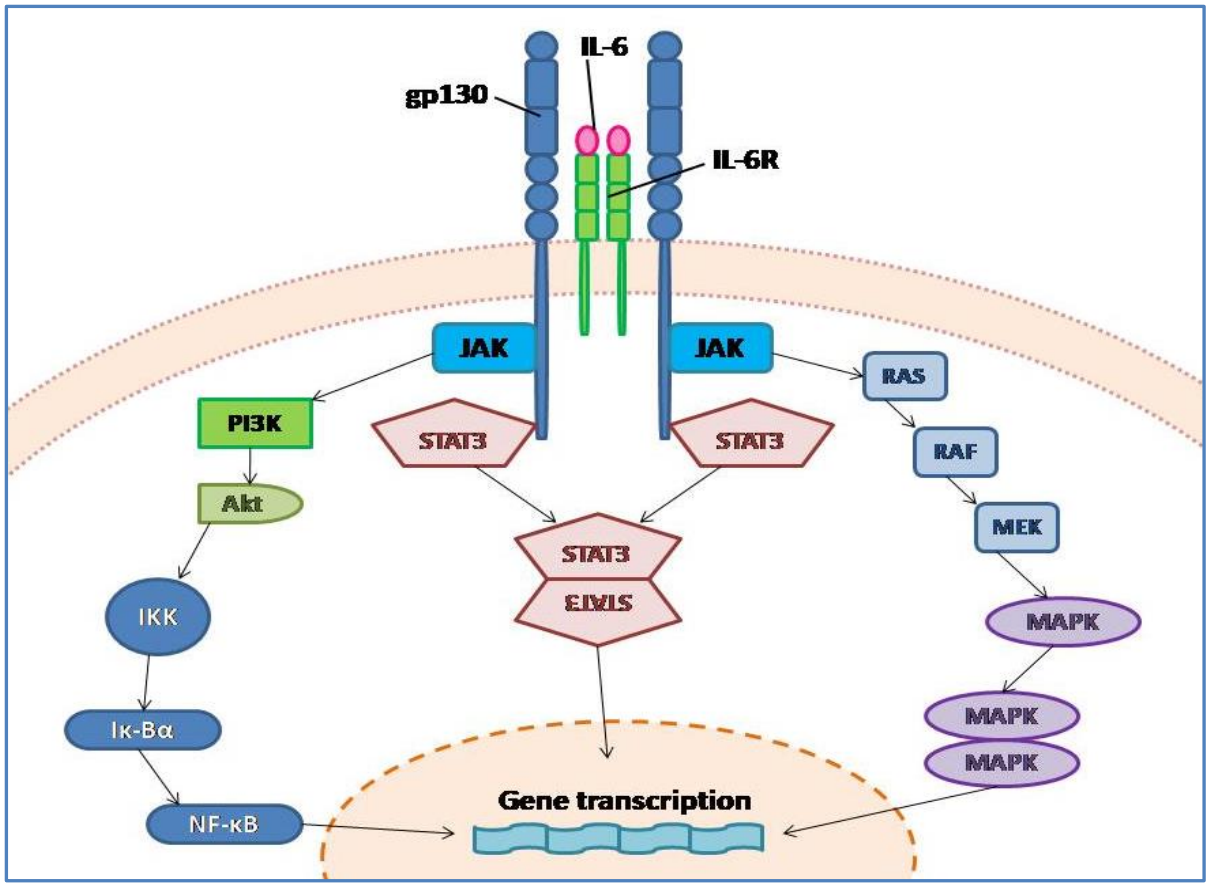


Figure 12: IL-6 signalling**.** Adapted from Masjedi et al. (2018)

The phosphorylation of JAK may also lead to the phosphorylation of tyrosine 759 residue on the IL-6R which results in the involvement of the SH2 domain in the PI3K enzyme. PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 then phosphorylates and activates serine/threonine kinase Protein kinase B also known as Akt. The activated Akt mediates transcription of genes for cell survival, proliferation and growth (Ataie-Kachoie et al. 2014; Masjedi et al. 2018).

In the Ras/MAPK pathway, the IL-6 ligand and IL-6R complex activates the small G protein Ras. Activation of Ras leads to the hyperphosphorylation of mitogen-activated protein kinase kinase kinase (MAPKKK) also known as Raf, followed by the phosphorylation and activation of mitogen-activated protein kinase kinase (MAPKK) also known as MAPK/ERK kinase (MEK) (Ataie-Kachoie et al. 2014). MEK then phosphorylates and activates of mitogen-activated protein kinase (MAPK) also termed ERK. Activated MAPK in turn activates transcription factors for cell growth, immunoglobulin synthesis and acute phase protein synthesis (Masjedi et al. 2018).

2.8.3 Physiology

Interleukin-6 has pro- and anti-inflammatory activities in the body. The major function of IL-6 is its role in inflammation and promoting the synthesis of acute phase proteins including CRP and serum amyloid A; thus also playing a role in fever development (Ataie-Kachoie et al. 2014). IL-6 enhances B lymphocyte maturation and consequently the synthesis and secretion of immunoglobulins (Igs) including IgM, IgG and IgA (Hirano et al. 1986; Schett 2018). Neutrophil recruitment is regulated by IL-6 during the inflammatory response through chemokine production and leukocyte apoptosis, signalled via gp130 (Fielding et al. 2008). Neutrophil recruitment leads to the production of inflammatory mediators such as cytokines, prostaglandins, ROS and proteases (Ataie-Kachoie et al. 2014). IL-6 is also involved in T lymphocyte activation and differentiation (Ataie-Kachoie et al. 2014). Cluster signalling of IL-6, also known as IL-6Rα, by dendritic cells prevent the premature initiation of IFN-γ expression in T cells (Heink et al. 2017). IL-6 promotes the differentiation of native T cells to pathogenic T helper 17 (TH17) cells and regulatory T cells (Schett 2018). IL-6 also induces endothelial expression of adhesion molecules VCAM-1, ICAM-1 and E-selectin, and endothelial production of MCP-1 (Barnes et al. 2011; Didion 2017). The migration and hypertrophy of vascular smooth muscle cells are enhanced by IL-6 (Ataie-Kachoie et al. 2014; Didion 2017). IL-6 also induces proliferation of vascular fibroblasts, contributing to arterial stiffness and hypertension (Didion 2017).

Regenerative activities of IL-6 includes neurogenesis (Erta et al. 2012), hepatocellular proliferation and osteoclast activation and differentiation (Schett 2018). IL-6 plays a role in haematopoiesis, together with interleukin-3 (IL-3) stimulating the production of blast cell colonies as well as macrophage and megakaryocyte differentiation (Heike & Nakahata 2002; Ataie-Kachoie et al. 2014).

2.8.4 Methods of detecting serum IL-6

An ELISA is the preferred and trusted method for IL-6 analysis. A monoclonal anti-human IL-6 antibody is adsorbed onto the microwells solid phase. Upon addition of the sample containing the corresponding antigen IL-6, it will bind to the solid phase antibody. The mixture is washed, leaving only the complexes on the solid phase. A biotin-conjugated anti-human IL-6 antibody then binds to the IL-6-antibody complex already bound to the microwell, forming a "sandwich-complex" of antibody-antigen-antibody. Another washing step is used to remove unbound antibodies. An enzyme substrate is added to react with the enzyme-labelled antibody producing a coloured product. The colour of the product is measured using a colourimeter and intensity thereof is proportional to the concentration IL-6 in the sample. A standards curve is drawn from the results of measured standards and the concentration of the samples are read from the curve (Immuno-Biological Laboratories International GmbH 2012a; Nimse et al. 2016; Rifai et al. 2018). The advantages and disadvantages of an ELISA method are discussed in 2.6.4.a.i., though the method is highly sensitive, it is expensive and requires a trained technologist.

2.8.5 Increased IL-6

In healthy individuals IL-6 is usually absent from body fluids and if it is present it constitutes a few nanograms per millilitre if detectable. Increased serum levels of IL-6 has been shown in the majority of diseases with an inflammatory component in the pathogenesis thereof, including CVD (Lokau et al. 2017). Due to its inflammatory role, IL-6 has been associated with rheumatoid arthritis, multiple sclerosis, Crohn's disease, Castleman's disease, adult onset Still's disease, Alzheimer disease, juvenile idiopathic arthritis and multiple sclerosis (Ataie-Kachoie et al. 2014; Schett 2018). IL-6 has also been linked to metabolic disorders including insulin resistance, diabetes and obesity (Ghanemi & St-Amand 2018).

Increased levels of IL-6 in serum have also been associated with aging, implicating IL-6 in inflammaging (Lin et al. 2014). Decreased cognitive ability is associated with increased IL-6 serum levels in elderly (Bezuch et al. 2019). IL-6 is associated with cancer as it promotes tumour growth and therapeutic resistance through its mediation of proliferation, apoptosis, metabolism, survival, angiogenesis and metastasis (Masjedi et al. 2018).

2.8.6 Increased IL-6 as a cardiovascular risk marker

The major IL-6 transcription factor, NF-κB is synthesized via TNF-α signalling (Lokau et al. 2017). IL-6 in turn promotes the synthesis of CRP (Ataie-Kachoie et al. 2014) which has been found to be a comprehensive marker of CVD (Mendall et al. 1997; Ridker et al. 1997; Ridker 2003; Abu-Farha et al. 2014; Ataie-Kachoie et al. 2014; Wang et al. 2016). IL-6 itself has been proven to be a marker of CVD for its role it plays in inflammation leading to atherosclerosis through endothelial activation, immune cell recruitment, vascular permeability, vascular hypertrophy, vascular fibrosis and endothelial dysfunction (Ataie-Kachoie et al. 2014; Didion 2017).

2.9 Molecular Biology

2.9.1 IL-6 gene

The IL-6 gene (Gene ID: 3569) has been located to the short arm of chromosome 7 at position 15.3 (7p15.3) (MacLeod & Nagel 2011; National Center for Biotechnology Information 2019). The gene has 6 exons and 5 introns as indicated in Figure 13. The exons are 375bp, 103 bp, 191 bp, 114 bp, 147 bp and 542 bp in length and the 5 introns are 920 bp, 162 bp, 1058 bp, 707 bp and 1745 bp in length (MacLeod & Nagel 2011). The functional promotor region of the IL-6 gene has been the focus for studies on IL-6 polymorphisms (Giannitrapani et al. 2013; Karaman et al. 2015; Ma et al. 2016).

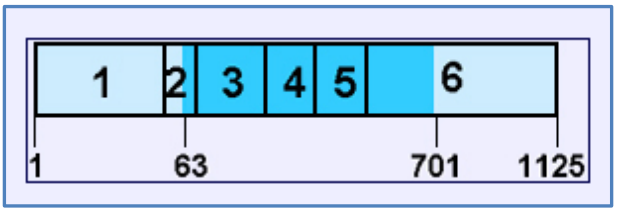


Figure 13: Interleukin-6 gene. (MacLeod & Nagel 2011)

2.9.2 C174G polymorphism

The C174G (rs1800795) polymorphism is a SNP mutation that causes a change from guanine to cytosine at position 174 in the promotor region of the IL-6 gene as indicated in red G/C in Figure 14 (Saxena et al. 2014; Yuepeng et al. 2019). In a study using HeLa cells, Fishman et al. (1998)) proposed that the C allele on position 174 is a binding site for nuclear factor-1 which supresses gene transcription in HeLa cells, but may not be the case for IL-6 transcription in

all cells. This study also found correlating results in vivo with CC genotype that produced lower levels of serum IL-6 compared to GC and GG genotypes.

The SNP has been proven to affect the transcription of IL-6, though studies are controversial as to which genotype is the high producer. The variation can be attributed to differences in ethnicity (Nadeem et al. 2013). Reviews in different ethnic populations have emerged with varying results; in some populations an increase in serum IL-6 levels is associated with the homogenous CC genotype, while in others increased serum IL-6 is associated with the G allele in CG and GG genotypes (Giannitrapani et al. 2013; Nadeem et al. 2013).

Various diseases have been studied in association with the C174G polymorphism; again, the findings vary based on different ethnic populations. In a review Nadeem et al. (2013) reported an association between the C174G polymorphism (G allele) and type 2 diabetes mellitus (T2DM) was present in Americans, Spanish Caucasians, Caucasian Danes, Taiwanese, Gujarati Indians, Japanese and Germans; However other studies found no association in Tunisians, Taiwanese, American, Japanese and Greek populations with the SNP. In another study, Herbert et al. (2005) reported the risk of T2DM associated with the GG genotype was lower relative to the GC and CC genotypes combined in American diabetic patients, whereas (Mohlig et al. 2004) reported a more than fivefold increase in the risk of developing T2DM in obese German subjects with the CC genotype than those with GG. Saxena et al. (2014) concluded that the G allele is associated with the risk of T2DM in a north Indian population. The C174G polymorphism has also been associated with other inflammatory diseases such as periodontits (Teixeira et al. 2014), liver diseases (Giannitrapani et al. 2013), systemic lupus erythrematosus (Katkam et al. 2017), irritable bowel syndrome (Dragasevic et al. 2017) and Alzeimer's disease (Yue et al. 2017). Cancers hepatocellular, colorectal, prostate, ovarian and breast cancers, as well as Hodgkin's lymphoma and neuroblastoma have been associated with the C174G polymorphism (Ataie-Kachoie et al. 2014).

Previous studies which evaluated the prevalence of the C174G polymorphism South Africans indicated that the C allele is the least common and the GG genotype the most common. The C allele was found more prevalent in Caucasians, though still much less common than the GG genotype (Meenagh et al. 2002; Phulukdaree et al. 2013; Joffe et al. 2014; Liu et al. 2015). Global studies indicate a higher allelic frequency of the G allele in Africans and African Americans, but also to an extent the majority of global studies. The GG genotype is reported as the most prevalent, except in European populations who are reported to have the highest prevalence of GC genotype (Giannitrapani et al. 2013; Mandal et al. 2014; Saxena et al. 2014; Yuepeng et al. 2019). The frequency of the G allele also varies in populations of different

ethnic backgrounds, emphasizing the importance of population-based studies and will be discussed further in 4.5.4. (Giannitrapani et al. 2013; Saxena et al. 2014).

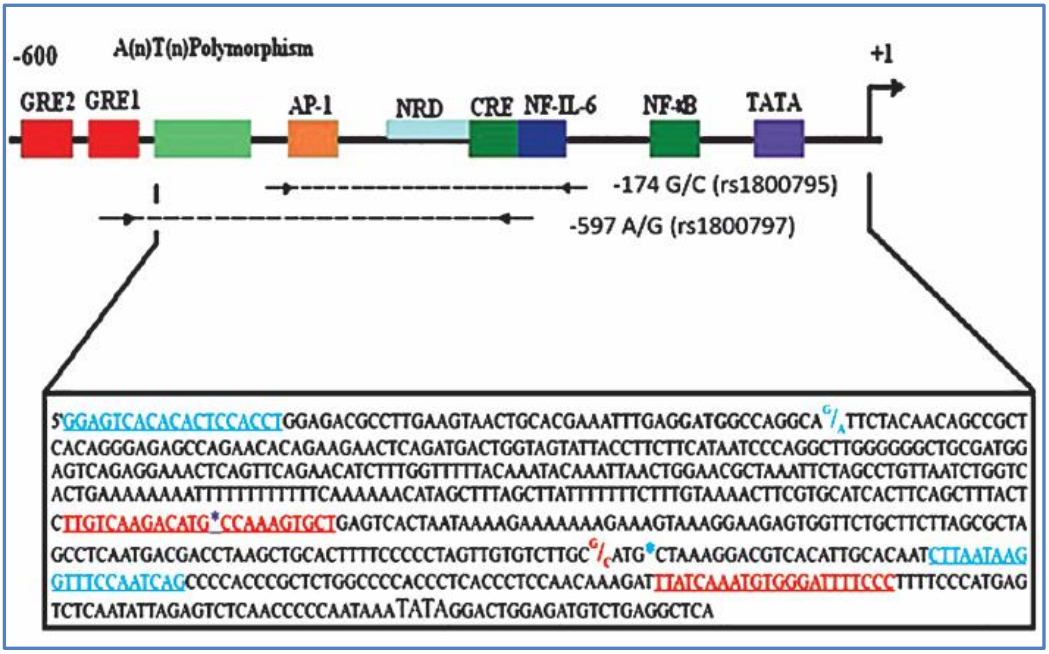


Figure 14: Promotor region of IL-6 with C174G polymorphism (Saxena et al. 2014)

2.9.3 Methods of detections

The detection and/or quantification of single nucleotide polymorphisms can be done using molecular techniques such as polymerase chain reaction (PCR), hybridization and/or sequencing. One of these techniques can be used or they can be used in combination.

