Interactions of CRP-SNPs with selected contributing factors in determining CRP concentrations in black South Africans

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In loving memory of my two dads, **Francois Myburgh** and **Dries van Coller**, to whom I dedicate my thesis.

It was a privilege learning to be a man from the both of you.

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ABSTRACT

Introduction: Non-communicable diseases, especially cardiovascular diseases, are on the rise globally, with low- and middle-income countries, including South Africa, at the epicentre of the increase in these conditions. The inflammatory origin of these diseases is increasingly being reported; however, most of the conducted research is focused on white populations. C-reactive protein (CRP) is of particular interest, as elevated concentrations, which are commonly observed in black individuals, are indicative of future cardiovascular disease risk, and limited information is available for this particular group.

Objectives: We investigated whether specific factors, identified from an initial literature review were associated with elevated CRP concentrations in black individuals (as compared to white individuals), and if these factors associated with *CRP* single nucleotide polymorphisms (SNPs) in a manner that could alter the CRP phenotype. These specific factors that were identified from a thorough review of the current literature included (i) factors pertaining to socio-economic status or SES, (ii) lower vitamin D status and (iii) anthropometric markers together with a metabolic determinant of CRP: interleukin-6 (IL-6).

Methods: This investigation was embedded in the Prospective Urban and Rural Epidemiology (PURE) study, where 2 010 apparently healthy participants were included at baseline in 2005. Data were collected on factors pertaining to their SES, dietary intake and various anthropometric, biochemical and physiological markers. Twelve *CRP* SNPs, previously analysed, were included in this study to investigate possible gene–environmental effects.

Results: Overall, women presented with higher CRP concentrations than men. Apart from attaining twelve or more years of formal education, none of the other SES factors resulted in individuals presenting with an altered *CRP* phenotype. We also reported for the first time that harbouring the variant allele at the rs3093068 locus was associated with an increased risk of developing elevated CRP in smokers compared with non-smokers.

No vitamin D x *CRP* SNP interactions were observed, although women co-presenting with low levels of vitamin D and high CRP concentrations were more likely to have poorer cardiovascular outcomes. However, some *CRP* polymorphisms were associated with an increased risk of presenting with an altered CRP phenotype when also harbouring this co-phenotype.

Both IL-6 and CRP concentrations were elevated in individuals with increasing body weight, and waist circumference (WC) was an important predictor of elevated CRP. CRP was determined to be elevated in certain genotypes, even when similar IL-6 concentrations were observed between these alleles, indicating that the genetic variation had a greater effect on the expression of the

CRP protein. It was established that WC, as a marker of abdominal adiposity, contributed substantially towards the elevated CRP concentrations.

Conclusion: Here we extended the literature by investigating non-biological and biological factors that we gleaned from the literature to be of importance when trying to shed light on the regulation of CRP concentrations. We ascertained that certain demographic characteristics – smoking, vitamin D and anthropometry – were all determinants of the CRP phenotype of black individuals. As certain of these determinants are modifiable, we recommend that healthcare providers reduce CRP by educating/treating patients to optimise their vitamin D status and manage their WC and thus possibly curb diseases contingent on inflammation. This study highlights the need for the development of population-specific preventative strategies to overcome the pandemic of cardiovascular disease.

Keywords: CVD, inflammation, socio-economic status, C-reactive protein, interleukin-6, waist circumference, 25-hydroxyvitamin D, nutrigenetics, single nucleotide polymorphisms, Tswana

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LIST OF ABBREVIATIONS

25(OH)D	25-hydroxyvitamin D
A	adenine
AIC	Akaike information criterion
AIDS	acquired immune deficiency syndrome
ANCOVA	analysis of co-variance
ANOVA	analysis of variance
ARV	anti-retroviral
AUTHeR	Africa Unit for Transdisciplinary Health Research
BMI	body mass index
BPM	beats per minute
C	cytosine
CEN	Center of Excellence for Nutrition
CRISPR	clustered regularly interspaced short palindromic repeats
CRP	C-reactive protein
CVD	cardiovascular disease
dbSNP	database of single nucleotide polymorphisms
DNA	deoxyribonucleic acid
G	guanine
GWAS	genome-wide association studies
HbA_{1C}	glycated haemoglobin
HDL-c	high-density lipoprotein cholesterol
HIV	human immunodeficiency virus
HREC	Health Research Ethics Committee
HWE	Hardy–Weinberg equilibrium
JAK	Janus kinase
JIS	joint interim statement
IJERPH	International Journal of Environmental Research and Public Health
IL-1	interleukin-1
IL-6	interleukin-6
IQR	interquartile range
LD	linkage disequilibrium
LDL-c	low-density lipoprotein cholesterol
LLMIC	low-income and lower-middle-income countries
LMIC	low- and middle-income countries
LOD	limit of detection
MRC	Medical Research Council

n	number of individuals
NCD	non-communicable disease
NHLS	National Health Laboratory Services
NRF	National Research Foundation
NS	not significant
NWU	North-West University
OR	odds ratio
PHRI	Population Health Research Institute
PRIMER	Profiles of Resistance to Insulin in Multiple Ethnicities and Regions
PURE	Prospective Urban and Rural Epidemiology
qFFQs	quantitative food frequency questionnaires
RAS	renin–angiotensin system
ROS	reactive oxidative species
RR	relative risk
rs	reference sequence
SAfrEIC	Sex, Age and Ethnicity on Insulin sensitivity and Cardiovascular function
SANPAD	South African Netherlands Partnerships in Development
SD	standard deviation
SES	socio-economic status
SMAC	sequential multiple analyser computer
SNP	single nucleotide polymorphisms
(p)-STAT3	(phospho) - signal transducer and activator of transcription factor 3
T	thymine
TB	tuberculosis
TC	total cholesterol
UVR	ultraviolet radiation
WC	waist circumference
WHO	World Health Organisation

DEFINITIONS

Age-specific rates of death: the total number of deaths to residents of a specific age residing in a specific geographic area, divided by the population of the same age in the same geographical area for a specific time, multiplied by 100 000.

Creative Commons Licensing: Allows creators to retain copyright, while allowing others to copy, distribute and make some uses of their work.

Single nucleotide polymorphism: A difference observed in a single DNA building block or nucleotide.

Polymorphic: occurring in several different forms.

Sympatric: within similar or overlapping geographical regions.

CHAPTER 1: INTRODUCTION

1.1 Background and rationale

Non-communicable diseases (NCDs) are the leading cause of death globally, with 78% of the nearly 40 million deaths in 2015 attributed to NCDs occurring in low- and middle-income countries, or LMICs (World Health Organisation, 2017). The disproportionate burden of NCDs in LMICs has been called the "social justice issue of our generation" with significant increases occurring in the number of NCD fatalities in these countries since 2000 (Horton, 2015; World Health Organisation, 2017). In September 2011, the United Nations General Assembly adopted a political declaration which included nine voluntary targets for the prevention and treatment of NCDs by 2025, namely:

1. A 25% relative reduction in the overall mortality from cardiovascular diseases (CVD), cancer, diabetes, or chronic respiratory diseases;
2. At least 10% relative reduction in the harmful use of alcohol, as appropriate, within the national context;
3. A 10% relative reduction in the prevalence of insufficient physical activity;
4. A 30% relative reduction in mean population intake of salt/sodium;
5. A 30% relative reduction in the prevalence of current tobacco use in persons aged 15+ years;
6. A 25% relative reduction in the prevalence of raised blood pressure or contain the prevalence of raised blood pressure, according to national circumstances;
7. Halt the rise in diabetes and obesity;
8. At least 50% of eligible people receive drug therapy and counselling (including glycaemic control) to prevent heart attacks and strokes; and
9. An 80% availability of the affordable basic technologies and essential medicines, including generics, required to treat major NCDs in both public and private facilities.

[*Verbatim* (World Health Organisation, 2011)]

The targets above have a strong focus on modifying behavioural risks associated with the development of NCDs. Most of the research conducted on the aetiology of NCDs has been done in high-income countries, which means that LMICs must not only incorporate the existing literature into regulations promoting healthy living, but should also investigate new and alternative ways to curb the global rise in NCDs for themselves in resource-limited regions (Checkley *et al.*, 2014). South Africa, as a member of the United Nations, has since developed a strategic plan to reduce NCDs (Department of Health, 2013). Based on this strategic plan, several new regulations have been passed, including the elimination of trans fats and reduction of sodium in foodstuffs.

In a recent meta-analysis among low-income and lower-middle-income countries (LLMICs), including 20 African countries, Allen *et al.* (2017) indicated that socio-economic status (SES) was a crucial determinant in identifying behavioural risks associated with NCDs. Populations that had a low SES had higher alcohol and tobacco usage and consumed fewer fresh vegetables and fruits and less fish and fibre, while populations that had a high SES were more likely to have reduced physical activity and consumed diets rich in processed foods, resulting in increased fat and sodium intake (Allen *et al.*, 2017).