2.9.3.1 Polymerase chain reaction

a) Principle

Polymerase chain reaction is an amplification technique used to increase the amount of a target DNA sequence by synthetic *in vitro* methods for quantitative or qualitative analysis (Burtis & Bruns 2015). PCR is the most commonly used amplification method and is considered the gold standard for DNA or ribose nucleic acid (RNA) quantification (Khodakov et al. 2016).

A PCR assay requires the target sequences, thermostable DNA polymerase, deoxynucleotide triphosphates (dNTPs) of each base and a pair of oligonucleotides (primers) complementary

to the opposite side flanking (preceding or following) the target sequences (Burtis & Bruns 2015).

The PCR assay is typically done in cycles of three steps, denaturation, annealing and extension. The analysis is done in a thermocycler, which is automated to change the temperature according to the three steps. Target double stranded DNA (dsDNA) is denatured to single stranded DNA (ssDNA) through exposure to heat. Upon cooling, primers anneal to the complementary sequence on the target DNA. The target DNA forms a template for DNA polymerase to continue extending the primers by addition of dNTPs in a 3'to 5'direction. The cycle starts again with the denaturing of the DNA by heat, now two dsDNA templates are denatured into 4 ssDNA. It is followed by a decrease in temperature to allow annealing and then extension of the strand by DNA polymerase. As the cycle is repeated, the number of DNA strands increase exponentially. The amplification products are then detected or quantified with electrophoresis, sequencing or hybridization (Burtis & Bruns 2015).

b) Real-time PCR

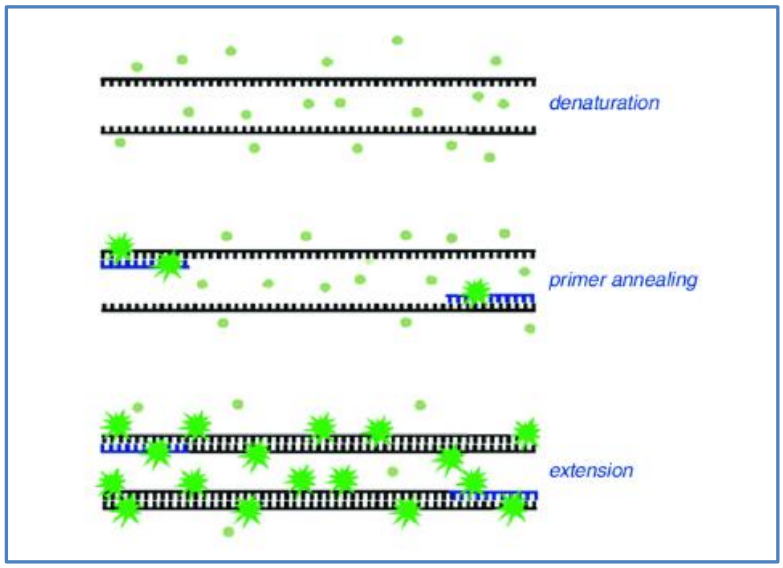


Figure 15: Intercalating dyes in qPCR (Fraga et al. 2014)

Quantification is most commonly done with real-time PCR (qPCR). A dsDNA intercalating dye such as SYBR ® green I, EvaGreen or ethidium bromide is added before amplification to allow the analyser to detect hybridization of complimentary DNA strands as it occurs real-time during amplification (Navarro et al. 2015). The dye is incorporated into minor grooves and is a non-specific detector of DNA amplification as seen in Figure 15 (Fraga et al. 2014).This procedure is done on a qPCR analyser which has a thermal cycler with an integrated light source, a

fluorescence detection system and software that displays the fluorescence detected as a DNA amplification curve. Dissociation of the double strand is detected as a drop in fluorescence (Burtis & Bruns 2015; Navarro et al. 2015).

Hybridization probes can be combined with PCR to quantify or detect a SNP. Hybridization is based on the principle that synthetic probes with known nucleic acid sequences will hybridize their complementary DNA strands. The hybridization probes will fluoresce when binding to the complementary DNA during the annealing or extension step and the amount of fluorescence is directly proportional to the amount of target sequence amplified during PCR (Navarro et al. 2015). The probes hybridize to the target DNA and are designed to do so in close proximity. The reporter or quencher probe is excited by and emits light at a different wavelength than the reporter or acceptor probe in order to differentiate between the two (Fraga et al. 2014). The donor is excited by the light source, and if in close proximity to the acceptor it will excite the accepter to emit light that is detected at a specific wavelength. This transfer is known as fluorescence resonance energy transfer (FRET) (Kim & Kim 2012).

Analysis of SNPs using hybridization probes are done with melting curves. As the temperature is increased the dsDNA will denature and will be detected via a decrease in fluorescence (Navarro et al. 2015). Hybridization occurs when the complimentary strand binds to form a stable duplex. Temperature is then used to melt or separate the DNA into two strands. When the fluorophore-labelled probe is bound to perfectly matched complementary DNA the double strand will denature at a higher temperature than those bound to mismatched DNA. The temperature at which the dsDNA denature is known as the melting temperature (Fraga et al. 2014; Navarro et al. 2015).

Hydrolysis probes emits fluorescence when dsDNA is degraded during the extension phase. The DNA polymerase such as Taq polymerase denatures the probe from the target DNA in a 5′–3′ direction. The 5’ end of the probe is labelled with a fluorescent reporter molecule and the 3’ end is labelled with a quencher molecule. Upon binding of the probe to the complementary strand the reporter and quencher are in close proximity, resulting in the quencher suppressing fluorescence of the reporter. DNA polymerase cleaves the reporter during the extension phase, as it is no longer in close proximity to the quencher the light emitted by the reporter can be detected. The fluorescence is measured at the end of the extension phase and is proportional to the amount of amplified target DNA (Navarro et al. 2015).

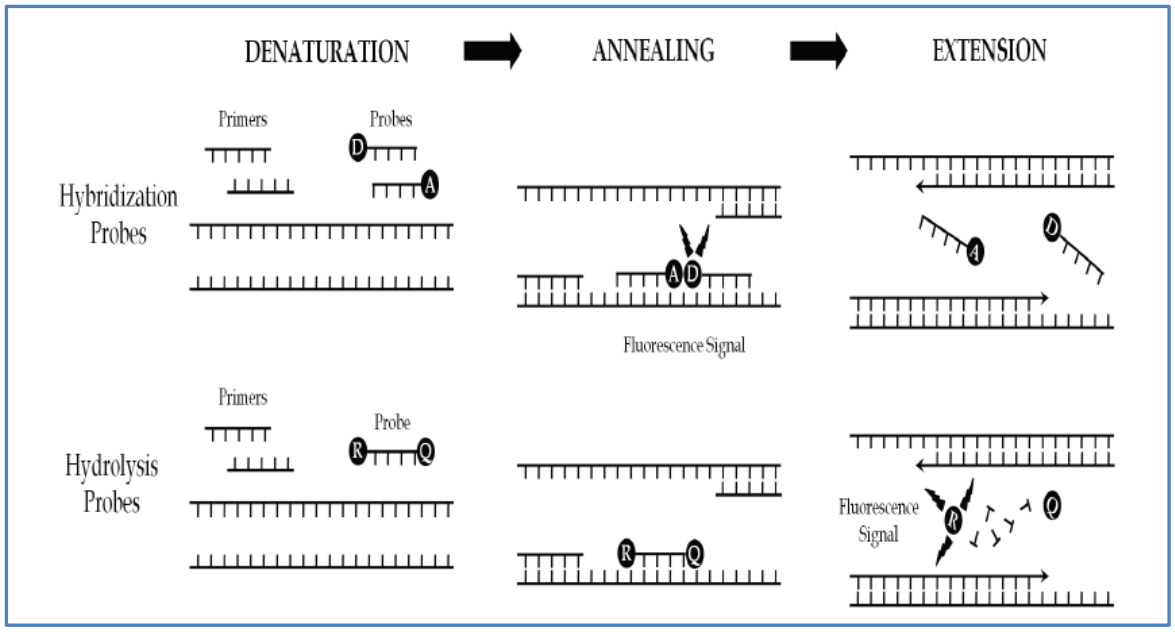


Figure 16: Hybridization and hydrolysis probes in qPCR (Yilmaz et al. 2012)

Discrimination techniques such as electrophoresis and mass spectrometry used to differentiate between two amplification products of PCR.

Gel electrophoresis can be used to separate and stain the PCR product on agarose or polyacrylamide gels according to its molecular weight and size for detection or comparison to standards for semi-quantification. Electrophoresis can be diagnostic for example in detecting bacterial, viral or fungal DNA in a human sample. Ethidium bromide is a widely used fluorescent stain for amplification products. Cyanine stains such as SYBR ® green I are also popular because they only fluoresce when bound to nucleic acids. Electrophoresis also a useful tool in quality control, to determine if a transcript product has formed and PCR was successful (Burtis & Bruns 2015; Navarro et al. 2015).

To determine if a SNP is present, restriction endonucleases can be used to target a specific sequence. If the sequence is present the DNA product will be cleaved into two shorter fragments and if the target is not present it will maintain the same length. After cleavage the products are separated via electrophoresis to determine if there are cleaved shorter products present and/or longer initial amplification products. For example, if the DNA alteration prevents cleavage by the endonucleases and is present as homozygous DNA only long uncut products will be detected. If the DNA is heterozygous the long uncut amplification products and shorter cleaved fragments will be detected. If the alteration is not present at all, endonucleases will cleave all amplification products and only cleaved fragments will be detected (Burtis & Bruns 2015).

Mass spectrometry may be used to discriminate DNA amplification products based on their size. DNA including the variant site is amplified. The extension primer is then added to bind at the polymorphic site for DNA extension. Termination of extension is accomplished by adding 3 dNTPs and one dideoxynucleotide (ddNTP) which will terminate extension, resulting in two different product lengths based on the DNA template. The Matrix-assisted laser-desorption time-of-flight (MALDI-TOF) mass spectrometry can then genotype sequence variants based on their allele specific different molecular weights (Burtis & Bruns 2015).

c) Advantages

Real-time PCR is more accurate and has a higher molecular sensitivity than hybridization and sequencing and it is done in a closed tube system which minimizes contamination. DNA intercalating dyes in qPCR are less expensive than fluorescent probes (Navarro et al. 2015). Agarose gels are able to separate smaller molecular products whereas polyacrylamide gels separate molecular products with higher resolution than agarose gels (Burtis & Bruns 2015).

d) Disadvantages

The main disadvantage of PCR is the formation of primer dimer when analysing multiple DNA sequences in one reaction (multiplex assays) or when primers attach to each other because they have complementary bases. Enzymes used are sensitive to pH changes and thus require a narrow range of acceptable buffers (Khodakov et al. 2016).

2.9.3.2 Hybridization

a) Principle

As discussed in 2.10.3.a.ii hybridization is most commonly used in conjunction with PCR; however, current advances in sensor technologies may allow future use of hybridization without PCR as a feasible alternative (Khodakov et al., 2016).

b) Advantages

Hybridization is a simple and robust method which allows multiplexing. The assay proceeds in many buffer conditions (Khodakov et al. 2016).

c) Disadvantages

Currently hybridization does not provide sufficient molecular sensitivity for practical use without combining it with PCR (Khodakov et al. 2016).

2.9.4 C174G polymorphism and cardiovascular risk

The polymorphism C174G has been studied in various populations to determine its association with CVD. As with the association of C174G with serum IL-6 levels, the results published were not consistent (Reichert et al. 2016b). Differences in the outcomes can once again be attributed to the different ethnic populations in which these studies were performed.

Various studies found an association between the presence of the C174G polymorphism and CVD or increased CVR. The polymorphism has also been found to be associated with an increased risk for further cardiovascular events in some populations (Reichert et al. 2016b) and not associated with others (Yuepeng et al. 2019).

The majority of the studies on the association between the C174G polymorphism and CVD were carried out in Asian and Caucasian populations. Mandal et al. (2014) found that the G allele is more prevalent in African Americans. Literature lacks evidence of the prevalence and association of the polymorphism with CVD in black, African populations. Epidemiologic studies show a variation in the significance of C174G on the serum levels of different populations. If this can improve the prevention of CVD in a specific population it will be noteworthy.

2.10 Conclusion

Since CVDs is a major non-communicable disease and cause of death, it demands to be investigated with the target at risk prediction, disease prevention and improved treatment (World Health Organization 2016b). As the major role player in the majority of CVDs, the pathogenesis of atherosclerosis has been studied and proven to be a complex inflammatory process (Viola & Soehnlein 2015). Vascular inflammation is not only triggered by risk factors of CVD but also an undeniable feature of aging (Franceschi & Campisi 2014). As research focused on the diverse role players of inflammation in atherosclerosis, it has become apparent that ethnic background and demographics have to be taken into consideration for risk profiling of populations for CVDs (Yusuf et al. 2014; Benjamin et al. 2017; Yuepeng et al. 2019).

CHAPTER 3
MATERIALS AND METHODS

3.1 Introduction

Inflammation has been established as the underlying mechanism of cardiovascular disease (CVD), particularly for the role it plays in the development atherosclerosis (Ross et al. 1977; Fredman & Tabas 2017). The relationship between inflammatory markers and CVD have thus been studied and HS-CRP, IL-6 and TNF-α found to be risk markers of inflammation (Jenny et al. 2002; Pearson et al. 2003; Cui et al. 2012; The Interleukin-6 Receptor Mendelian Randomisation Analysis Consortium 2012; Ziv-Baran et al. 2017). The physiology of aging causes a low-grade inflammation and this phenomenon is known as inflammaging. The resultant chronic low-grade inflammatory state of the elderly increases their risk for CVD (Franceschi & Cossarizza 1995; Badimon et al. 2016; Franceschi et al. 2017).

The correlation between IL-6 serum levels and the C174G polymorphism has been proven to have various outcomes in different populations (Giannitrapani et al. 2013; Nadeem et al. 2013; Ramirez Garcia 2017). With CVD as the leading cause of non-communicable diseases, it is a priority to research improved risk profiling, possible prevention or treatment specific aspects (World Health Organization 2016b). Research on single nucleotide polymorphisms (SNPs) and their relationship to CVD is a growing focus area in the field of CVD. Further research is needed to establish genomic profiles in different populations (Fiatal & Adany 2017; van der Ende et al. 2017). This chapter will discuss the methods used in the study, as well as the principles behind them.

3.2 Ethical Consideration

As mentioned in chapter 1, the study formed part of a multi-disciplinary Sharpeville Integrated Nutrition Programme managed by the Centre of Sustainable Livelihoods at VUT. The nutritional programme was ethically approved by University of the Witwatersrand, Johannesburg (M070126), and the study of cardiovascular risks with its related genetic polymorphisms was approved by the ethics committee of VUT (20140827-1ms).