These behaviours have previously also been associated with increases in the inflammatory marker termed C-reactive protein, or CRP (Azak *et al.*, 2014; Ma *et al.*, 2006; Tonstad & Cowan, 2009; van Bussel *et al.*, 2015). C-reactive protein is by far the most widely investigated marker of chronic low-grade inflammation, as it has a stable half-life, is easily quantifiable and, if elevated, has been associated with various NCDs (Ridker, 2016). CRP also carries a risk score for the development of CVD similar to that of total (TC) and/or high-density lipoprotein cholesterol (HDL-c) (Ridker, 2016). These observations, including CRP measurements in longitudinal studies such as the Prospective Urban/Rural Epidemiological (PURE) study, add value to a retrospective analysis, such as the current study, because it is possible to draw upon baseline findings and therefore advance the current understanding of the aetiology of CVDs. Although some debate exists as to whether CRP is actively involved in the development of CVDs, its use as a prognostic marker cannot be refuted (Ridker, 2016; Yousuf *et al.*, 2013). However, an understanding of how CRP concentrations predict markers of cardiovascular health, especially in LMICs and non-European ethnicities, enables recommendations to be made.

CRP is known to differ substantially depending on the ethnic background of individuals, with black individuals often presenting with increased concentrations of this cytokine (Nazmi & Victora, 2007). It is, therefore, crucial to investigate CRP's prognostic value in predicting future CVD risk in black populations, as well (Fonseca & de Oliveira Izar, 2016; Nazmi & Victora, 2007), although this is not the primary focus of this thesis. South Africa, being classified as a middle-income country, has a majority of black individuals known to have elevated CRP concentrations (Kruger *et al.*, 2013) residing within its borders. This, together with a higher number of CVD risk factors observed in black and mixed-ethnicity communities (Phaswana-Mafuya *et al.*, 2013), results in the South African setting being the perfect milieu in which to undertake research investigating the association between CRP and cardiovascular health.

We hypothesised that certain modulators exist that affect the CRP status in black individuals, based upon their living environments and behavioural choices, and that these factors may alter the effects of specific *CRP* polymorphisms known to affect CRP concentrations. The presented work further investigates and builds upon previous work published by our laboratory to

characterise the CRP phenotype of black South Africans (Nienaber-Rousseau *et al.*, 2014; Swanepoel, 2013). In searching for these modulators of CRP concentrations, we aim to address the prevalence of elevated inflammation in black individuals by making recommendations as to the modifiable risks contributing to increased chronic inflammation and, in doing so, presenting much-needed information for clinicians and other healthcare providers in the fight against CVDs.

The genetic contribution towards CRP concentrations has been estimated to be as much as 40% of CRP variation (Pankow *et al.*, 2001; Reiner *et al.*, 2012); *ergo*, 60% of variation in CRP concentrations can be accounted for by modifiable factors[1]. Various other determinants are known to affect CRP status in black adults. These include age, body weight and, especially, abdominal adiposity, diet, glucose control and physical activity (Nienaber-Rousseau *et al.*, 2014); however, these factors are observed to be independent of ethnicity. We, therefore, aimed to investigate the social, phenotypic and cultural factors identified from literature that are specific to black individuals and are reported to be associated with CRP status. This aim was extended in order to characterise whether these factors interacted differently within twelve specific *CRP* genotypes, certain of which have previously been determined to affect CRP status in this study cohort (Nienaber-Rousseau *et al.*, 2014).

We identified three factors known to affect CRP status, for which differences based on ethnicity have also been previously reported. First, the PURE study included an investigation into the effects of SES on general health, and as such, individuals were included with differing SES markers. Black South Africans have a history of social oppression, the remnants of which persisted during the study's sampling (Micklesfield *et al.*, 2013). Low SES often co-presents with elevated CRP concentrations, and individuals with a low SES in childhood frequently have elevated CRP in adulthood (Kershaw *et al.*, 2010; Koster *et al.*, 2006; Liu *et al.*, 2017). Low levels of SES have also been indicated to affect CVD risk, which can be offset by improving the SES of the individual (Egbujie *et al.*, 2016; Min *et al.*, 2017). Therefore, in identifying specific SES factors as significant contributors towards CRP variation, these factors could be targeted by interventions that result in better health.

Secondly, there is a major phenotypic difference between our population and white individuals, i.e. that of skin colour. Black individuals are more likely to be vitamin D-deficient or -insufficient, owing to the increased exposure time to ultra-violet radiation (UVR) needed to initialise the production of 25-hydroxyvitamin D (25(OH)D) in darker-skinned *vs* light-skinned individuals. This is especially true for black persons living further away from the equator, where atmospheric

[1] Although genetic modification using gene-editing techniques exist, these techniques are still under ethical debate for use *in vivo* in non-life-threatening disorders (Mulvihill *et al.*, 2017).

interference reduces UVR. A reported negative association exists between 25(OH)D and CRP, although the biological mechanisms for this association remain vague (Liefaard *et al.*, 2015). Further, CVD risk can be mitigated by maintaining sufficient 25(OH)D concentrations (Rosen *et al.*, 2012), underlining the need for research on this subject. If 25(OH)D were to be associated with CRP concentrations in our study populations, recommendations could be made on safe exposure to UVR and vitamin D supplementation, thereby increasing 25(OH)D concentrations and improving health.

A review by Micklesfield *et al.* (2013) observes there is a preference for a larger body size among black women. Thus, we also investigated the effects that this culturally accepted norm in relation to anthropometry in black cultures may have on CRP concentrations. Previously, Nienaber-Rousseau *et al.* (2014) reported that, for our population, various anthropometric markers interacted with *CRP* polymorphisms to alter CRP concentrations. In the current analysis, we included the metabolic precursor of CRP, namely interleukin-6 (IL-6), which is an adipocytokine which is increased in viscerally obese individuals (Furukawa *et al.*, 2017). We also compared two WC classifications for increased cardiometabolic risk in relation to the effectiveness of stratifying individuals based upon their inflammation status. By investigating the interplay between IL-6, anthropometric markers, *CRP* genetics and CRP concentrations, we would be able to address the effects of increased adiposity on the inflammation cascade and make recommendations based upon the observed data in terms of inflammation.

1.2 Aims and objectives

The central aim of this research project was to investigate whether specific factors (i.e. SES, vitamin D status and/or IL-6 concentrations mediated by anthropometric markers) could explain the CRP phenotype of black South African populations and whether these factors interact on specific *CRP* polymorphisms in a manner which could mitigate CRP concentrations. This cross-sectional analysis was embedded in the PURE-SA study, for which sampling occurred in 2005. The aim was investigated by means of the following objectives:

- Determining how measured markers of SES associated with the inflammatory state, as measured by circulating CRP concentrations, in different *CRP* genotypic backgrounds.
- Determining how CRP associates with circulating 25(OH)D concentrations, and if any interactions are observed with the twelve *CRP* genotypes.
- Determining how anthropometric markers, in particular body mass index (BMI) and waist circumference (WC), associate with IL-6 and CRP concentrations in relation to different CRP genotypes.

1.3 Ethics

Ethical clearance (Ethics number 04M10) for the South African arm of the Prospective Urban and Rural Epidemiology study was obtained from the Health Research Ethics Committee of the Faculty of Health Sciences, North-West University (HREC). The study was conducted in accordance with the recommendations of the Declaration of Helsinki. Participants were well informed about the study and gave signed, informed consent before enrolment. For the affiliated study presented in this thesis, ethical approval was also obtained from HREC (NWU-00004-17-S1, Annexure 1).

1.4 Team

The research team that contributed to the articles contained in this thesis are as follows. Permission is hereby also granted by the co-authors to use these manuscripts for examination purposes.

Mr P.H. Myburgh

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PhD student involved in the presented study. Conceptualised the research questions, wrote the protocol and applied for ethical clearance. Performed all the statistical analyses on baseline data. Wrote the manuscripts as the first author and compiled the thesis. Was part of the team conducting follow-up sampling of PURE participants in 2013 and 2015, was responsible for the laboratory set-up and management, for maintaining sample integrity and several laboratory analyses of 2015 samples.

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Supervision and quality control of anthropometry data, critically revised Chapter 5.

X

Prof. H.S. Kruger
Co-author

1.5 Outline of thesis

This thesis is presented in article format, and comprises six chapters. Chapter 1, 2 and 6 are presented in British English, while Chapters 3–5 are presented in American English. A reference list is included at the end of each chapter. The format and citation styles for Chapters 3–5 (the three articles) are in accordance with the scientific journals identified for publication and included as addenda. Uniformity was achieved by maintaining a similar font type and size within this document. Publications cited are presented at the end of each chapter.

Chapter 1 provides a situational analysis and background information pertaining to the importance of the presented work, states the aim and objectives of the presented study, and describes the structure of the thesis as well as the roles of the team members.

Chapter 2 provides background on the study and the factors known to influence CRP concentrations. Topics included deal with the role of inflammation in the aetiology of NCD and, in particular, CVD; the genetics of the *CRP* gene; and reasons for our choice of specific factors for further investigation.

Chapter 3 presents the first article manuscript and details especially how education and smoking associates CRP concentrations in a group of black South Africans. This article will be submitted to the *International Journal of Public Health* under the proposed title: "Education, smoking and CRP genetics in relation to CRP concentrations in a group of black South Africans".

Chapter 4 pertains to the influence of vitamin D status, as measured by 25(OH)D, in relation to CRP concentrations. This article was published in the *International Journal of Environmental Research and Public Health (IJERPH)* and entitled: "CRP Genotypes Predict Increased Risk to Co-Present with Low Vitamin D and Elevated CRP in a Group of Healthy Black South African Women*"* (Myburgh *et al*., 2018)*.*

Chapter 5 is the third manuscript, in which we compare two anthropometric thresholds of WC proposed for sub-Saharan Africans that are indicative of future cardiometabolic risk in relation to IL-6, *CRP* genotypes and CRP concentrations. This manuscript will be submitted to the journal *Cytokine* under the working title, "Low-grade inflammatory markers in black South Africans: interplay between anthropometry, IL-6 and CRP polymorphism in relation to CRP".

Chapter 6 concludes the thesis and summarises the main findings. The PhD candidate also provides a critical review of our study's limitations and strengths and makes recommendations for future research.

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CHAPTER 2: LITERATURE REVIEW

The WHO defines NCDs as chronic diseases not caused by an infectious agent, which tend to be of long duration and are caused by a combination of genetic, physiological, environmental and behavioural factors (World Health Organisation, 2017c). Currently, the term “non-communicable disease” is being debated, as the term is simply indicative of that which does not cause these diseases. The prefix “non” may also prompt an erroneous association with “not important”, which may explain the disproportionate expenditure in global development funding on communicable disease, while NCDs remain an important cause of death and morbidity (Allen, 2017; Allen *et al.*, 2017). Suggestions have been made that the term “non-communicable disease” be changed to “socially transmitted conditions”, as this terminology would not underplay the importance of these conditions (Allen & Feigl, 2017); however, because the terminology is still under debate, and the term “non-communicable disease” is still being used, this thesis will use NCDs to refer to the collection of chronic diseases not caused by a pathogenic vector.

A shift in the leading global causes of death was observed during the period ranging from 2000 to 2015 (Figure 2.1; World Health Organisation (2017f)). Although the increased population size was not factored into the statistics provided, an increased incidence of death due to ischaemic heart disease, stroke, chronic obstructive pulmonary disease and cancers associated with the lungs was documented. Overall, the incidence of communicable diseases decreased during the same period, with HIV/AIDS-related morbidities no longer being among the leading causes of death. Two new NCDs were, however, included within the top ten leading causes of death, namely Alzheimer’s disease and diabetes mellitus (World Health Organisation, 2017f).

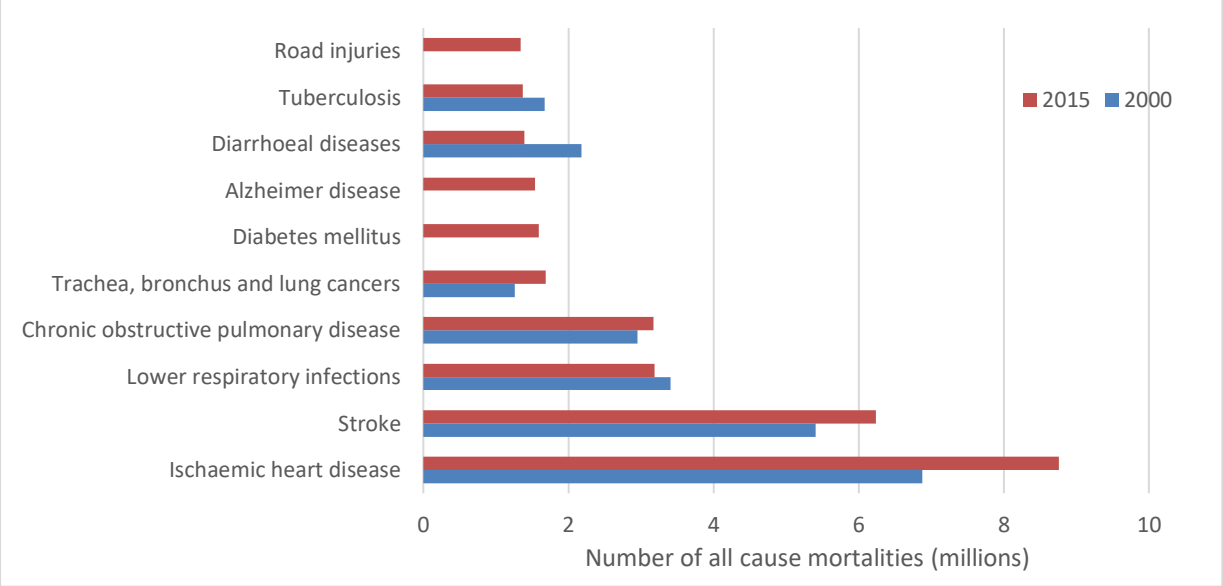

Figure 2.1: **Comparison of leading global causes of death between 2000 and 2015.** Data are sorted in ascending number of deaths for 2015. Reworked from data courtesy of the World Health Organisation (2017f).

The leading cause of NCD-related mortality globally is attributable to CVD (~44.3% including ischaemic heart disease, cerebrovascular disease,etc.), which, together with cancers (~22.0%), respiratory disease (~10%) and diabetes (~4%), constitute the top four leading causes of NCD-related mortality (World Health Organisation, 2017c). Economic forecasts predict that NCDs, excluding mental health disorders, will cost the global economy more than US$ 30 trillion for the period between 2011 and 2031, with a further cumulative output loss of US$ 47 trillion for the same period (Bloom *et al.*, 2012). Although NCDs are responsible for 60% of the global disability-adjusted life-years and 70% of global deaths, only 2% of overseas development assistance towards health is allocated to NCDs. Contrastingly, allocations made to combat HIV/AIDS amount to 30% of overseas development assistance, with HIV-related conditions accounting for only 3% of global disability-adjusted life-years or DALYs (Allen, 2017). This relatively high investment in HIV has, fortunately, paid off, as the prevalence of this communicable disease is subsiding; however, this highlights the need for economic assistance in order to decrease the risk of developing NCDs.

LMICs, including South Africa, share a considerable burden of NCDs, with more than 31 million NCD-related deaths recorded in LMIC countries in 2015 (World Health Organisation, 2017c). The economic burden of NCDs on the South African economy was estimated to amount to a loss in gross domestic product of US $1.88 billion for the period ranging between 2006 and 2015 (Abegunde *et al.*, 2007; Hofman, 2014). The South African morbidity data, however, differed significantly from the global estimates. In South Africa, 38.9% of deaths were directly attributable to NCDs in 2010 (Nojilana *et al.*, 2016); however, this had increased

to 55.5% of all deaths by 2015 (Statistics South Africa, 2017). Although a decline in the number of tuberculosis(TB)-related deaths was observed between 2013 and 2015, this communicable disease was still the leading cause of death in the country (Statistics South Africa, 2017). It should be noted, however, that diabetes mellitus, cerebrovascular disease, hypertensive disease and chronic lower respiratory diseases, which were the leading NCD-related causes of death, had all been discovered to have increased in incidence during the 2013–2015 timeframe (Statistics South Africa, 2017). By 2015, ischaemic heart disease also found its way onto the top ten causes of death in South Africa (Statistics South Africa, 2017). Owing to CVD being the leading cause of death worldwide, with an increasing prevalence in African populations (Keates *et al.*, 2017), it is critical that we investigate the potential origins of this disease to enable us to develop therapeutic strategies to stem the tide of this pandemic, especially in developing countries such as our own.

2.1 Cardiovascular disease — at the forefront of the global NCD pandemic

The WHO defines CVDs as “disorders of the heart and blood vessels and include coronary heart disease, cerebrovascular disease, rheumatic heart disease and other conditions” (World Health Organisation, 2017b). Global estimates have indicated that 17.7 million deaths in 2015 were due to CVDs, which included 7.4 million deaths due to coronary heart disease (CHD) and 6.7 million deaths due to strokes (World Health Organisation, 2017b). The Global Burden of Disease study investigated the increasing trends of mortality from CVD, with results (Table 2.1) indicating that the increase was due to a combination of global population increase, ageing populations as well as epidemiological changes in CVD risk (Roth *et al.*, 2015).