The study was conducted within the Constitution of the Republic of South Africa, Act 108 of 1996 Section 12(2)(c), which states: “Everyone has the right to bodily and psychological integrity, which includes the right not to be subjected to medical or scientific experiments

without their informed consent" (Constitutional Assembly 1996). Ethical guidelines according to South African Medical Research Council (SAMRC) were followed and incorporates the guidelines of:

- National Health Act 61 of 2003, Ethics in Health Research: principles, structures and processes: Department of Health 2015 (South African Department of Health 2003)
- Council for International Organizations of Medical Sciences (CIOMS) guidelines (Council for International Organizations of Medical Sciences & World Health Organization 2016)
- Declaration of Helsinki (World Health Organization 2001 2001)

The SAMRC Guidelines to the Responsible Conduct of Research promote four basic principles of biomedical ethics. Autonomy being the first means that all research should be conducted with respect for the person and with human integrity. The research should be beneficial to the research participants. The research should be conducted with non-maleficence, absence of harm to the research participants. Lastly it should be just, with equal distribution of risks and benefits between communities (South African Medical Research Council 2018).

3.3 Sampling Strategy

3.3.1 Sample size

A total of 84 samples were collected on the same day (within 2 hours) from the elderly volunteers at the day-care centre in Sharpeville who met the inclusion criteria. To determine the sample size required to evaluate the prevalence of C174G polymorphism in the Sharpeville population, the sample size calculation for qualitative variables was used (Naduvilath et al. 2000; Charan & Biswas 2013). Based on previous studies in black South African populations, the expected proportion of the population to have the C allele is 2% (Meenagh et al. 2002; Phulukdaree et al. 2013; Joffe et al. 2014).

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

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

p = 0.02 (2% expected proportion in the population)

d = 0.05 (precision/absolute error)

Therefore:

$$n = \frac{1.96^2 \times 0.02\,(1 - 0.02)}{0.05^2}$$

$$n = 30$$

A minimum of 30 samples were required for a significant statistical representation of the black Sharpeville community for the determination of the C174G polymorphism prevalence. However, as part of the collaborative study the total of 84 samples collected from volunteers were analysed in this study.

3.3.2 Inclusion criteria

Participants included in the study met the following criteria:

a) Participant aged 60 years or older
b) Voluntary attendants of the Sharpeville day care centre
c) Informed consent given
d) Participants suffering from conditions that characterise cardiovascular risk markers such as diabetes and hypercholesterolemia will not be excluded from the study.

3.3.3 Exclusion criteria

Persons who were not able to provide substantial information to complete the consent process due to conditions like dementia were excluded from this study.

3.4 Study Design

The research was conducted in the field of Biomedical Sciences. The study quantitative, cross-sectional, analytical observational design (Fathalla & Fathalla 2004). It involved collection of samples from volunteers in a purposively selected population. A total of 84 samples were collected but not all samples were of sufficient volume to perform all the tests.

3.5 Data Collection

Data was collected by a research team as part of the collaborative study with CSL. Health Professionals Council of South Africa (HPCSA) registered medical technologists, a qualified dietician, qualified phlebotomist, HPCSA registered student medical technologist and trained field workers were involved with data collection at the day care centre in Sharpeville. The collaborative study entails blood pressure, weight and height measurements as well as a

panel of blood parameters. This study will only report on HS-CRP, TNF-α, IL-6 and C174G analysis. Fieldworkers explained the aim, objectives and methodology of the study to participants in Sotho, which is the home language of the majority of residents in Sharpeville (Statistics South Africa 2011). They also assisted volunteers who wanted to participate in the study with the written informed consent (Annexure E). Participants were treated sensitively, helpfully and with respect to ensure their dignity. All actions, at all times were non-maleficent.

The data collection procedure as indicated in Figure 17 started with ethical approval as well as approval form management of the day care centre to conduct the study. A numbering system was used in order to ensure that participants are unknown to the laboratory personnel, as well as to the data analyst to assure confidentiality of the participants at all times. A specific number was allocated to each individual on the control list. A file which contained questionnaires and blood collection tubes (in a re-sealable clear plastic bag) and was labelled with the corresponding number was given to the participant by the field workers. Participants rotated between the allocated stations (anthropometric, questionnaires and phlebotomy) Fasting blood samples were collected by a trained phlebotomist using a vacutainer system from the vena cephalica. Two 7 ml samples of clotted blood were collected from each individual to obtain serum samples for analysis, and one 5 ml ethylenediaminetetraacetic acid (EDTA) sample for whole blood analysis. Blood samples were placed in a cooler box ±8^{o}C and protected against direct sunlight. The individuals were requested to return to their initial starting point, where files were handed in and the control list verified to assure that all the data collection processes were completed. The participants were then served breakfast.

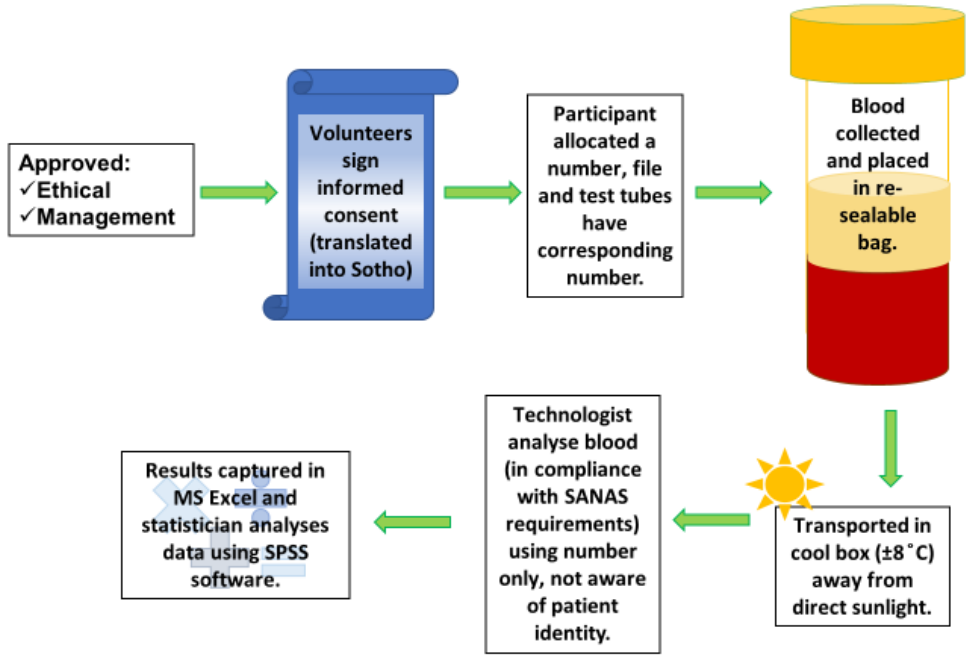


Figure 17: Figure showing steps in data collection for this research**.**

3.6 Blood Analysis

Samples were transported to the laboratory in the cooler boxes, protected from direct sunlight. The serum separator tube (SST) samples were centrifuged at 3000 rpm for 10 minutes, and aliquoted into test tubes labelled with the corresponding sample number. The EDTA samples were used for DNA extraction upon arrival at the laboratory. The DNA was then stored in 2 ml Eppendorf tubes labelled with the corresponding sample number. Serum samples were stored at -20ºC and extracted DNA at -80ºC until analysis was performed. Standard laboratory procedures were followed in order to comply with South African National Accreditation System (SANAS) requirements, including procedures performed to ensure reliability and validity for each analyte.

3.6.1 High sensitivity C-reactive protein analysis

3.6.1.1 Principle

High sensitivity C-reactive protein was measured using an automated microparticle enhanced immunoturbidimetry procedure. Immunocomplexes form when CRP in buffered serum samples combine with microparticles coated with anti-human CRP (sheep) in the reagent. The absorbance was measured at 540 nm when the reaction reached its end-point. The change in absorbance measured is directly proportional to the concentration of CRP in the serum sample (Thermo Fisher Scientific 2011).

3.6.1.2 Method

Serum samples were analysed with the clinical chemistry auto-analyser, Thermo Scientific Konelab™ 20 (Massachusetts, United States) using Thermo Scientific CRP High Sensitivity reagent kits and controls.

3.6.1.3 Validation

The analyser was calibrated using the HS CRP Calibrator included in the Konelab™ kit with a value of 11.81 mg/L. The true value of CRP was assigned using the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) CRM 470 as the primary reference. The autoanalyzer used the standard curve obtained from the calibration to calculate results. The detection range was 0.25-40 mg/L. CRP High Sensitivity Controls for were run and

documented daily, and upon opening and using a new reagent (Thermo Fisher Scientific 2011).

3.6.1.4 Reference range for high sensitivity C-reactive protein

Serum HS-CRP levels of less than 1 mg/L is considered a low risk of CVD. A HS-CRP level of 1-3 mg/L indicates a medium/average risk, and levels greater than 3 mg/L a high risk. Patients with HS-CRP levels >5mg/L have the highest risk of CVD (Pearson et al. 2003; Shrivastava et al. 2015b). Those with medium risk have a 50% greater CVR than those with who fall within the low risk. Having high risk for CVD based on HS-CRP, means having a risk twice as large as those at low risk (Salazar et al. 2014).

3.6.2 Tumour necrosis factor alpha analysis

3.6.2.1 Principle

The Immuno-Biological Laboratories Co. Ltd (IBL) International enzyme-linked immunosorbent TNF-α assay kit contains microwell plates with anti-human TNF-α antibody adsorbed onto the microwells. During incubation of samples and standards in these wells, human TNF-α binds to the antibodies coating the microwells. A biotin-conjugated anti-human TNF-α antibody then binds to the TNF-α-antibody complex already formed on the microwell. Unbound antibody is removed through washing. Streptavidin-HRP is added, which binds to the biotin-conjugated anti-human TNF-α antibody during incubation, and excess is removed through washing (Immuno-Biological Laboratories International GmbH 2012b).

Biotinyl-tyramide is added to the wells, starting the amplification phase. After incubation excess biotinyl-tyramide is removed. Streptavidin-HRP is added converting biotinyl-tyramide into highly reactive free radicals, it is incubated, and excess removed through washing. Substrate solution (tetramethyl-benzidine) with HRP is added.

A coloured product is produced, and the colour intensity is directly proportional to the concentration of TNF-α is the sample or standard. The reaction is stopped by addition of 1M phosphoric acid and read immediately at 450 nm (Immuno-Biological Laboratories International GmbH 2012b).

3.6.2.2 Method

Serum TNF-alpha concentration in the samples were measured using the manual TNF-alpha high sensitivity ELISA kit form IBL International GmbH (Hamburg, Germany). Manual preparation was done using calibrated pipettes, plate washing was done using the EMP W206 Microplate Washer (Sichuan, China) and the Rayto RT-2100C Microplate Reader (Shenzhen, China) was used to read the absorption.

3.6.2.3 Validation

Highly purified recombinant human TNF-α which was evaluated using the International reference standard National Institute for Biological Standards and Control (NIBSC) 87/650 was used to calibrate the immunoassay. Seven standards with concentrations 20.00 pg/ml, 10.00 pg/ml, 5.00 pg/ml, 2.50 pg/ml, 1.25 pg/ml, 0.61 pg/ml and 0.31 pg/ml were measured in duplicate to obtain a standard curve. The standard curve was used to determine the concentration of the controls and samples measured. Low and high TNF-α controls were run in duplicate and within the reference range (Immuno-Biological Laboratories International GmbH 2012b).

3.6.2.4 Reference range for tumour necrosis factor alpha

The normal reference range for IL-6 was 0.00 - 3.22 pg/mL (Immuno-Biological Laboratories International GmbH 2012b)

3.6.3 Interleukin-6 analysis

3.6.3.1 Principle

The IBL International enzyme linked immunosorbent IL-6 assay kit contains a microwell plate with monoclonal anti-human IL-6 antibody adsorbed onto the microwells. Upon incubation the IL-6 present in the standard or sample that is added to the microwells will bind to the antibody coating the microwells. A biotin-conjugated anti-human IL-6 antibody then binds the human IL-6-antibody complex bound to the microwell during incubation. Excess unbound biotin-conjugated anti-human IL-6 antibodies are removed by washing. Streptavidin-HRP is added and binds to the biotin-conjugated anti-human IL-6 antibody during incubation, and excess is removed through washing.

The amplification phase is achieved by adding biotinyl-tyramide to the wells, excess is removed after incubation. Streptavidin-HRP is added which converts biotinyl-tyramide into highly reactive free radicals during incubation, and excess reagent removed through washing. Substrate solution (tetramethyl-benzidine) containing reactive HRP is added and incubated.

A coloured product develops, the intensity of the colour being directly proportional to the concentration of IL-6 present in the sample or standard. The reaction is stopped by adding 1M phosphoric acid and read immediately at 450 nanometre (nm) (Immuno-Biological Laboratories International GmbH 2012a).

3.6.3.2 Method

Serum samples were used to measure IL-6 using the manual Interleukin-6 high sensitivity ELISA kit form IBL International GmbH. Calibrated pipettes were used for manual preparation, plate washing was done using the EMP W206 Microplate Washer and the absorption was read with the Rayto RT-2100C Microplate Reader.

3.6.3.3 Validation

The immunoassay was calibrated with highly purified recombinant human IL-6, evaluated against and equivalent to the International reference standard NIBSC 89/548. Seven standards with concentrations 5.00 pg/ml, 2.50 pg/ml, 1.25 pg/ml, 0.63 pg/ml, 0.31 pg/ml, 0.16 pg/ml and 0.08 pg/ml were measured in duplicate. A standard curve was derived from the calibrators and used to determine the concentration of the controls and samples measured. Low and high IL-6 controls were run in duplicate and within the reference range (Immuno-Biological Laboratories International GmbH 2012a).

3.6.3.4 Reference range for interleukin-6

The normal range for interleukin-6 concentration in serum was 1.1 - 14.3pg/mL (Immuno-Biological Laboratories International GmbH 2012a).

3.6.4 C174G polymorphism

3.6.4.1 Principle

The SNP genotyping assay is made up of a locus-specific PCR reaction to detect the C174G (rs1800795). After unwinding double stranded DNA to form a template, the segment of DNA containing the IL-6 gene is multiplied using forward (ACGTTGGATGAGCCTCAATGACGACCTAAG) and reverse (ACGTTGGATGGATTGTGCAATGTGACGTCC) primers. The resultant product is extended by a single base extension using mass-modified dideoxynucleotide terminators of an oligonucleotide primer (GTGACGTCCTTTAGCAT) that anneals at the polymorphic site (Herbert et al. 2005).

The MALDI-TOF mass spectrometer measures the mass of the extended primer in order to identify the SNP. The DNA extension products crystallize within the matrix of the SpectroCHIP. Upon radiation of the extension products, molecules undergo desorption and ionization. This results in positively charged DNA molecules accelerating up the vacuum tube towards a highly sensitive detector. The speed of the molecules is inversely proportional to the mass of the individual extension products, the lightest ions will reach the detector first. The software processes the time-of-flight and differentiates the variants by their mass, producing a mass spectrum (Agena Bioscience Inc 2018).

3.6.4.2 Method

DNA was extracted from EDTA samples according to manufacturer's instruction using the Quick-DNA™ Universal kit manufactured by Zymo Research (California, United States). Genomic DNA samples of 20 nanogram per microlitre (ng/µl) were prepared using the kit. The following primers and probes were purchased form Inqaba Biotech (Pretoria, South Africa) at concentrations of 10 µM:

RS1800795_W1_Forward: ACGTTGGATGAGCCTCAATGACGACCTAAG
RS1800795_W1_Reverse: ACGTTGGATGGATTGTGCAATGTGACGTCC
RS1800795_W1_Extension: GTGACGTCCTTTAGCAT

Polymerase chain reaction amplification of the target loci involves amplifying that specific fragment of genomic DNA, which was then genotyped on the Agena MassARRAY (San Diago, United States) platform. Multiplex PCR cocktail was prepared according to Table 5.