Table 2.1: Observed and counterfactual changes in global CVD related deaths (1990–2013)

Disease	Deaths in 1990	Deaths in 2013	% change 1990-2013	% change due to population growth	% change due to ageing populations
Ischaemic heart disease	5,737,483	8,139,852	41.7	23.6	52.5
Ischaemic stroke	2,182,865	3,272,924	50.2	21.6	62.1
Haemorrhagic stroke	2,401,931	3,173,951	30.7	26.8	59.5
Hypertensive heart disease	622,148	1,068,585	74.1	29.5	63.6
Cardiomyopathy and myocarditis	293,896	443,297	51.4	27.5	37.4
Rheumatic heart disease	373,493	275,054	–26.5	31.8	42.8
Total	**12,279,565**	**17,297,480**	**40.8**	**25.1**	**55.0**

Table 2.1 adapted from Roth *et al.* (2015).

Roth *et al.* (2015) estimated that 25.1% of the rise in CVD-related deaths was attributable to increases in the global population size, while 55% was caused by ageing. Ischaemic heart disease and strokes accounted for the majority of CVD-related mortalities. Deaths related to ischaemic strokes increased more rapidly (50.2%) during the period than those related to haemorrhagic strokes (30.7%). In their study, changes in the distribution of CVD-related deaths within specific regions were also noted. The most substantial increase (97.4%) in CVD deaths was reported for South Asia, with decreasing trends observed only in Central Europe (-5.2%) and Western Europe (–12.8%; Roth *et al.,* 2015). In sub-Saharan Africa, excluding southern sub-Saharan Africa, most of the increases in CVD-related deaths were attributable to the population growth. However, southern sub-Saharan Africa, including South Africa, had substantial relative increases in CVD mortality, which were mostly attributable to increasing population sizes and ageing, which was moderated by a decline in the number of age-specific rates of deaths that were observed (Roth *et al.*, 2015).

Although we are experiencing an increase in the occurrence of CVD, most of these premature CVD deaths are preventable (World Health Organisation, 2017b). As mentioned in the rationale for this study, the WHO has embarked on a global campaign to reduce the number of NCDs, including CVD-related deaths (World Health Organisation, 2011). Colloquially, the target set by the WHO is called the “25x25”, where a 25% reduction in premature deaths (defined as between the ages of 30 and 70), should be achieved by 2025 (Kontis *et al.*, 2014). Six target areas were identified as lifestyle changes at individual level i.e. reducing alcohol and tobacco use, increasing physical activity, reducing sodium intake and controlling blood pressure, as well as maintaining healthy body weight and avoiding the development of diabetes.

2.2 How "25x25" targets for individuals aim to reduce systemic inflammation

The multifactorial aetiology of CVDs necessitates a multi-pronged approach to treat these conditions successfully. However, the adage "prevention is better than cure" is of utmost importance in the fight against CVDs, as preventative measures would cost the global economy less and reduce the number of premature preventable deaths (Kelly & Fuster, 2010). Although no mention is made of inflammation within the six voluntary targets aimed at individual lifestyle changes set by the WHO, achieving these targets will result in population-wide reduced levels of chronic inflammation.

2.2.1 Reducing harmful use of alcohol by 10%

Harmful alcohol usage in middle-income countries was reported as the 5th highest risk factor for death in 2004, a year before the commencement of the presented study (Parry *et al.*, 2011). More recently, alcohol-related mortality in Africa was estimated at 6.4% of all deaths, while 4.7% of all DALYs are attributable to alcohol abuse (Ferreira-Borges *et al.*, 2016). The WHO estimates that South Africa is the 25th highest alcohol-consuming nation in the world, with individuals consuming 11.2 L of pure alcohol per annum; almost twice the global average of 6.1 L (World Health Organisation, 2017a).

Indications are that a large segment of the South African population starts using alcohol during adolescence (Morojele & Ramsoomar, 2016). Alcohol use in South Africa is, however, disparate, as a large segment of the population abstains (42%), whereas those who do consume alcohol are often involved in excessive drinking (Morojele & Ramsoomar, 2016). To reduce alcohol intake in South Africa, an inter-ministerial committee was established in 2010. Proponents of an "individual responsibility" approach were outweighed by the "public health" factions, and, therefore, the focus on limiting the influence of alcohol was addressed using public health messages. Included in the approach was increased police presence and the closing of illegal "shebeens" or alcohol houses. Alcohol producers are also now required to have a public health warning on the labels of all alcoholic products sold in South Africa (World Health Organisation, 2014).

Excessive alcohol consumption is also associated with an increased risk of developing CVDs. In their review on the effects of alcohol consumption and its associations with CVDs, O'Keefe *et al.* (2014) indicated the four primary effects of heavy alcohol use as:

1. the most common determinant of reversible hypertension;
2. accounting for a third of non-ischaemic dilated cardiomyopathies;
3. resulting in frequent atrial fibrillation; and
4. markedly increasing the risk of either ischaemic or haemorrhagic strokes.

Excessive chronic alcohol consumption is also associated with changes in gut microbiota composition, impaired liver function and increases in gut microflora-derived lipopolysaccharides which, in turn, induce inflammation (Wang *et al.*, 2010). Chronic alcohol use impairs gut and liver function that can consequently result in persistent systemic inflammation and organ damage (Wang *et al.,* 2010).

However, in moderate use (maximum two units per day) alcohol has cardio-protective properties, as it has been reported that both abstainers and heavy drinkers presented with weaker endothelial function (van Bussel *et al.*, 2017) than moderate drinkers. The cardioprotective effects of moderate alcohol intake may be attributed to reduced inflammatory markers, including CRP (Bell *et al.*, 2017; Imhof *et al.*, 2001). More research on the association of moderate alcohol use with cardio-protective mechanisms is needed (Bell *et al.*, 2017). Excessive alcohol often coincides with other poor lifestyle decisions, such as making poor dietary choices and being physically inactive (Farhud, 2015), which may also increase the risk of CVDs.

2.2.2 A 10% reduction in the prevalence of insufficient physical activity

Physical inactivity is associated with various chronic diseases and premature deaths (Ding *et al.*, 2016). Conservative estimates are that physical inactivity cost the global economy US$ 53.8 billion for the year 2013, and was responsible for 13.4 million global DALYs (Ding *et al.*, 2016). Current research on lifetime NCD risk and physical activity are limited to one report, which indicated that those with poor physical activity were more likely to succumb to CVD. Physical activity was also determined to attenuate the mortality risks associated with extensive periods of sitting (Ekelund *et al.*, 2016).

In a study comparing physical activity in older individuals (defined as above 50 years) in six countries (China, Ghana, Mexico, India, Russia and South Africa), South Africans were found to have the highest levels (59.7%) of physical inactivity (Wu *et al.*, 2015). Increasing physical activity in South African men was achieved using theory-based, culturally congruent interventions (Jemmott *et al.*, 2014), which could be used by government to increase physical activity levels in the population. Educating the broader South African public about NCDs and the beneficial effects of physical activity in reducing the risks of CVDs, especially, is necessary (Makamu, 2014).

It is thought that physical activity reduces the risks of developing CVDs by preventing obesity, reducing blood pressure and improving lipid profiles (Mora *et al.*, 2007). Increased physical activity has also been shown to reduce inflammatory markers, including CRP (Mora *et al.*, 2007). Although CRP concentrations are elevated immediately following vigorous exercise,

both cross-sectional and longitudinal indications are that individuals who often exercise have better CRP profiles (Kasapis & Thompson, 2005; Palmefors *et al.*, 2014). A recent meta-analysis indicated that CRP concentrations was lowered in individuals engaging in regular exercise training, with greater improvements in individuals achieving a healthier BMI (Fedewa, 2017). Even individuals who had previous episodes of myocardial infarction could reduce their basal inflammation levels substantially by exercising four times per week, following a well-balanced diet, reducing their body weight and avoiding tobacco use (Booth *et al.*, 2014).

2.2.3 A 30% relative reduction in prevalence of current tobacco use in persons aged 15+ years

In 2015, more than 1.1 billion individuals, mostly men, smoked tobacco products, with increasing trends observed in the Eastern Mediterranean countries and most of the African countries (World Health Organisation, 2017e). The effects of smoking on the global economy and health are reviewed in depth by Jha and Peto (2014). Importantly, their work indicates that this WHO target would result in more than 200 million deaths being prevented by the end of the current century. In South Africa, a general downward trend in the number of smoking individuals was observed between 2000 and 2015, with 3.9% fewer men and 6.0% fewer women being identified as smokers (Figure 2.2). The decline in the number of smokers is attributable to governmental interventions, including increases in excise taxation and increased public health messaging, as well as a ban on tobacco advertising and sponsorships (Delobelle *et al.*, 2016).

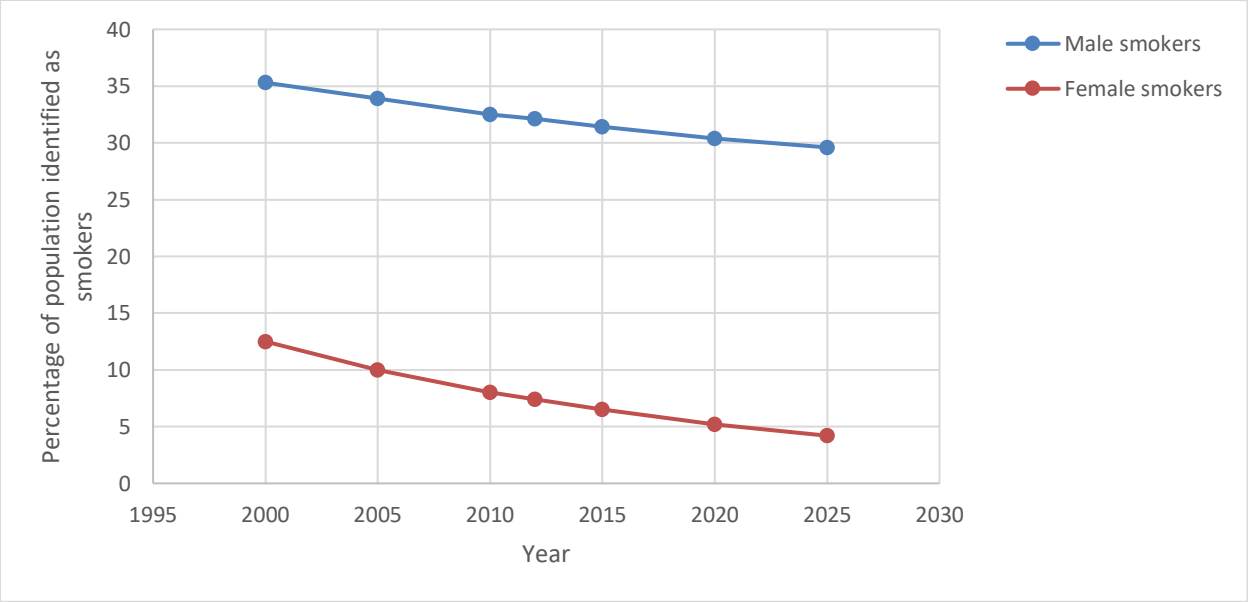

Figure 2.2: Decreasing trend in and forecast of the number of South African men and women identified as smokers (2000–2025).
Data reworked from World Health Organisation (2017e).

The pathophysiology of tobacco smoke in the aetiology of CVD is discussed in great detail by Rigotti and Clair (2013). In summary, the effects of smoking are described here. In the brain, nicotine binds to nicotinic cholinergic receptors, where it acts as a sympathomimetic agent, releasing catecholamines which increase heart rate and blood pressure (Benowitz, 2009; Benowitz, 2010). Nicotine also results in vasoconstriction, which induces endothelial dysfunction, especially in coronary and cerebral blood vessels (Favero *et al.*, 2014). Hypoxaemia due to carbon monoxide's higher affinity for binding with haemoglobin also drives ischaemic events, which is further exacerbated by hypoxaemic-mediated hypercoagulation (Nielsen *et al.*, 2013). Smoking also increases other risk factors for CVD, such as increasing low-density lipoprotein cholesterol (LDL-c) and triglycerides, and lowers HDL-c (Rigotti & Clair, 2013; Taylor *et al.*, 1981).

Moreover, cigarette smoking also increases inflammation in the human body, of which the cellular and molecular mechanisms were reviewed by Lee *et al.* (2012). Several reactive oxidative species (ROS) are produced by burning tobacco, which damages epithelial cell linings in the airways. Damaged cells activate oxidative-sensitive cellular pathways, with resultant DNA damage (Valavanidis *et al.*, 2009) and increased inflammation. Cigarette smoke also contains trace amounts of bacterial lipopolysaccharides, inducing inflammation at the mucosal linings (Lee *et al.*, 2012).

Interleukin-6 (IL-6) concentrations were determined to be elevated in smokers (Aldaham *et al.*, 2015). CRP increases immediately following the smoking of a cigarette; however, concentrations return to pre-smoking concentrations within 35 minutes (van Dijk *et al.*, 2013). When correcting for other factors, CRP concentrations were similar between apparently healthy long-time smokers and former smokers (Aksu *et al.*, 2013; Aldaham *et al.*, 2015), which we also report to be the case in our study (Chapters 3–5). However, smokers with metabolic syndrome were at increased risk of co-presenting with elevated CRP concentrations compared with non-smokers (Jamal *et al.*, 2014). The combined effect of localised inflammation, the vasoconstrictive effects of nicotine and increased heart rate results in raised blood pressure, which, combined with a diet high in sodium, can result in an increased risk of developing CVD.

2.2.4 A 30% relative reduction in mean population intake of salt/sodium and a 25% relative reduction in the prevalence of raised blood pressure

The WHO included reductions in sodium intake as a pertinent strategy for NCD prevention, as excessive sodium intake has been linked to an increased incidence of CVD-associated mortality (Cogswell *et al.*, 2016). Powles *et al.* (2013) calculated that in 2010 the global mean dietary sodium intake was 3.95 g.day^{-1}, while the WHO recommended 2.00 g.day^{-1}. Regional

stratification indicated that Eastern and Central Asian countries, together with Eastern European countries, had the highest sodium intake, while Sub-Saharan Africa and Latin America had the lowest mean sodium intake at 3.3 $mg.day^{-1}$ (Powles *et al.*, 2013). More recently, Swanepoel *et al.* (2017b) found that, in three different South African communities, the mean sodium intake was 7.8 $g.day^{-1}$, with 92.8% of all participants exceeding the WHO recommended daily allowance. Moreover, South Africans are among those with the highest prevalence of hypertension in the world (Lloyd-Sherlock *et al.*, 2014).

Sodium is an essential element, actively involved in muscle function, neural transmission and the homeostatic balance of extracellular fluid (Dötsch *et al.*, 2009). Blood sodium concentration is highly regulated by the kidneys, with excretion in urine normally equalling the amount ingested. Although a limited number of studies exists investigating the effects of high sodium diets in participants with no history of chronic kidney disease, evidence is mounting to indicate that excessive sodium reduces renal function (Farquhar *et al.*, 2015; Smyth *et al.*, 2014), resulting in chronic elevated blood pressure. Various meta-analyses concluded that reducing the intake of sodium reduced blood pressure, irrespective of sex and ethnicity, with no adverse effects on blood lipid profiles or renal function, while reducing the risk of strokes and fatal CHD in adults (Aburto *et al.*, 2013; He *et al.*, 2013).

To curb the increase in the number of individuals developing NCDs, and especially CVDs, the South African Department of Health took decisive steps to address sodium content in the foods available. In 2011, all trans fats were banned from mass-produced foods (Department of Health, 2011). Two years later, South Africa became the first country to implement mandatory regulation of sodium in certain foodstuffs, with implementation taking place in 2016 (Department of Health, 2013; Swanepoel *et al.*, 2017a). The author of this thesis formed part of a team that investigated industry compliance with this regulation, and found that industry generally complied well with these regulations (see Annexure 3 for full-text; Swanepoel *et al.,* 2017b). Reports on whether sodium reduction efforts resulted in decreased prevalence of hypertensive cases are still awaited.

Mechanisms exist by which excessive sodium intake and increased blood pressure result in chronically elevated systemic inflammation. Individuals diagnosed as hypertensive also have increased basal IL-6 and CRP concentrations (Chamarthi *et al.*, 2011). A direct linear response has been reported, where a 100 mmol increase in blood sodium concentrations resulted in a 1.20 $mg.L^{-1}$ elevation in CRP concentrations. When adjusting for body mass index (BMI), this linear response subsided to an increase in CRP of 1.06 $mg.L^{-1}$ per 100 mmol increase in sodium (Fogarty *et al.*, 2009). The intertwined effect of sodium intake and body mass in eliciting inflammatory responses was also described in adolescents, independent of

total energy intake and sugar-sweetened soft drink consumption (Zhu *et al.*, 2014). Increased sodium intake also augments the risk of developing metabolic syndrome (Soltani *et al.*, 2017), characterised by obesity and hyperglycaemia, *inter alia* (Furukawa *et al.*, 2017).

2.2.5 Halt the rise in obesity and diabetes

The global number of cases of both obesity and diabetes has reached epidemic proportions (Bhupathiraju & Hu, 2016). Estimates indicate that, in 2014, more than 1.9 billion individuals were overweight or obese, while 422 million individuals were living with diabetes (Organisation, 2017; Saltiel & Olefsky, 2017). Such is the rise in global body weight that most countries reported increased population BMI values between 1975 and 2014 (NCD Risk Factor Collaboration, 2016). The epidemic rise of these two conditions is, however, not limited to developed nations, as most African countries also presented with increasing numbers of individuals that were overweight or obese, as well as an increasing trend in those diagnosed with diabetes (NCD Risk Factor Collaboration, 2016; Organisation, 2017; Steyn & McHiza, 2014).