Table 5: Multiplex PCR cocktail

Reagent	Concentration in 5µl	Volume (1 run)
Nanopure water	N/A	1.8 µL
10x PCR Buffer with 20mM $MgCl_2$	1 (2 mM $MgCl_2$)	0.5 µL
$MgCl_2$ (25 mM)	2 mM	0.4 µL
dNTP mix (25 nM each)	500 µM	0.1 µL
Primer mix (500 nM each)	100 Nm	1.0 µL
PCR enzyme (5 U/µL)	1.0 U per run	0.2 µL
	Total volume	**4.0 µL**

$MgCl_2$ = Magnesium chloride, dNTP = deoxy nucleotide triphosphate, millimolar (mM) nanomolar (nM), Units per microlitre (U/µL)

After centrifuging 4 µL of multiplex cocktail and 1 µL extracted DNA together in the 96-well microtiter plate, the PCR was performed using the temperature cycles in Table 6.

Table 6: PCR temperature cycles

Step	Temperature	Duration	Phase
1	94 ºC	2 minutes	Initial denaturation
2	94 ºC	30 seconds	Denaturation
3	56 ºC	30 seconds	Annealing
4	72 ºC	60 seconds	Extension
5	Repeat step 2 – 4 for 45 cycles		
6	72 ºC	5 minutes	Final extension
7	4 ºC	Continued	

The enzyme Shrimp Alkaline Phosphatase (SAP) was used to clean up remaining primers. SAP dephosphorylates unused dNTPs through cleavage of the phosphate group from the 5' terminal. The SAP enzyme solution was made up of nanopore water, SAP buffer and SAP enzyme according to follows indicated in Table 7.

Table 7: SAP solution

Reagent	Volume per run
Nanopure water	1.53 µL
SAP Buffer	0.17 µL

SAP enzyme (1.7 U/µL)	0.30 µL
Total volume	2.00 µL

After addition of 2 µL SAP solution to each microwell, the temperature cycles were run according to Table 7.

The PCR clean-up was ran at cycles of 37 ºC for 40 minutes, 85 ºC for 5 minutes and 4 ºC continued. After PCR clean-up, the primer extension reaction cocktail was added to the amplification products for the single-base extension reaction, vortexed and then centrifuged. This solution contained the reagents and volumes stipulated in Table 8.

Table 8: Primer extension reaction cocktail

Reagent	Concentration in 9 µL	Volume per run
Nanopure water	N/A	0.619 µL
iPLEX Buffer Plus (10x)	0.222X	0.200 µL
iPLEX Termination mix	1x	0.200 µL
Primer mix (8 µM: 10 µM: 15 µM)	0.52 µM:1.04 µM: 1.57 µM	0.940 µL
iPLEX enzyme	1x	0.041 µL
	Total volume	2000 µL

Micromolar (µM)

The temperature program in Table 9 was used for thermocycling conditions.

Table 9: Primer extension temperature program

Step	Temperature	Duration	Phase
1	94 ºC	30 seconds	Initial denaturation
2	95 ºC	5 seconds	Denaturation
3	52 ºC	3 seconds	Annealing
4	80 ºC	3 seconds	Extension
5	Repeat step 3 – 4 for 5 cycles		
6	Repeat step 2 - 4 for 60 cycles		
7	72 ºC	3 minutes	Final extension
8	15 ºC	Hold	

To optimize mass spectrometry analysis, a resin was added to the primer extension reaction products to remove salts such as sodium, potassium and magnesium ions according to manufacturer's instructions. The resultant product was then transferred to the SpectroCHIP for analysis by the MALDI-TOF. The SpectroCHIP is a silica chip with matrix spots that allows dispensing of nano-volumes through capillary action.

The extended products were measured using the MassARRAY Compact mass spectrometer and Agena real-time detection software.

3.6.4.3 Validation

A no-template control was included in every PCR reaction to detect false positive reactions. To prevent contamination and ensure optimal amplification, filter tips were used and discarded after use (not re-used). A clean laboratory coat was worn, clean gloves and all work surfaces were cleaned with 70% ethanol and 5% sodium hypochlorite solution.

3.6.4.4 Results interpretation for C174G polymorphism

The following genotypes were detected; GG (homozygous normal), CG (heterozygous) and CC (homozygous mutant) and the Hardy-Weinberg exact test reported.

3.7 Data Analysis

After analysis of the samples, raw data was captured on Microsoft Excel. The IL-6 and TNF-α concentration of samples were calculated from a standard curve in Microsoft Excel. Data was imported to IBM SPSS® Statistics version 25 software for descriptive and inferential statistical analysis as well as figures. Medians, interquartile ranges, coefficients of variance and correlations were reported.

3.8 Conclusion

The study aimed to evaluate the inflammatory profile of the elderly population in Sharpeville, in order to contribute to population specific CVR profiling. The study was conducted in a representative sample of the population as calculated using the power calculation. Ethical procedures were followed throughout the study, taking care to ensure autonomy, beneficence, non-maleficence, respect and confidentiality. Good laboratory practice was applied in all procedures.

CHAPTER 4
RESULTS AND DISCUSSION

4.1. Introduction

The aim of this study was to evaluate the prevalence of the C174G polymorphism, in correlation with the inflammatory biomarkers, HS-CRP, TNF-α and IL-6 as CVR markers in elderly black South Africans. The inflammatory markers, tumour necrosis factor-alpha, interleukin-6 and high sensitivity C-reactive protein, were examined to evaluate the inflammatory status. These inflammatory markers have been associated with an increased risk for CVD in other populations (Pearson et al. 2003; Biasillo et al. 2010; Hartupee & Mann 2013; Ofstad et al. 2013; Yu et al. 2016). The results will be presented, analysed and discussed in this chapter using graphs and inferential statistics.

4.2. Socio-Demographic

The 37599 residents of Sharpeville live in 12170 households (Statistics South Africa 2011). A poverty rate of 43.1% was determined using the Slabbert's method which compares the individual households poverty line with the total income of that household (Sekatane 2013). In a study conducted by Grobler (2015) on the Sharpeville day care attendees it was reported that all participants received state pension which is the main source of income in the majority of the households. Grobler determined that nearly all participants lived in brick houses and all of them had access to safe water, sanitary facilities and waste removal. Food insecurity was also reported in the Sharpeville population. Of the 84 participants in this study, 15 (18%) are male and 69 (82%) are female which correlates to the gender ratio distribution of the above mentioned study (15% males and 85% females). Grobler (2015) also found that although 63% of participants did seek medical attention at the clinic when required, 64.3% had to walk to the clinic. Of all the CVR markers, the clinic only tested blood pressure and capillary glucose.

4.3. Quality Assurance

Instruments were calibrated and controls were run together with samples. Kits were calibrated by the manufacturer using NIBSC standards as discussed in Chapter 3. Samples were accepted when controls were within the normal range as indicated in Table 10, which were supplied by the manufacturers. The means of controls were calculated and compared to the target means, control results also adhered to Westgard rules and coefficient variations (CVs)

were less than 15%. The CV for HS-CRP was 4.17%, TNF-α was 8.5% and for IL-6 was 6.0% as per table 11.

Table 10: Quality control results

Control	Range (pg/mL)	Target mean (pg/mL)	Actual mean (pg/mL)
TNF-α low	15 - 50	33	38.709
TNF-α high	250 - 750	500	503.031
IL-6 low	4 - 10	7	5.314
IL-6 high	50 - 150	100	83.162
PCR No-template control		Negative	Negative

Table 11: Quality assurance statistics

Biomarker	Method	Coefficient variation (CV)
HS-CRP	Immunoturbidimetry	4.17%
TNF-α	ELISA	8.5%
IL-6	ELISA	6.0%

4.4. Results

4.4.1. High sensitivity C-Reactive protein

The results have been analysed using IBM SPSS ® Statistics 25. The HS-CRP results were positively skewed (2.475) as indicated by the bell curve in Figure 18. The skewness was also confirmed by the Shapiro-Wilk normality test (p=0.000). The data was leptokurtic (7.745) as seen in Figure 18 and there was a high frequency of distribution around the median with a heavy tail towards the right with outliers.

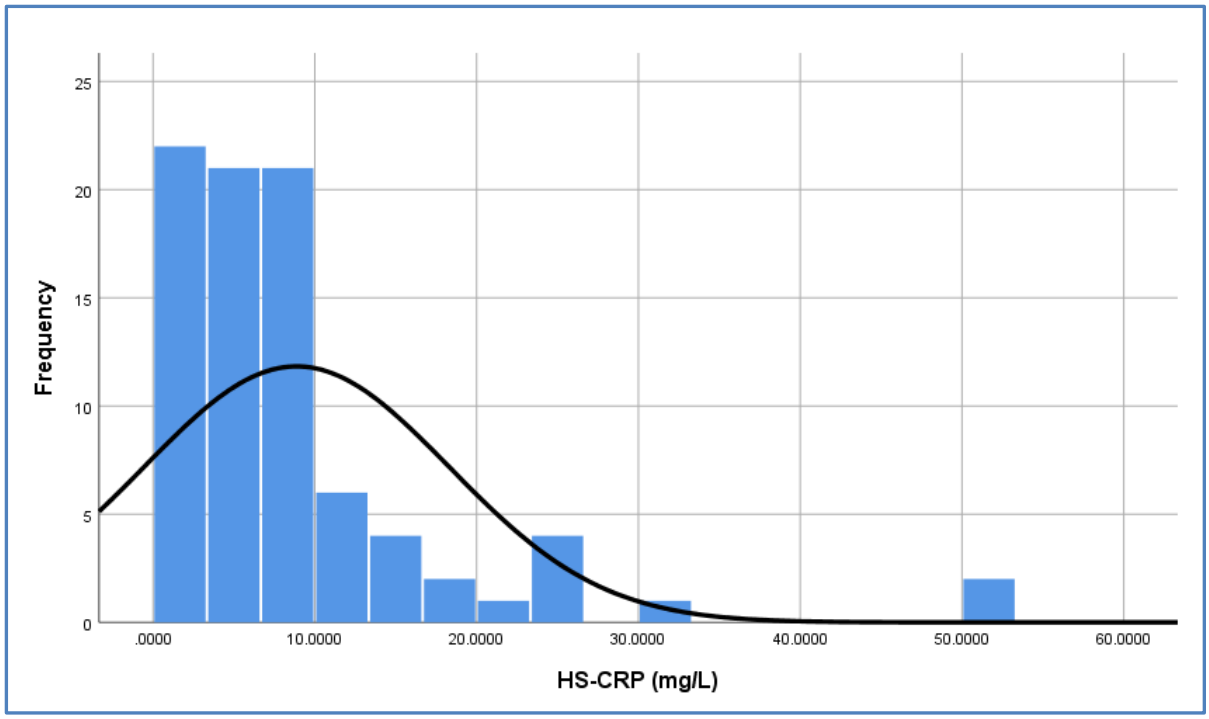


Figure 18: Frequency distribution histogram of HS-CRP concentrations

The outliers (* indicates results above 3SD) were confirmed and can also be clearly visualized in Figure 19 on boxplot. The boxplot also illustrates the HS-CRP median of 6.44 mg/L and the interquartile range (IQR) of 2.82 - 9.86 mg/L. The median was in the category of highest risk for CVD.

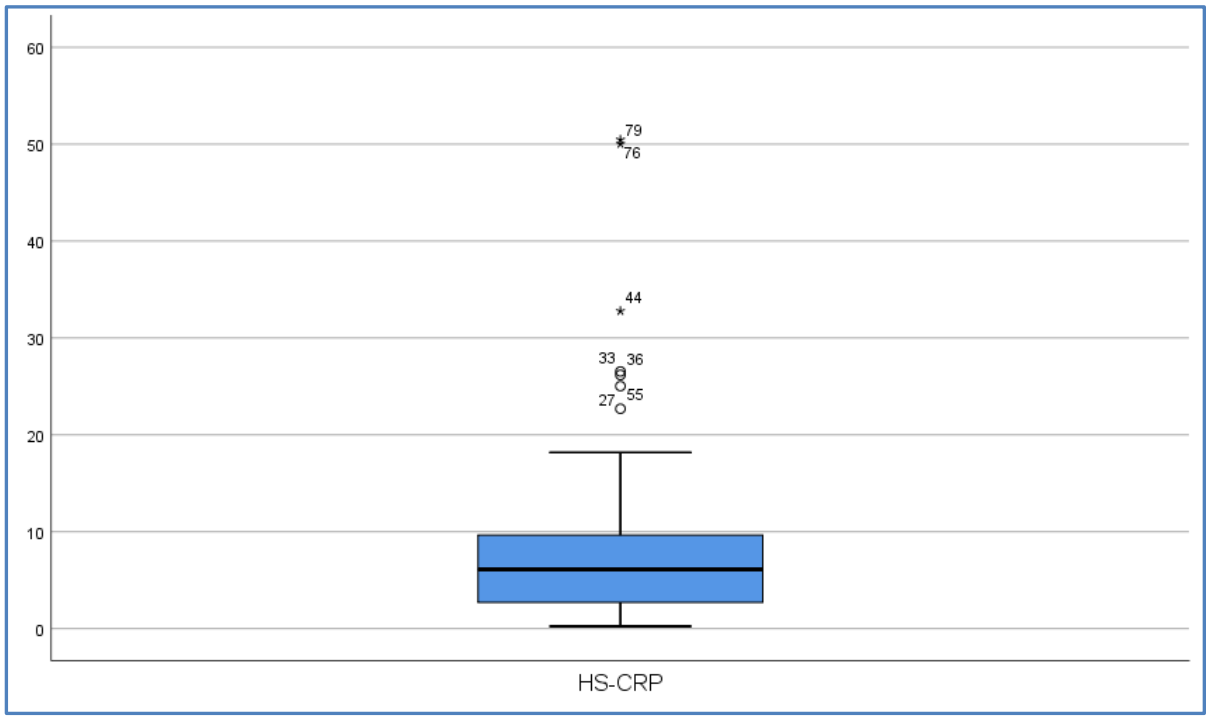


Figure 19: Boxplot of HS-CRP concentrations with the median and interquartile range

The results were clustered into the different risk categories and illustrated in Figure 20. The low risk category (<1 mg/L) consists of 15.5% (n=13) of participants, 9.5% (n=8) fall within the medium risk category (1 – 3 mg/L) and 15.5% (n=13) fall within the high risk category (3.01 – 5 mg/L). As indicated with red in the pie chart, 59.5% (n=50) of participants were at very high risk (>5mg/L) of developing CVD (Pearson et al. 2003; Shrivastava et al. 2015a).

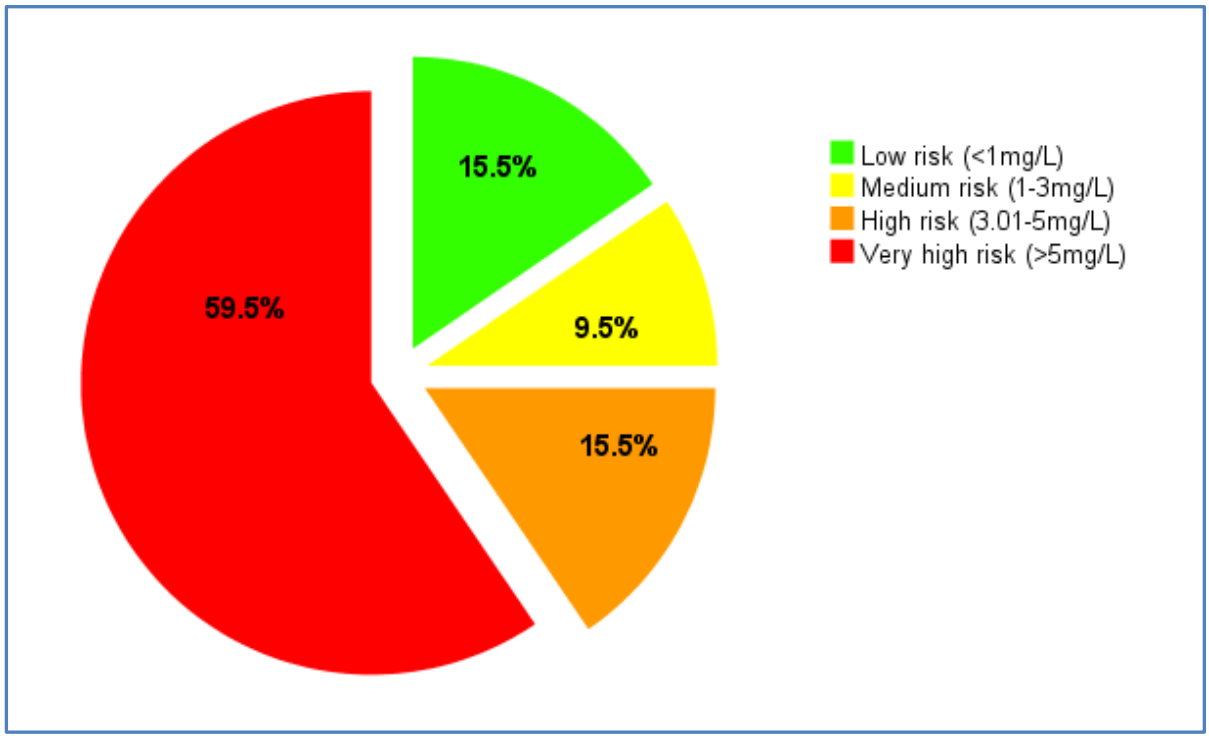


Figure 20: Pie chart of HS-CRP concentrations clustered into risk categories

4.4.2. Tumour necrosis factor alpha

As indicated in Figure 21, TNF-α results were positively skewed (6.519). The Shapiro-Wilk normality test confirmed skewness (0.000). The leptokurtosis (43.446) can be seen on the histogram with a bell curve. This is due to the majority of results being below 2 pg/mL, with two outliers at 34.9 pg/mL and 56.4 pg/mL.