Similarly, the body weight of South Africans is expanding, with nearly a third of the population being obese in 2017 (Figure 2.3). Proportionally, obesity rates have almost tripled in South Africa since 1975, with the majority of the population now being defined as overweight (World Health Organisation, 2017d).

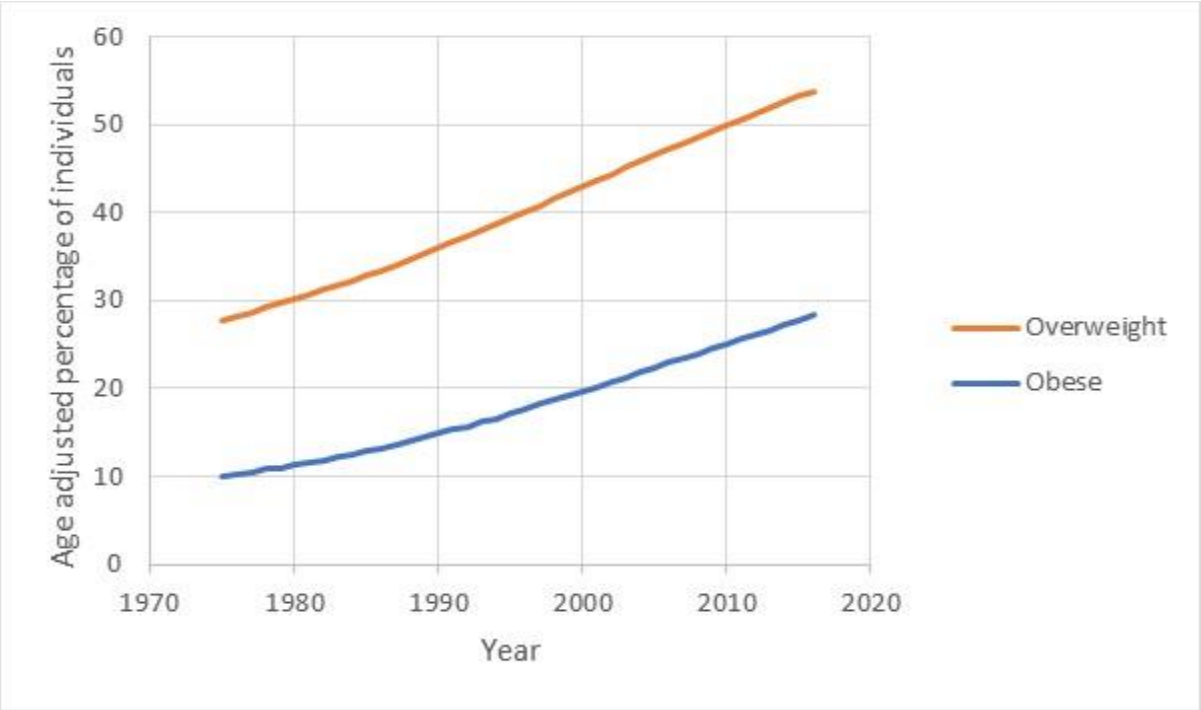

Figure 2.3: Age-adjusted percentage of overweight and obese individuals in South Africa for the period 1975–2017.
Reworked from data obtained from World Health Organisation (2017d).

The role of increased body mass, as measured by BMI, in the aetiology of CVD is still highly debated. This is mostly attributable to the lack of sensitivity towards lean *vs* fat body mass afforded by the use of the BMI (Bastien *et al.*, 2014). Although obesity, as defined by the BMI, is linked with the development of both CVD and diabetes, various studies have reported that body fat distribution better predicts the risk of developing CVD (Goossens, 2017; Kopelman, 2000). As global body mass increased, so did the prevalence of diabetes, with the incidence in most sub-Saharan countries doubling between 1980 and 2014 in both men and women (Figure 2.4; (NCD Risk Factor Collaboration, 2016)).

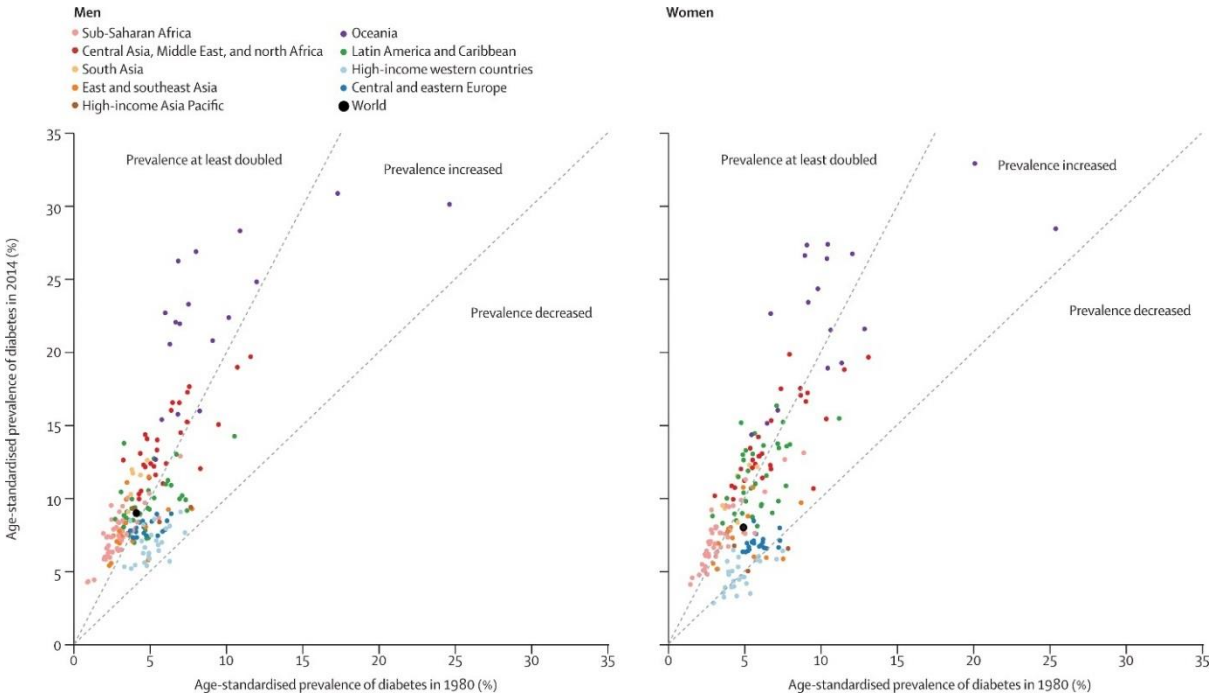

Figure 2.4: Shifts in the age-standardised prevalence of diabetes between 1980 and 2014.
Original artwork under Creative Commons licencing (NCD Risk Factor Collaboration, 2016).

The co-burden of increased weight and reduced insulin sensitivity is often observed, justifying its combined inclusion as one of the areas the WHO aims to address. With the exception of congenital diabetes, the aetiology of diabetes is generally considered to be due to a combination of genetic susceptibility, as well as behavioural and environmental factors, such as poor diet (Olokoba *et al.*, 2012) and increased body weight.

Both excessive weight and decreased insulin sensitivity are traits associated with the metabolic syndrome, together with hyperlipoproteinaemia, hyperuricaemia, and steatosis hepatitis, as first defined by Haller (1977). The major driving force in the aetiology of the metabolic syndrome, however, remains obesity (Furukawa *et al.*, 2017). Fat accumulation increases systemic oxidative stress, with *in vitro* results obtained from studying cultured

adipocytes indicating that oxidative stress causes the dysregulation of the production of adipocytokines such as adiponectin and IL-6, the latter of which controls the production of CRP (Furukawa *et al.*, 2017). It has been determined that losing excess body fat by correcting diet and increasing physical activity significantly reduces systemic inflammation and includes reductions in CRP concentrations (Mavros *et al.*, 2014).

The degree of obesity related directly to systemic inflammation, and, with a high degree of certainty, correlations with insulin resistance, have been determined, which will ultimately lead to the development of type II diabetes mellitus (Reilly & Saltiel, 2017). Obesity-induced insulin resistance also leads to peripheral arterial dysfunction, hampered blood flow, micro- and macro-angiopathy, cardiomyocyte and endothelial dysfunctions, as well as hypertension, thereby increasing the risk of developing CVD (Patel *et al.*, 2016).

Achieving the aforementioned six targets by 2025 will result in the prevention of 31 million premature deaths, most of which would be in LMICs (Kontis *et al.*, 2014). Furthermore, a global reduction in premature deaths attributed to the four leading NCDs is estimated at 22% in men, and 19% in women (Kontis *et al.*, 2014). Although no mention is made specifically of inflammation with regard to the six voluntary targets aimed at individual lifestyle changes set by the WHO, it can be deduced from the previous discussion that achieving these targets will simultaneously result in reduced levels of chronic inflammation across the South African population.