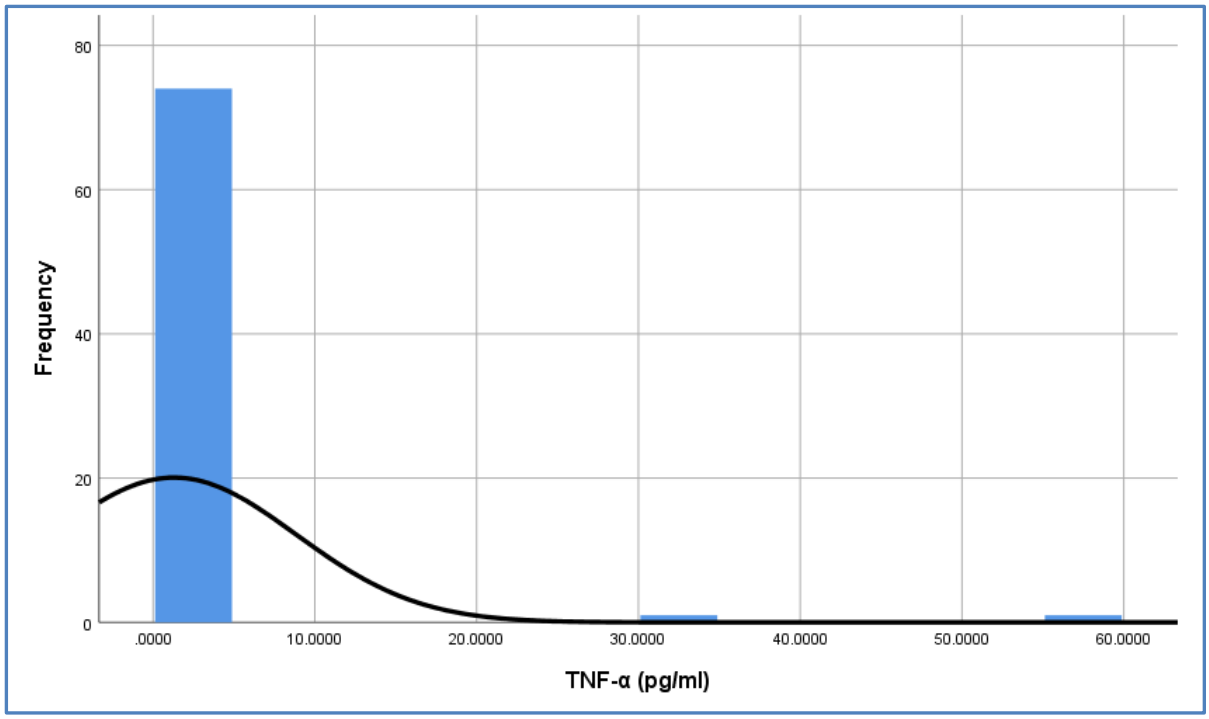


Figure 21: Frequency distribution histogram of TNF-α concentrations

As seen in Figure 22, majority of the results were distributed around the median of 0.00 pg/mL (green line). The two outliers were omitted from the scatterplot to better visualize sample distribution. The interquartile range of TNF-α was 0.00 – 0.05 pg/mL.

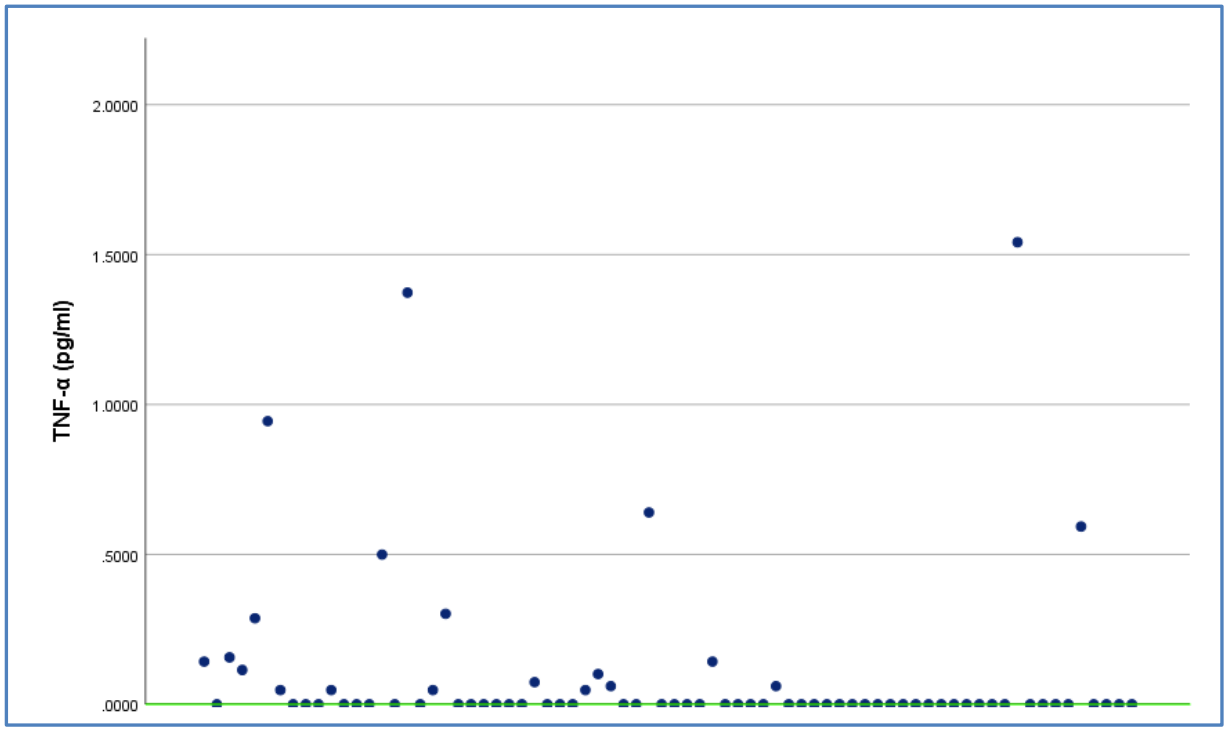


Figure 22: Scatterplot of TNF-α concentrations around the median excluding outliers

The pie chart in Figure 23 illustrates the distribution of results according to the normal range. Majority of results, 97.4% (n=74) fall within the normal TNF-α range of 0.0 - 3.22 pg/mL. Only 2.6% (n=2) of results were high, and 8 samples had insufficient serum for analysis of TNF-α.

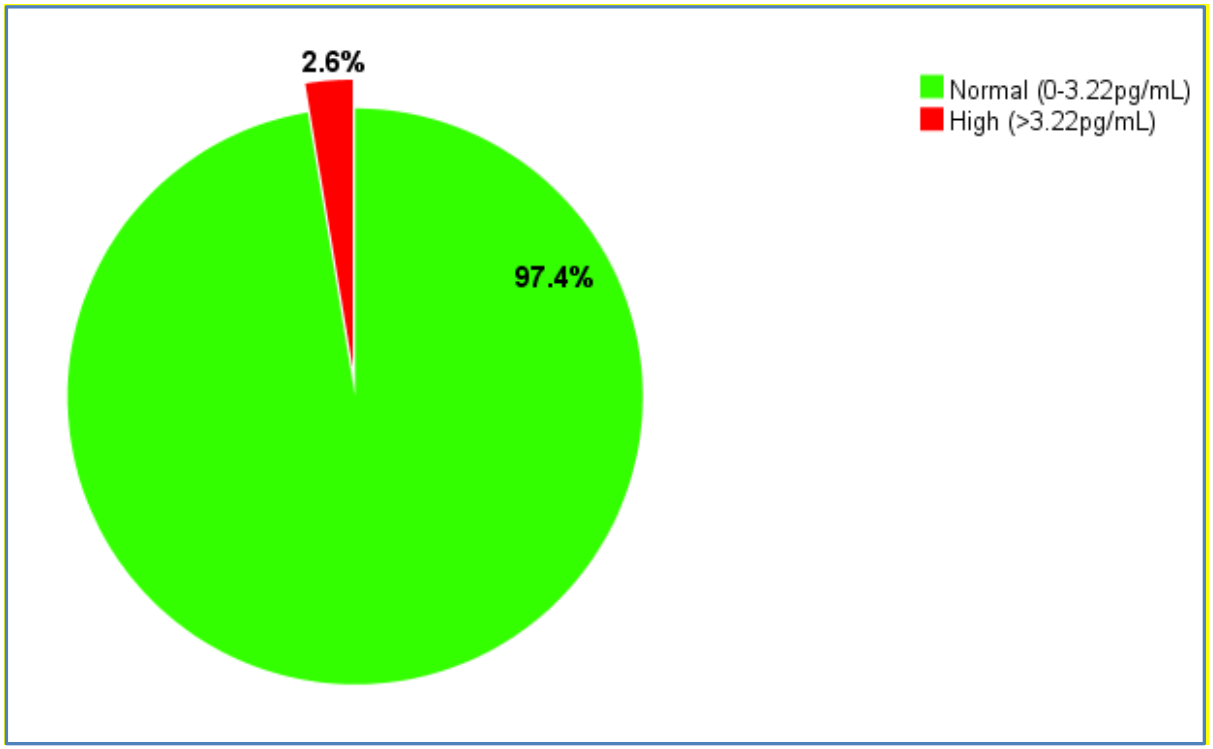


Figure 23: Pie chart of clustered TNF-α concentrations

4.4.3. Interleukin-6

The bell curve in Figure 24 shows IL-6 results were skewed positive (3.683). The skewness was confirmed by the Shapiro-Wilk normality test (0.000). The graph also illustrates leptokurtosis (16.085) which indicates the high frequency of results around the median with a heavy tail resulting from outliers.

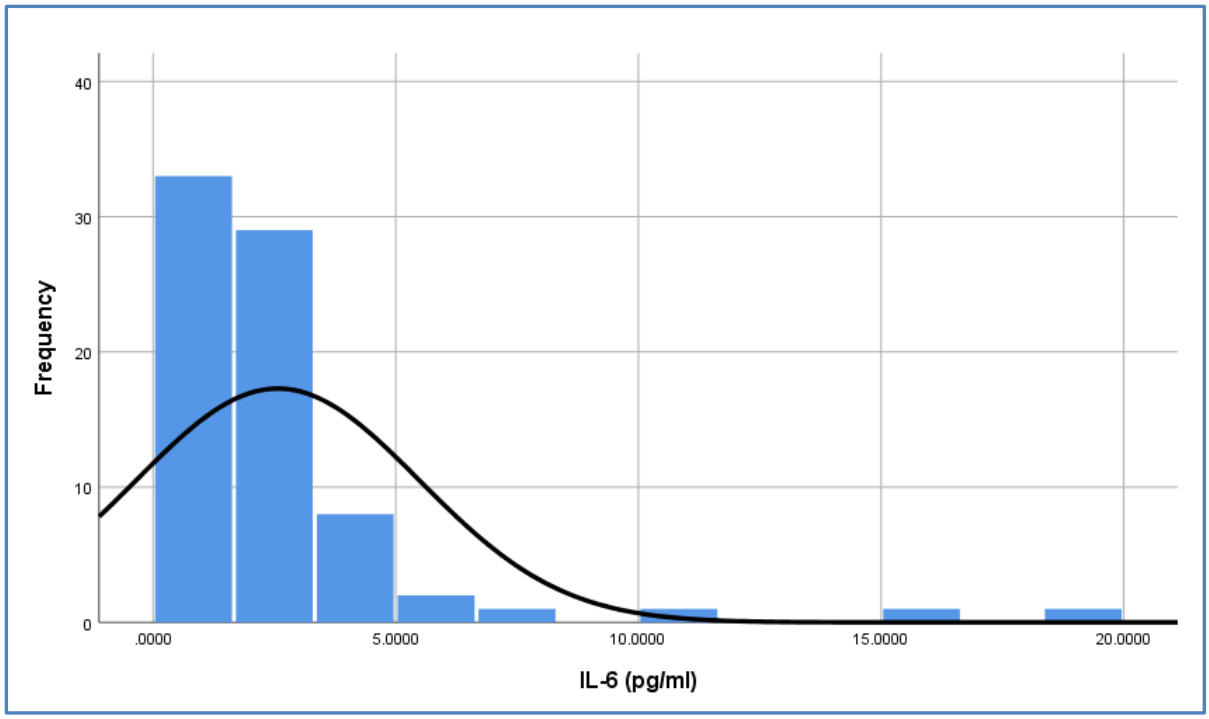


Figure 24: Frequency distribution histogram of IL-6 concentrations

The median (green line) of 1.92 pg/mL is indicated in figure 25 and falls within the normal range of 1.1 – 14.3 pg/mL. The interquartile range for IL-6 was 0.99 – 2.79 pg/mL.

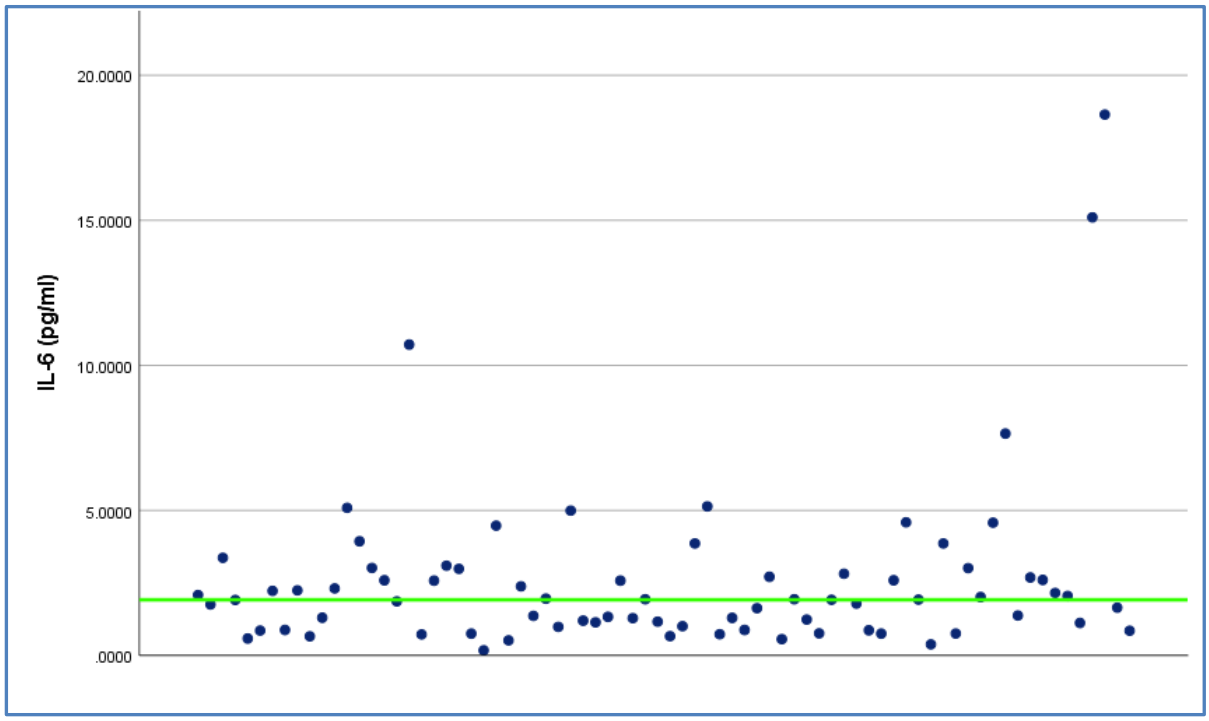


Figure 25: Scatterplot of IL-6 concentrations around the median

The pie chart illustrates 26.3% (n=20) of the participants had low serum IL-6 concentrations. A total of 71.1% (n=54) of participants had IL-6 concentrations within the normal range of 1.1 – 14.3 pg/mL. The category at risk of CVD with a high IL-6 serum concentration comprised of 2.6% (n=2) of participants.

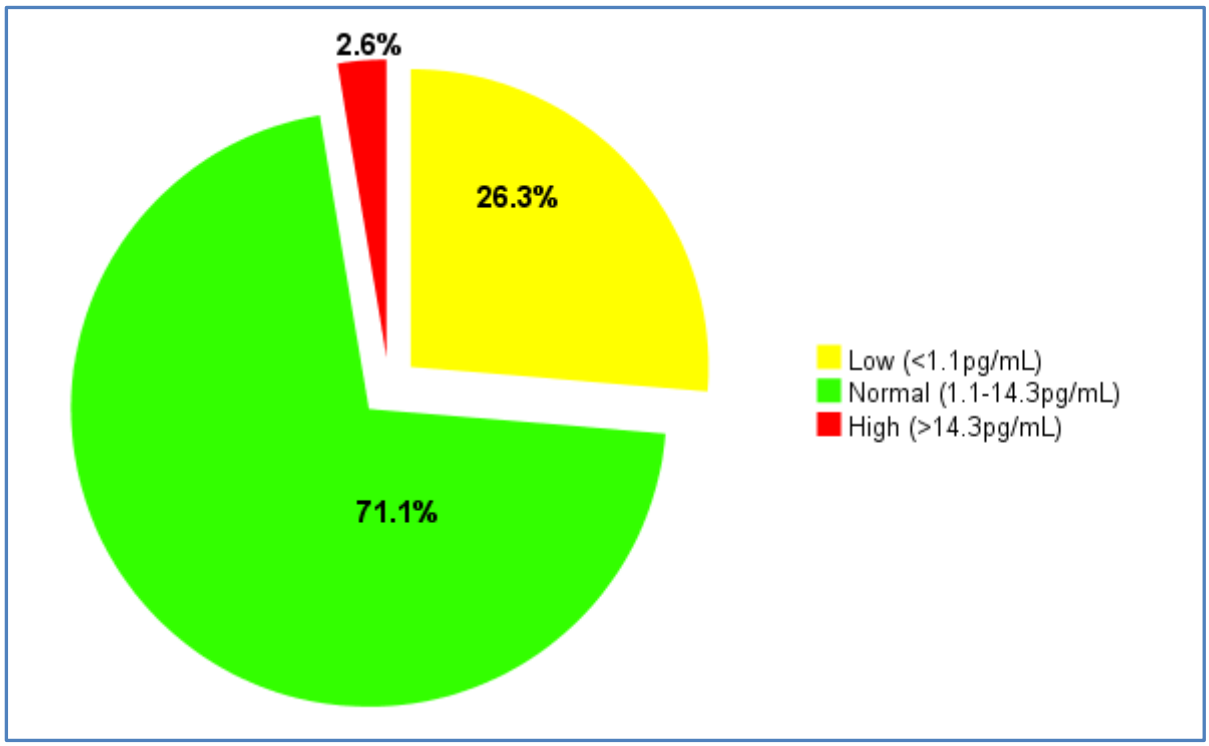


Figure 26: Pie chart of clustered of IL-6 concentrations

4.4.4. C174G polymorphism

In the sample population only one participant (1.4%) had the heterozygous genotype CG. There were no participants with the CC genotype and as indicated in Figure 27, 98.6% (n=69) of participants had the homogenous GG genotype. The chi-squared P value was 0.95 which is higher than 0.05 and therefore consistent with the Hardy-Weinberg equilibrium.

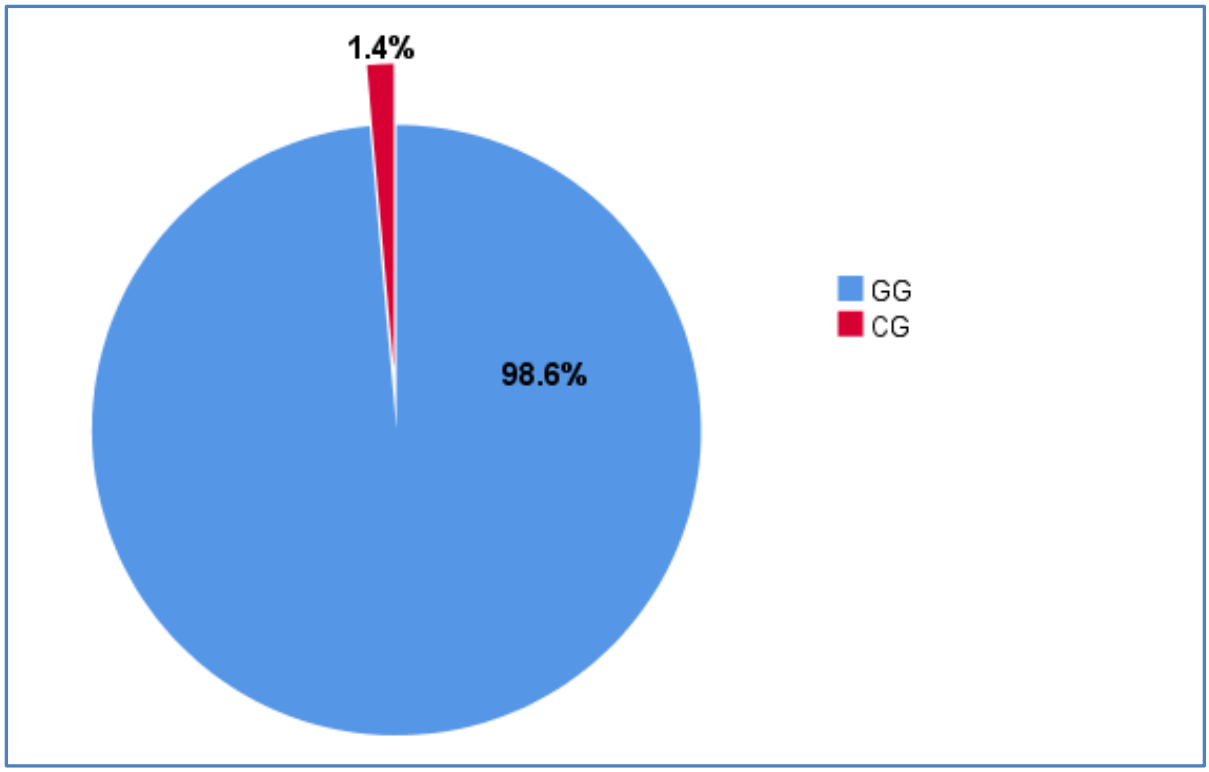


Figure 27: Pie chart – frequency distribution of C174G polymorphism

4.4.5. Correlations

Correlations were analysed in IBM SPSS Statistics version 25 using the Spearman's rho (also known as Spearman's rank correlation coefficient) since HS-CRP, TNF-α and IL-6 results were not distributed normally. A probability (p) value of less than 0.05 was considered a significant correlation. There was a correlation between HS-CRP and IL-6, with a p-value of 0.001. No correlation was found between HS-CRP and TNF-α. There was also a correlation between IL-6 and TNF-α with a p-value of 0.048, as seen in Table 13.

Table 12: Correlation coefficient values for HS-CRP, IL-6 and TNF-α

	HS-CRP	TNF-α	IL-6
HS-CRP		0.330	0.001*
TNF-α	0.330		0.048
IL-6	0.001*	0.048*	

* correlation is statistically significant at ($p < 0.05$)

The medians of the GG genotype participants are compared to the medians of the participant with the CG genotype in Table 14, using listwise exclusions.

Table 13: Median values of HS-CRP, IL-6 and TNF-α for GG and GC alleles (listwise exclusion)

	GG	GC
HS-CRP	6.32mg/L	4.13mg/L
TNF-α	0.00pg/mL	0.06pg/mL
IL-6	1.9pg/mL	1.14pg/mL

The medians of both the GG and GC genotypes for HS-CRP fall within the very high risk category. The medians for TNF-α and IL-6 for both genotypes fall within the normal ranges of these analytes. Though a very small percentage of participants had the C allele, the prevalence is supported by literature and the serum IL-6 levels of the different genotypes were correlated. Though the medians for serum HS-CRP and IL-6 serum for the GG group were higher than that of the GC group, the difference is not significant ($p = 0.623$ and $p = 0.627$, respectively).

4.5. Discussion

The study aimed to determine the prevalence of the C174G polymorphism in an elderly black South African population. It also determined the inflammatory status of the population by measuring not only the serum HS-CRP concentration of participants, but also serum TNF-α and IL-6 concentrations. The study further correlated the genotypes of the polymorphism with serum IL-6 concentrations. There are significant variations in the prevalence of CVR factors in populations of different ethnicity, demographics and environmental factors, highlighting the need for population-based risk analysis (Kurian & Cardarelli 2007; Graham 2014).

As an independent CVR marker, HS-CRP was measured to determine the inflammatory state of the sample population. The median CRP concentration of 6.44 mg/L was within the category at very high risk of CVD with 75% of the population at high or very high risk for CVD. The results confirm the findings of Grobler (2015) that this population is at risk for CVD due to their inflammatory state, who reported the mean concentration of this population to be 6.28 mg/L with 68% of the population at high or very high risk of developing CVD. It is noted that the inflammatory status of this elderly population has worsened since the study conducted by Grobler, the mean HS-CRP has increased and there was an increase in the prevalence of individuals at high or very high risk of CVD. In another study conducted in the South West Township of Johannesburg in South Africa the majority of the African participants of the Nguni and Sotho chiefdoms were also reported to be low risk (25.2), moderate risk (18.1%) and high

risk (56.7%) of developing CVD based on their elevated HS-CRP levels (Redelinghuys et al. 2011).

The Profiles of Obese Women with Insulin Resistance Syndrome (POWIRS) study which was performed on urban African women and Caucasian women found that though CRP levels were elevated in both ethnic groups; however, more so in African women compared to Caucasian women (Tolmay et al. 2012). Black South Africans also had a higher risk of CVD based on increased serum HS-CRP levels than Caucasian South Africans in the South African investigation on the role of Sex, Age and Ethnicity on Insulin sensitivity and Cardiovascular function (SAfrEIC) study (Kruger et al. 2013) and the Sympathetic Activity and Ambulatory Blood Pressure in Africans (SABPA) study (van Vuren et al. 2016). Black South Africans are at higher risk of CVD based on increased HS-CRP serum levels than their Caucasians counterparts in the same demographic setting. Thus, HS-CRP is a risk marker which is influenced by ethnic background more than demographics. This once again emphasizes the need to risk profiling that incudes environmental, demographic, ethnic and genetic factors.

A study conducted in South African children also reported a higher median HS-CRP in African than in Caucasian children. In comparison to this study, the black South African children have a lower median HS-CRP than the elderly black South Africans. These results are supported by literature which shows that Africans overall have higher serum levels of HS-CRP due to ethnic or genetic factors. The elderly population has an even higher HS-CRP level due to the presence of an additional CVR factor, inflammaging (Rensburg et al. 2012).

High-Sensitivity C-reactive protein is a CVR marker that should not be ignored as highlighted in the South African component of the international Prospective Urban and Rural Epidemiology (PURE) study, in which it was concluded that increased serum CRP levels correlated with cardiovascular death and the means of non-survivors were higher than the mean serum levels of the survivor (Botha 2015). It can thus be utilized as an independent marker for CVR especially in black and elderly individuals. The fact that both the elderly population and the African population is at the highest risk based on their inflammatory status as measured by HS-CRP, should be a very important focus point in CVD prevention and treatment by health professionals of South Africa.

As found in this study, various studies globally reported that the median HS-CRP of African American participants was elevated (Effoe et al. 2015; Lin et al. 2016). Previous studies in other populations, worldwide, found that the serum HS-CRP of African Americans was higher than those of Caucasian participants (Slopen et al. 2010; Windham et al. 2016) and the

median was higher than that of European participants (Halcox et al. 2014). This may be attributed to the ethnic link between African Americans and black South Africans.

A study conducted in South African participants with a low-grade inflammatory state (CRP > 3 mg/L) reported that Africans had higher fibrosis values, but lower serum IL-6 and TNF-alpha levels than Caucasians (Jansen van Vuren et al. 2016). This study also found that the serum IL-6 of 1.92 pg/mL and TNF-α of 0.00 pg/mL are normal in the sample population, even though the population had an increased risk of CVD. Therefore, this suggests that these factors could be used as independent CVR markers in black South Africans.

Other studies that were conducted globally reported higher serum IL-6 and TNF-α levels in African Americans than in Caucasians and with higher means than that of the elderly Sharpeville population (Slopen et al. 2010; Pine et al. 2016; Windham et al. 2016). In a study conducted in Europe on elderly participants which compared myocardial infarction (MI) and stroke cases with controls, it was reported that the mean serum TNF-α of the MI cases were significantly ($p < 0.001$) higher than the controls and it was higher than that found of the black elderly Sharpeville population. Stroke cases had higher IL-6, but not higher serum CRP levels than the controls (Jefferis et al. 2009) . Taking these studies and the current study into consideration it can be reasoned that IL-6 and TNF-α might be more susceptible to behavioural, environmental, social factors or demographics than HS-CRP, as these are not linked to ethnicity.

In a study by (Jefferis et al. 2009), TNF-α showed a positive association ($p < 0.05$) with IL-6 and CRP. TNF-α in this study did not correlate with HS-CRP and IL-6, which may suggest that TNF-α does not increase when CVR occurs in the black South African population as found in global studies (Cui et al. 2012; de Gonzalo-Calvo et al. 2012).

A study evaluated prevalence of the C174G polymorphism in cases of spontaneous abortion and controls in South African females, of the controls 211 (74%) had the genotype GG, 63 (22%) had genotype GC and 10 (4%) had genotype CC. Of the participants, 171 (20%) had the GG genotype, 95 (34%) had GC genotype and 18 (6%) had CC genotype (Liu et al. 2015). Though the study did not report race, the high prevalence of GG genotype and low prevalence of GC fairly relates to the Sharpeville population. A study conducted in 86 randomly selected pure descendants of the Nguni speaking Zulu's in Kwa-Zulu Natal South Africa reported that only one participant (1.2%) had the C allele in the GC genotype and none of the participants had the CC genotype (Meenagh et al. 2002). Another study reported that only 2% of the young African males studied had the C allele (Phulukdaree et al. 2013). Joffe et al. (2014) conducted

a study using African and Caucasian obese and normal weight participants (age 18-45 years) in Cape Town, South Africa. A total of 97% of African participants had the GG genotype, 3% the GC genotype and none the CC genotype. Of the Caucasian participants, 26.5% had the GG genotype, 55.5% had the GC genotype and 18% had the CC genotype. The current study in the black Sharpeville population also found that 1.4% had GC genotype and none had the CC genotype. These results support literature in that genetic and ethnic diversity plays a role in allele frequencies.