The importance of chronic systemic inflammation in the development of NCDs, and especially CVD, cannot be overstated (Mangge *et al.*, 2014). By understanding the potential modulators of inflammation, public health efforts could focus on reducing inflammation levels and, thereby, reduce the burden of CVD-associated morbidities and mortalities. Understanding the driving forces of chronic low-grade inflammation will not only aid healthcare practitioners in suggesting therapeutic actions but will also lead to a better quality of life for the people of South Africa. Although the inflammatory cascade has various biochemical factors, none has been as widely studied as CRP, a cytokine which, when elevated, is known to be predicative of future CVD risk (Ridker, 2014).

2.3 C-reactive protein: Marker or mediator of NCD?

Chronic low-grade inflammation, especially as measured by the widely used inflammatory marker CRP, is considered to have mild to strong associations with several NCDs, and in particular with CVD (Ridker, 2014). Understanding the history of CRP, as well as the biochemical pathways that are involved and associated with CRP, provides a better

understanding of the role of this cytokine in CVDs. Understanding the mechanisms by which CRP associates with CVD will enable suggestions for therapeutic interventions.

2.3.1 A brief history of C-reactive protein

Tillett and Francis (1930) first reported the existence of the protein in 1930, which they identified from the serum of patients with acute pneumococcal infection. CRP is a major non-specific acute-phase plasma protein, which is rapidly synthesised mainly by hepatocytes following the release of cytokines such as IL-6 at sites of inflammation, infection or tissue injury (Lau *et al.*, 2005; Pepys & Hirschfield, 2003). The protein has been determined to be highly conserved, with several homologues present in a variety of animals (Kumar *et al.*, 2011; Magor & Magor, 2001).

Figure 2.5 represents our current understanding of how IL-6 induces CRP synthesis. IL-6 is transported to the liver, where it binds to IL-6 receptors and activates various signalling pathways, including transcriptional activation of *CRP* mediated *via* signal transducer and activator of transcription factor 3 or *STAT3* (Bode *et al.*, 2012; Zhang *et al.*, 1996).

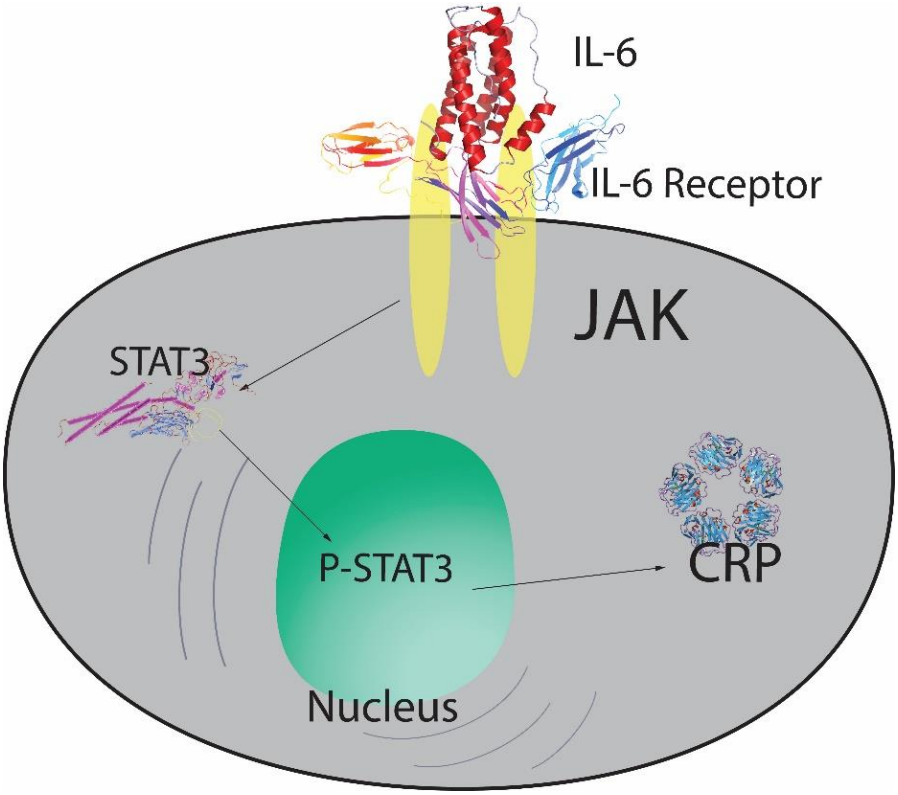

Figure 2.5: Schematic diagram of IL-6-induced synthesis of CRP in a hepatocyte.
Abbreviations: CRP, C-reactive protein; IL-6, interleukin-6; JAK, Janus kinase, (P)-STAT3, (phospho)-signal transducer and activator of transcription. Image based upon the works of Bode et al. (2012) and Zhang et al. (1996).

Each CRP molecule consists of a planar ring of identical non-covalently linked monomers arranged pentamerically (Figure 2.6). These subunits need to undergo calcium ion (Ca^{2+})-

mediated transformation before binding can occur. CRP binds with high affinity to phosphocholine, which is expressed on several bacterial and necrotic cells. It has also been determined that it binds with chromatin, fibronectin, histones and small nuclear ribonucleoproteins (Abernethy & Avery, 1941; Du Clos *et al.*, 1991; Du Clos *et al.*, 1988; Sui *et al.*, 1999; Thompson *et al.*, 1999). CRP's binding to phosphocholine activates the complement system, which in turn enhances phagocytosis by macrophages.

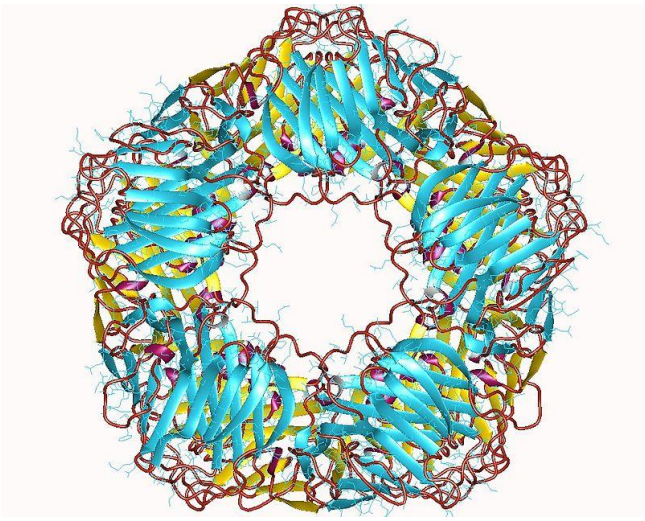

Figure 2.6: Three-dimensional structure of the CRP molecule, indicating the pentameric structure.
Image in public domain. Image sourced from the European Bioinformatics Institute (www.ebi.ac.uk).

CRP has diverse biological functions, which are mainly derived from its binding properties (Ablij & Meinders, 2002). Not only is CRP actively involved in the innate host defence against microorganisms, but it also functions in the non-inflammatory clearance of nuclear host material and apoptotic cells, modulates the inflammatory process, prevents adhesion of neutrophils to endothelial cells, and enhances inflammation in the vascular wall (Du Clos, 2013; Gewurz *et al.*, 1995).

Normal high-sensitivity CRP concentrations range between <1.0 and 3.0 $mg.L^{-1}$ (Teidel, 2015); however, in disease states, CRP levels can increase upwards of 500 $mg.L^{-1}$ (Pepys & Hirschfield, 2003). Various meta-analyses have concluded that individuals with a baseline CRP concentration of more than 3 $mg.L^{-1}$ are at a higher risk of developing events related to CHD (as reviewed by Strang & Schunkert, 2014).

2.3.2 CRP and its association with CVD

Several studies have reported that associations between NCDs and inflammation exist. King *et al.* (2003) reported that elevated CRP concentrations are observed with increasing glycated haemoglobin (HbA_{1c}) levels, which was indicative of an association between glycaemic control

and CRP in adults with diabetes. In a cohort of 7,350 British civil servants, Tabák *et al.* (2010) found that CRP concentrations were higher in individuals who subsequently developed diabetes or died from CVD. C-reactive protein is often used as a prognostic marker for CVD risk, and although it is somewhat controversial (Yousuf *et al.*, 2013), it is still applicable (Shrivastava *et al.*, 2015). Elevated CRP concentrations are also observed in acute asthma (Razi *et al.*, 2012), various cancers (Allin & Nordestgaard, 2011; Szkandera *et al.*, 2014), periodontitis (Paraskevas *et al.*, 2008) and chronic kidney disease (Fox *et al.*, 2010). Whether NCDs cause inflammation or *vice versa* is still highly debated.