Ibrahim et al. (2017) found that 57% of Egyptian adolescent participants had the GG genotype and 43% had the GC genotype. In a study evaluating the prevalence of genetic polymorphisms in systemic lupus erythematosus, the genotypes of the normal controls reported to be 75.6% GG, 23.5% GC and 0.9% CC. (Talaat et al. 2016). The prevalence distribution of alleles in Africans resembles that of South Africa as the prevalence of the CC genotype is also very low.

The Cardiovascular Health Study (CHS) reported that the genotype distribution of African Americans (aged 65 years and older) resemble that found in South Africa. The majority of African American participants (85%) had the GG genotype, followed by the GC genotype (15%) and no participants had the CC genotype (Jenny et al. 2002). A study performed in the USA using 138 African Americans and 140 Caucasians reported a similar genotype distribution. The African American participants primarily (74%) had the GG genotype, 22% had the GC genotype and 6% had the CC genotype. A highly significant difference ($p=0.0001$) was observed in the Caucasian participants of which 19% had the GG genotype, 34% had the GC genotype and 17% had the CC genotype (Mandal et al. 2014). The genetic similarity in allelic distribution can be observed between South Africans, Africans and Africans Americans.

Adler et al. (2016) reported the prevalence of the C174G polymorphism in a Polish sample population of 297 participants aged 63.5 ± 5.9 years. Of the 297 participants, 81 (27%) had the GG genotype, 147 (50%) had GC genotype and 69 (23%) had CC genotype. The results correlate with a study conducted in a Polish population by Popko et al. (2008) in which 44 (25%) participants had the G genotype, 86 (48%) had the GC genotype and 49 (27%) had the CC genotype. A study in an Irish population by Meenagh et al. (2002) reported 30 participants with the GG genotype, 45 participants with the GC genotype and 22 participants with the CC genotype. Reichert et al. (2016a) performed a study using 895 participants in central Germany of which 29.7% had the GG genotype, 48.8% had the GC genotype and 21.5% had the CC genotype. This indicates that Europeans had the highest prevalence of the GC genotype. iGiannitrapani et al. (2013) reported that Asian populations have a lower frequency of the C

allele than Caucasian populations. In a north Indian population, the CC genotype was rare, and GG was most prevalent (Saxena et al. 2014).

In a meta-analysis of the C174G polymorphism and susceptibility to CVD it was reported that ethnic differences had an effect on heterogeneity. Their evaluation of the polymorphism in Asian and Caucasian populations showed that the GG genotype is the most prevalent in both ethnicities, followed by less prevalent GC genotype and the least prevalent CC genotype. (Yuepeng et al. 2019). The same pattern of prevalence was reported by Barartabar et al. (2018) for the Irian population.

Not only is there a similarity between the allelic distribution of South Africans, Africans and African Americans, but to an extent the majority of global studies. Global studies also showed a higher prevalence of the G allele except in Europeans who had highest prevalence of GC genotype.

The C174G, leading to the substitution of guanine with cytosine at position –174 has had varying effects on serum levels of IL-6 in different populations. The relationship of the C174G polymorphism with increased or decreased serum IL-6 levels different amongst ethnic groups as well as geographical regions (Fishman et al. 1998; Ma et al. 2016).

A study performed in young male African and Indians in South Africa reported that participants with CC genotype had higher serum IL-6 levels than other genotypes. Healthy Indian controls in this study showed significantly ($p=0.0001$) higher serum IL-6 levels for participants with the CC genotype compared to that of GC and GG genotypes (Phulukdaree et al. 2013).

Jenny et al. (2002) reported that the C allele showed a trend ($p=0.163$) towards increased serum IL-6 and no significant difference was observed between IL-6 serum levels of Caucasians and African Americans in the CHS cohort. Bezuch et al. (2019) found no significant differences between the plasma IL-6 levels of participants with the GG genotype and C allele carriers of a European population. Another study in a European population also found no significant difference in the serum IL-6 level of the different genotypes (Mohlig et al. 2004). On the contrary a study performed in the United Kingdom, Fishman et al. (1998) found that Caucasian participants with the GG genotype had serum IL-6 levels almost twice that of the participants with GC genotype. In support, Popko et al. (2010b) also reported the highest serum IL-6 levels in control group participants with the GG genotype, lower IL-6 levels in those with the GC genotype and the lowest serum IL-6 levels in participants with homozygous CC. The overall serum IL-6 levels of the obese group were higher than that of the control group.

In a meta-analysis performed by Bashashati et al. (2017), there was no significant difference in the prevalence of the C allele in controls and irritable bowel syndrome cases. However, there was a significant difference in the serum IL-6 level between experimental and control samples. The researchers concluded that the increased serum IL-6 levels are not caused by the presence of the C or G allele, but rather the presence of irritable bowel syndrome itself. Similarly, Testa et al. (2006) reported that the C allele did not contribute to increased serum IL-6 in a study of an Italian population, but rather that the presence insulin resistance resulted in increased serum IL-6 levels. The results of the current study suggest that the high producer IL-6 genotype was the GG genotype in the black elderly Sharpeville population. However, it should be taken with caution as only one participant had the GC genotype.

As supported by the literature review, the results from other studies performed globally shows evidence that elderly individuals have a higher risk of CVD based on their increased inflammatory state caused by physiological changes of aging. This is also evident in the current study, emphasizing the importance of inflammatory markers during CVR profiling of an elderly individual. These studies also shed light on the ethnic and genetic links between populations when risk profiling CVD, with a clear link between black or African populations who have a higher inflammatory profile than the European descendants. The allelic distribution once again relates to that of studies in populations of similar ethnicity. The varying outcomes of the high or low IL-6 producing genotypes in different populations emphasizes the realization of the complexity of CVR of the numerous factors involved. Though the role of genetics in CVR is undeniable, so is that of behavioural, demographic and environmental factors.

4.6. Conclusion

The median serum HS-CRP of 6.44 mg/L of the elderly black Sharpeville population fell within the highest risk (>5mg/L) of CVD. A total of 75% of participants were at high (3.01-5 mg/L) or very high (>5mg/L) risk of CVD based on their HS-CRP results. The median TNF-α was within the normal range (0.0-3.22 pg/mL) and only 2.6% of participants had high serum TNF-α levels. The median serum IL-6 level also fell within the normal range (1.1-14.3 pg/mL) with only 2.6% of participants having high serum IL-6 levels.

Only one participant had the C allele as heterozygous GC, which is consistent with findings in other African populations. The median serum IL-6 level of the homozygous GG group (6.51 mg/L) was higher than the 4.13 mg/L IL-6 of the heterozygous GC participant. A significant (p=0.001) correlation was found between HS-CRP and IL-6 of the elderly black Sharpeville population.

CHAPTER 5
CONCLUSION AND RECOMMENDATIONS

5.1. Introduction

Through addressing the aims and objectives, the findings of this study brought insight to the prevalence of the C174G polymorphism and inflammatory status of the elderly black South African population of South Africa. Together with the literature review it also highlighted the role that ethnicity and genetics play in risk profiling of CVD in different populations. With CVD as the major cause of deaths in non-communicable diseases it is important to study factors affecting CVD in all the different populations, regardless how big or small, rich or poor, young or old (World Health Organization 2016b). Risk profiling of the elderly black South African population in Sharpeville is even more valuable taking into consideration that the majority of these elderly individuals are living in poverty, yet they are the heads of households and caregivers of children (Grobler 2015).

5.2. Researcher's Contribution

This study was conducted as a part of a multidisciplinary, multi-nutritional programme of the CSL at VUT under the leadership of Prof W.H. Oldewage-Theron. The team of which the researcher was part during this study included a nutritionist, dietician, phlebotomists, statistician and medical technologists. Funding was acquired through National Research Foundation for the various studies focussing on cardiovascular disease as a component of the programme, under leadership of Dr C.J. Grobler. Ethical clearance was obtained from VUT and WITS as mentioned in Chapter 1.

The researcher is a HPCSA registered medical technologist and took responsibility for the planning, analysis and reporting of this study, starting with problem identification, study design and proposal writing. The researcher was also involved in application for funding of the study. Furthermore, the researcher contributed in planning of fieldwork and took part in data collection during fieldwork. The researcher is personally responsible for blood analysis and received technical guidance form Dr. J. Lebea at Council for Scientific and Industrial Research (CSIR). Data analysis and statistical interpretation of results were performed by the researcher with use of IBM SPSS® Statistics version 25 software. With guidance of the supervisor Dr C.J. Grobler, the researcher analysed and interpreted the results, wrote this dissertation towards obtaining MTech Biotechnology qualification and wrote articles to be published in future. The researcher presented a component of the findings at the Medical Laboratory Professionals

(MLP) congress in 2015 and submitted an abstract for presentation at the MLP congress in 2019.

5.3. Limitations of the Study

Phlebotomists experienced some challenges during blood collection resulting in some samples having insufficient sample volume for all parameters, HS-CRP was performed on the total 84 samples, TNF-α and IL-6 had 76 samples and the C174G analysis for performed using 70 samples. The missing samples were addressed through pairwise exclusion of data in analysis of correlations. Though samples were less for C174G prevalence determination, the number of samples analysed were more than the minimum number of 30 samples required based on the sample size calculation as indicated in 3.3.1. of the dissertation.

Gender-based comparisons were not done in this study due to the unequal gender distribution of participants. Only 18% of participants were male, compared to the 82% female participants.

The study did not take into consideration the presence of inflammatory diseases such as arthritis or use of anti-inflammatory medication in the study population, which could affect the serum level of inflammatory markers. Other CVR factors such as smoking, diabetes, increased cholesterol and hyperhomocysteinaemia among others which contribute to increased inflammatory state were also not considered in the study.

The evaluation of median serum IL-6 levels based on the genotype was done with only one participant that had the C allele, compared to the 69 participants with homozygous GG genotype. However, the low prevalence of the C allele corresponds to literature.

5.4. Main Findings

The main findings of this study are outlined as follows:

5.4.1. Problem and setting

- The elderly population has grown worldwide in past years and is expected to continue growing substantially, with the rate of population growth in developing countries surpassing that of developed countries (Chatterji et al. 2015; United Nations 2015b).

- It is common in South Africa that households with three generations rely on the pension income of an elderly due to lack of resources (Oldewage-Theron & Slabbert 2010; van der Pas et al. 2015).
- The number of elderly headed households increased by 5.6% from 14,6% in 1996 to 20,2% in 2011 (Statistics South Africa 2014).
- The elderly population of Sharpeville lives in poverty (Grobler 2015).
- The elderly Sharpeville population are at risk of CVD as the majority are overweight or obese and have increased serum HS-CRP (Oldewage-Theron et al. 2015).
- The C174G polymorphism plays a role in IL-6 production, though the prevalence and stimulatory effects thereof varies in studies done in different populations (Kumar et al. 2015).
- Published data on the prevalence of the C174G polymorphism in black South Africans is very limited (Meenagh et al. 2002; Phulukdaree et al. 2013; Joffe et al. 2014).

5.4.2. Literature review

- Cardiovascular diseases are the leading cause of deaths globally (World Health Organization 2016b).
- The term CVDs refers to a group of diseases which affect the heart and vascular system (World Health Organization 2011).
- The majority of CVDs are caused by atherosclerosis, an inflammatory process which results in plaque formation in the coronary arteries and decreased blood supply to the heart due to failed resolution of inflammation (Viola & Soehnlein 2015).
- Cardiovascular risk factors such as smoking, hyperglycaemia, dyslipidaemia, hyperhomocysteinaemia, hypertension and old age act as atherogenic stimuli, triggering the inflammatory response through endothelial injury, leading to plaque formation and atherosclerosis (Park & Park 2015).
- The physiological changes caused by aging, especially in the vascular endothelium increases the risk of CVD in elderly.
- Atherosclerosis and plaque formation are characterized by endothelial dysfunction, vascular inflammation and accumulation of modified lipids, inflammatory cells and cellular debris in the plaques within the vascular wall (Viola & Soehnlein 2015).
- Different risk prediction formulae are used to determine a patient's risk of developing CVD, the common systems currently used are the Framingham Risk Score, Reynolds Risk Score and the Systematic Coronary Risk Evaluation (Liao & Solomon 2013).

- Risk factors are classified as modifiable or non-modifiable based on the premise that an individual can change the presence/effect of the risk factor (Douglas & Channon 2014).
- Cardiovascular risk markers used to evaluate CVR factors include physical biometric measurements and biochemical measurements (Mensah 2013).
- Population based risk stratification are important not only as genetic factors that influence CVR, but also environmental factors (Kumar et al. 2015).
- Inflammation is a process protecting against infective and tissue injury, with the end result being healing. However, when inflammation becomes chronic or is not resolved, it causes damage (Franceschi & Campisi 2014).
- Inflammatory mediators are responsible for initiation, intensification and resolution of inflammation (Porth 2015).
- Changes due to aging places the elderly in a chronic state of low grade inflammation, which has become known as “inflammaging” (Franceschi et al. 2000a).
- C-reactive protein is an acute phase protein, primarily synthesized by the liver in response to IL-6 stimulation and to a lesser extent TNF-α (Thiele et al. 2015).
- Even slight increases in serum CRP levels, within the ranges which were previously thought to be normal, were associated with the risk of developing CVD (Ridker et al. 1997; Salazar et al. 2014).
- The term HS-CRP is used when referring to testing CRP levels with sensitivity below 5 mg/L to detect low grade inflammation (Ziv-Baran et al. 2017).
- Elevated serum levels of CRP have been proven to be an independent risk marker of CVD (Greenland et al. 2010; Abu-Farha et al. 2014).
- TNF-α is a cytokine that primarily exerts pro-inflammatory functions, though it also performs anti-inflammatory functions (Blandizzi et al. 2014).
- Increased serum TNF-α has been associated with traditional CVR factors and CVD (Mendall et al. 1997; Cui et al. 2012).
- The cytokine IL-6 is a pro-inflammatory cytokine which plays a major role in the production of acute phase reactants, especially CRP (Ataie-Kachoie et al. 2014).
- Increased serum IL-6 levels have been associated with CVD (Mendall et al. 1997; Didion 2017).
- The C174G (rs1800795) polymorphism is a single nucleotide polymorphism (SNP) at position 174 in the promotor region of the IL-6 gene, the mutation causes a change from guanine (G) to cytosine (C) (Yuepeng et al. 2019).

  Reviews in different ethnic populations have emerged with varying results as to which genotype is regarded as the high producer of IL-6. In some populations an increase in

serum IL-6 levels is associated with the homozygous CC genotype, while in others increased serum IL-6 is associated with the G allele in CG and GG genotypes (Nadeem et al. 2013).

5.4.3. Methodology

- This study was ethically approved and applied principles of autonomy, beneficence, non-maleficence and equal distribution of risk throughout the study.
- The sample size was greater than the minimum sample size required to determine the statistical significance of the prevalence of the polymorphism in the sample population, as calculated using the power calculation.
- The study was performed on volunteers of 60 years and older who gave consent to participate during their attendance at the Sharpeville day care centre and did not suffer from any conditions which prevented them from given the required information for this study.
- The study was quantitative, cross-sectional, and analytical observational design.
- Serum HS-CRP was measured using the Thermo Scientific Konelab™ 20 chemistry auto-analyser, IL-6 was measured using the IBL International enzyme linked immunosorbent IL-6 assay, TNF-α was measured using the IBL International enzyme-linked immunosorbent TNF-α assay kit and detection of the C174G polymorphism was done with PCR, MassARRAY Compact mass spectrometer and Agena real-time detection software.
- Raw data was captured on Microsoft Excel and imported to IBM SPSS® Statistics version 25 software for descriptive and inferential statistical analysis.

5.4.4. Results and interpretation

The median serum level of HS-CRP was found to be 6.44 mg/L (IQR 2.82 - 9.86 mg/L), which fell within the category of very high risk for CVD. The median serum TNF-α level was within the normal range of 0.0-3.22 pg/mL and measured at 0.00 pg/mL (IQR 0.00 – 0.05 pg/mL). The median serum IL-6 level also fell within the normal range of 1.1 – 14.3 pg/mL and was found to be 1.92 pg/mL (IQR 0.99 – 2.79 pg/mL).

Determination of the prevalence of the C174G polymorphism resulted in the genotype distribution as follows, 98.6% of participants had the homozygous GG genotype, 1.4% had the heterozygous CG genotype and no participants had the homozygous CC genotype.

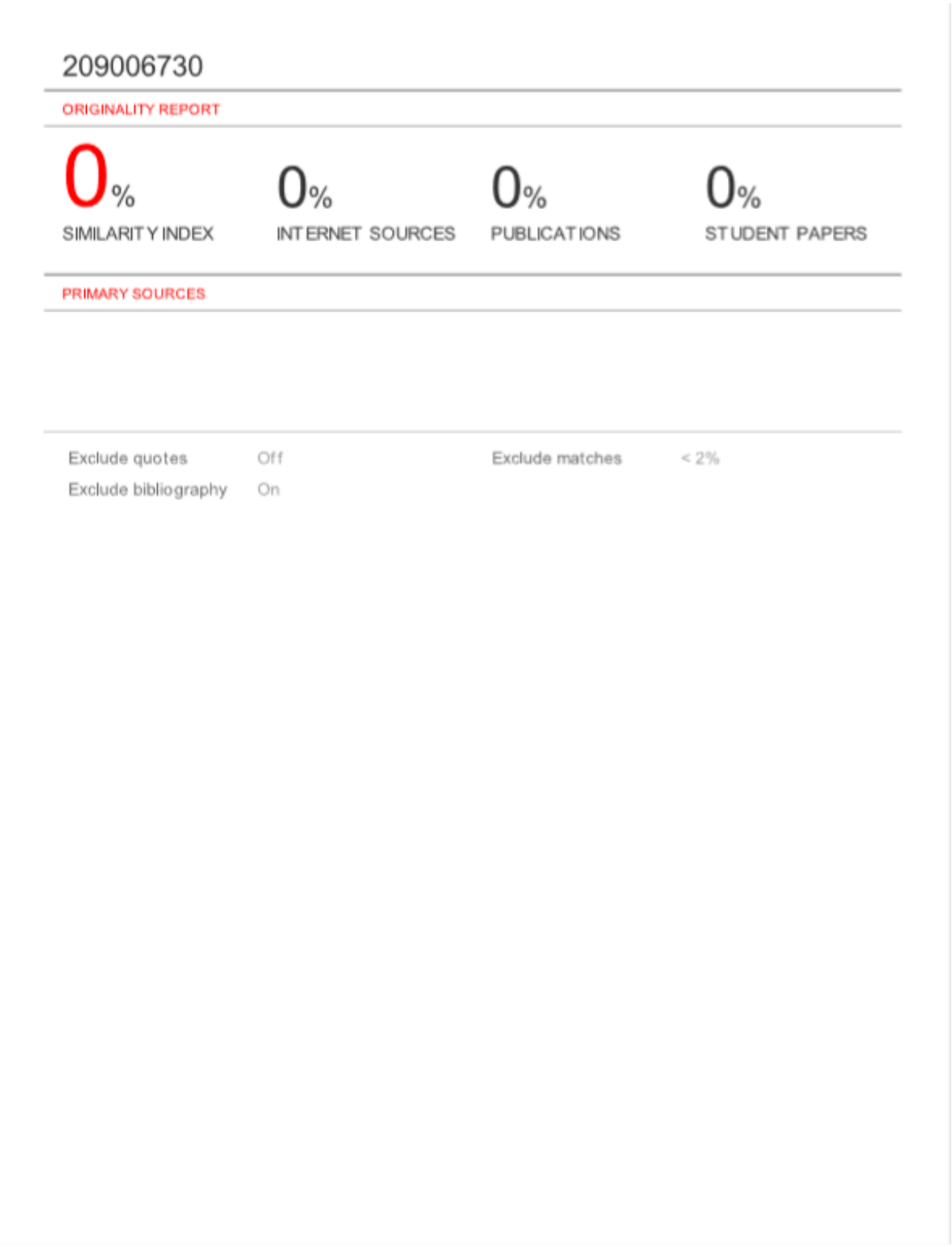
Though a very small percentage of participants had the C allele, the prevalence is supported by literature and the serum IL-6 levels of the different genotypes were correlated. The median serum IL-6 level of the homozygous GG group (6.51 mg/L) was higher than the 4.13 mg/L IL-6 of the heterozygous GC participants, however, the difference in IL-6 should be considered with caution as only one participant had the C allele. There was a statistically significant ($p=0.001$) correlation between HS-CRP and IL-6 of the elderly black Sharpeville population. There was also a correlation between IL-6 and TNF-α ($p=0.048$), but there was no correlation between HS-CRP and TNF-α.

5.5. Conclusion and Recommendations

In conclusion the elderly black South African population residing in Sharpeville are in an increased inflammatory state. Being in a state of increased inflammation places these elderly residents at risk of CVD. There is a low prevalence of the C174G polymorphism C allele in the study population; therefore, it should be considered with caution that the more prevalent G allele is indicated as the higher producer of serum IL-6 ion this population. The study achieved its aim of evaluating the prevalence of the C174G polymorphism, in correlation with the inflammatory biomarkers, HS-CRP, TNF-α and IL-6 as CVR markers in the elderly black Sharpeville population. Quality measures and good laboratory practices were implemented to ensure reliable and reproducible results. The outcome of the study will be communicated to the Sharpeville day care centre management and attendees.

It is recommended that from this study, that future studies include a questionnaire to determine the presence of other inflammatory conditions in the elderly and the presence of other CVR factors including but not limited to smoking, hypertension, diabetes and dyslipidaemia which could be the underlying cause of inflammation. Further studies should also take into consideration the use of anti-inflammatory medication. From the results of the study it is recommended that further studies be done as intervention studies to decrease the inflammatory state of the elderly. Further studies should be conducted in South African populations similar to the elderly Sharpeville population where elderly are the breadwinners and caregivers, to motivate policy changes for the use of more accurate CVR profiling markers to be used at clinics.

Annexure A



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PRIMARY SOURCES

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Annexure B

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** [redacted]
(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 C

VAAL UNIVERSITY OF TECHNOLOGY

RESEARCH & INNOVATION

ETHICAL CLEARANCE CERTIFICATE

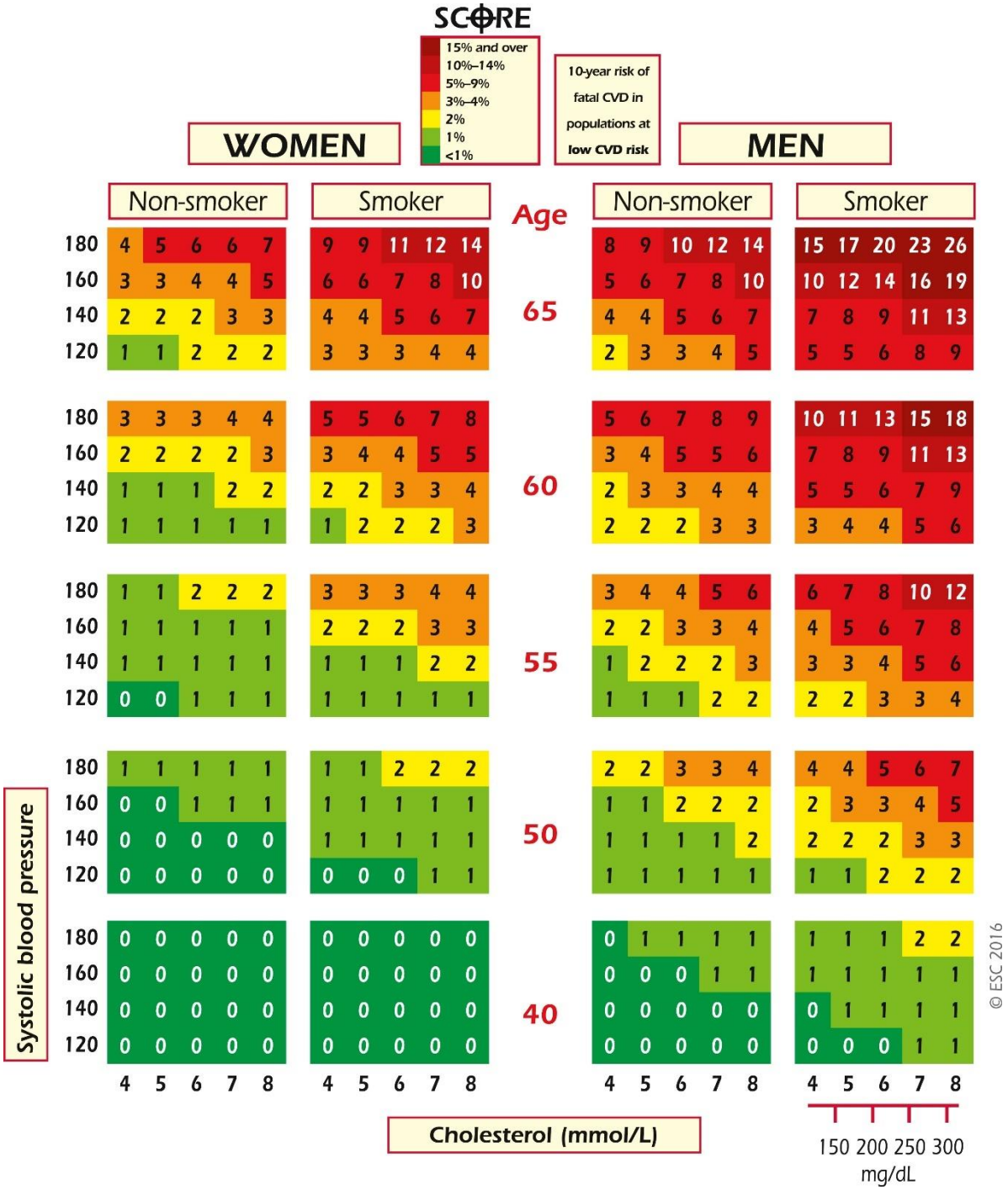
Applicant:	Mrs. Christa Grobler
Project:	To establish the effect of vitamin B6 supplementation on the cardiovascular risk (CVR) and to determine the prevalence of genetic polymorphisms in correlation with CVR markers, in a black elderly community in Sharpeville.
Institution:	Vaal University of Technology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

Approved by: BJ Johnson Date: 21/8/14

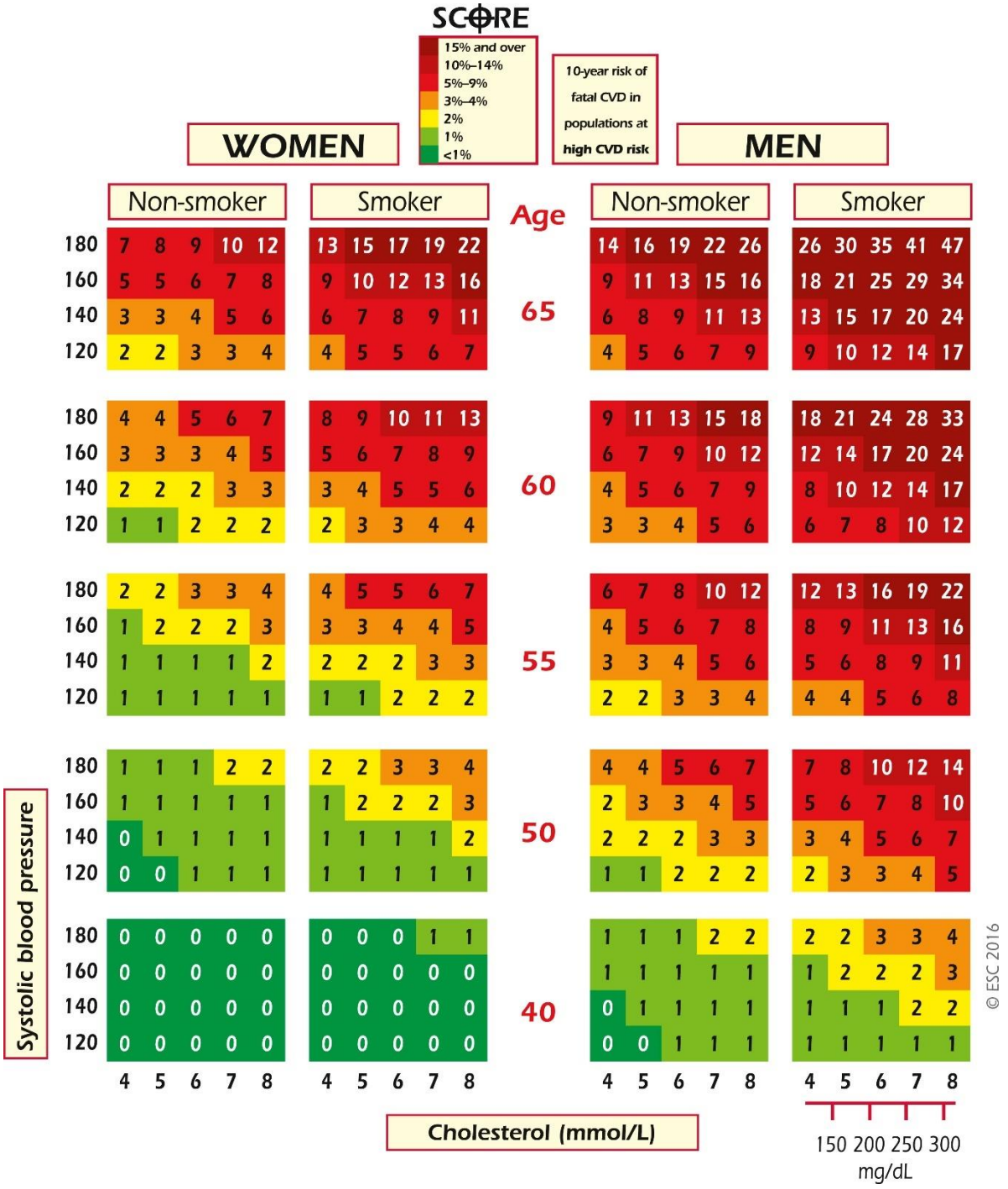
Dr. B.J. Johnson
Chairperson: Research & Innovation Ethics Committee

Private Bag X021 – Vanderbijlpark – 1900
Andries Potgieter Boulevard – South Africa
Tel: +27 16 950 9000 – www.vut.ac.za

Annexure D


SCORE
15% and over
10%–14%
5%–9%
3%–4%
2%
1%
<1%
10-year risk of fatal CVD in populations at low CVD risk
WOMEN
MEN
Non-smoker
Smoker
Age
Non-smoker
Smoker
65
60
55
50
40
Systolic blood pressure
Cholesterol (mmol/L)
150 200 250 300
mg/dL
© ESC 2016

Annexure E


SCORE
15% and over
10%–14%
5%–9%
3%–4%
2%
1%
<1%
10-year risk of fatal CVD in populations at high CVD risk
WOMEN
MEN
Non-smoker
Smoker
Non-smoker
Smoker
Age
65
60
55
50
40
Systolic blood pressure
180 160 140 120
4 5 6 7 8
Cholesterol (mmol/L)
150 200 250 300
mg/dL
© ESC 2016

Annexure F

Informed Consent Form

Person to contact in the event of any problems or queries:

Dr C.J. Grobler	Prof W.H. Oldewage-Theron
016 950 9210	016 930 5085

Statement of agreement to participate in the research study:

I _____ (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 _____ (fieldworker name) 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.

Name: _____ Signature: _____

Date: _____

Researcher's Name: _____ Signature: _____

Date: _____

Witness Name: _____ Signature: _____

Date: _____

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