By far the most convincing evidence for the usefulness of CRP as a prognostic marker in any disease is present in its association with CVD. Some of the first reports of CRP being elevated in cases of myocardial infarction date back to the 1950s (Levinger *et al.*, 1957). However, it was not until the mid-1990s that cardiovascular interest in CRP re-emerged. The first definitive study to address the importance of CRP as a prognostic marker of future CVD risk was the Physicians Health Study (Ridker *et al.*, 1997). Their work indicated that individuals within the highest quartile of CRP concentrations had a three times higher risk of experiencing a myocardial infarction and were twice as likely to suffer from ischaemic strokes (Ridker *et al.*, 1997). Similarly, the risk of developing CVD-related conditions was investigated in apparently healthy women, with CRP being the most reliable predictor of future cardiovascular incidents. The relative risk was estimated to be 4.4 times higher in women in the highest CRP quartile than those in lower quartiles (Ridker *et al.*, 2000). Since then, numerous studies have been conducted in various populations to quantify the prognostic value of CRP. These studies were summarised in a meta-analysis of American and European individuals, which indicated that the relative adjusted risk ratio associated with a 1-standard deviation (SD) higher $\log_e$ CRP concentration has been estimated at 1.37 for CHD, 1.27 for stroke, and 1.55 for vascular mortality (The Emerging Risk Factors Collaboration, 2010). It was also stated that the vascular risk associated with increased CRP was the same as that of HDL-c (Ridker, 2016a).

The difficulty of using this marker in studies investigating the aetiology of CVD is that a clear causal link between CRP and CVD has not yet been established (Ridker, 2016a). CRP is produced in a wide variety of cells beyond hepatic cells, including human coronary artery smooth muscle cells and adipocytes, which some claimed to be indicative of a causative effect of CRP in CVD (Calabro *et al.*, 2005; Calabro *et al.*, 2003; Shrivastava *et al.*, 2015). However, genetic studies have failed to implicate genetic polymorphisms located in the *CRP* gene in increased odds of developing CVD (Elliott *et al.*, 2009; Zacho *et al.*, 2008). Infusing healthy individuals with pharmaceutical-grade human CRP was also reported not to be proinflammatory, with no adverse cardiovascular effects observed (Lane *et al.*, 2014).

Furthermore, strong evidence originating from Mendelian randomization studies confirms the prognostic value of CRP to predict future cardiovascular events, while also being indicative of CRP not being causally associated with CVD (as reviewed in Ridker, 2016b). Current research efforts are therefore aimed at implicating upstream inflammatory markers, such as IL-6, as the causative agents for CVD, possibly explaining the association between elevated CRP and increased CVD risk (Ridker, 2016a). However, more research is needed on these upstream markers, especially with regards to the validation and standardisation of different assays in different populations. Although causality has not been established, the use of CRP as a diagnostic test in primary and secondary prevention of CVD cannot be argued against (Ridker, 2016a).

The core motif of this thesis, however, is based on the observation that most individuals of African descent tend to have a higher circulating CRP concentration than their white counterparts (Nazmi & Victora, 2007; Ridker, 2016b). In a South African context, the SAfrEIC (Sex, Age and Ethnicity on Insulin sensitivity and Cardiovascular function) study reported mean CRP concentrations of 1.73 (0.007–22.86) $mg.L^{-1}$ in African men as opposed to 1.02 (0.007–9.22) $mg.L^{-1}$ in individuals of European descent (Kruger *et al.*, 2013b). As part of the Prospective Urban Rural Epidemiological (PURE) study, Nienaber-Rousseau *et al.* (2014) reported a median CRP level of 3.29 $mg.L^{-1}$ in a healthy, mixed-gender black population. Although black individuals tend to have elevated basal CRP concentrations, to our knowledge, no alternate cut-off values for future CVD risk apart from the 3 $mg.L^{-1}$ exist. Therefore, an increased risk of developing CVD is predicted for black South Africans in the PURE population, which is substantiated by the increased prevalence of CVD seen in the South African population, as detailed earlier. However, by investigating the potential modulators of CRP concentrations in black South Africans, one would be able to suggest where intervention efforts could be focused, and by reducing the general inflammatory load, prevent new cases of CVD. As complex interactions exist between genetic factors predisposing individuals to certain phenotypes and the environmental and behavioural aspects of human beings, any investigation considering modifiable factors should also consider those factors which are currently non-modifiable as well, especially when such genetic factors have been determined as being associated with CRP levels. In this matter, CRP is no different as its concentration can be influenced by environmental, clinical and genetic factors. Using data obtained from twin and family studies, a substantial heritability of between 35 and 40% has been observed for CRP concentrations (Pankow *et al.*, 2001). Therefore, the genetics of the *CRP* gene plays a crucial role in determining the concentration of circulating CRP as well.

2.3.3 Genetic variation in the *CRP* gene

The human *CRP* gene is located on chromosome 1 and spans a 2,301 base pair stretch localised to 1q21–1q23 (Floyd-Smith *et al.*, 1986; Lei *et al.*, 1985). Carlson *et al.* (2005) first reported 31 single nucleotide polymorphisms (SNPs) in the *CRP* gene in 47 individuals (24 were African American or AA and 23 European or EA), of which 30 were polymorphic in AAs and 13 polymorphic in EA. Using Bayesian statistical methods, 18 common *CRP* haplotypes were inferred. To date, 441 SNPs have been identified in the *CRP* gene (Database of Single Nucleotide Polymorphisms (dbSNP), National Center for Biotechnology Information, National Library of Medicine, Bethesda (MD), Date of Access: 22 May 2018).

It has been determined that circulating CRP concentrations are influenced by SNPs located on the *CRP* gene (Hage & Szalai, 2007; Jones *et al.*, 2009). The association of the *CRP* gene with circulating CRP concentrations is further accentuated by a number of genome-wide association studies (GWAS) that have been undertaken (Doumatey *et al.*, 2012; Reiner *et al.*, 2012). An example is harbouring a C allele at position 159713648 (rs1800947, Genome Reference Consortium Human Genome Build 38), which is associated with lower circulating CRP concentrations. Similarly, harbouring the minor alleles at rs1130864, rs1417938 and rs1800947 has been associated with lower CRP concentrations in elderly, homozygous French men (Ancelin *et al.*, 2015).

There is, however, very little known about the genetics of *CRP* in African populations, and how this relates to circulating CRP (Carlson *et al.*, 2005; Reiner *et al.*, 2012). Israelsson *et al.* (2009) investigated three SNPs in the Fulani and sympatric ethnic groups in Western Africa as a possible evolutionary defence mechanism against infection with *Plasmodium falciparum,* for which the Fulani are known to have a reduced susceptibility. They concluded that the tri-allelic polymorphism, rs3091244, may be a contributing factor to the lower susceptibility to malaria observed in the Fulani. The Fulani have a lower prevalence of the A allele at this position compared with other ethnic groups in the region.

Nienaber-Rousseau *et al.* (2014) first reported on the black South African population under investigation in this thesis with regard to the interactions observed between markers of nutritional status and various *CRP* genotypes on circulating CRP concentrations. Table 2.2 summarises the SNPs identified within the *CRP*-gene that were investigated in the PURE population, as well as their effects in other populations. Their work indicated, *inter alia,* that individuals harbouring a minor allele at rs3093058 and rs3093062 (SNPs in strong linkage disequilibrium) had higher CRP concentrations, which increased with increasing triglyceride or cholesterol intake, thus establishing the effects that diet may have on circulating CRP concentrations, mediated by CRP genetics. Marked genetic differences were also observed

in GWAS studies comparing African American and white individuals, although the *CRP* gene remained the most important locus for altering CRP concentrations in both populations (Doumatey *et al.*, 2012). Altered genotypic frequencies also exist when comparing *CRP* genetics between African Americans and individuals of European ancestry (Doumatey *et al.*, 2012), indicative of a possible genetic origin of the observed differences in CRP phenotype. The resultant expression of a gene can, therefore, be modulated to some extent by the environment and behaviour of an individual, which may alter the phenotype observed (Reiss *et al.*, 2013).

Table 2.2: Summary of the associations of the minor alleles within the *CRP* SNPs investigated in the PURE population

CRP* genotypes** ***rs number	**ΔCRP in the PURE study**	**ΔCRP in white individuals**	**ΔCRP in African American populations**	**ΔCRP in other populations**
rs7553007ϕ	↓	↓ (Elliott *et al.*, 2009)	↓ (Reiner *et al.*, 2012)	↓ Filipinos (Rhodes *et al.*, 2008)
rs1341665ϕ	↓	↔ (Wang *et al.*, 2006)	---	---
rs2027471ϕ	↓	---	---	↓ Filipinos (Rhodes *et al.*, 2008)
rs3093058§	↑	---	↑ (Lange *et al.*, 2006)	↑ CRP in non-Hispanic black Americans (Crawford *et al.*, 2006)
rs3093062§	↑	---	↓ (Szalai *et al.*, 2005)	↑ CRP in non-Hispanic blacks (Crawford *et al.*, 2006)
rs1417938	↔	↓ French elderly men (Ancelin *et al.*, 2015)	---	↓ Mainly individuals of European descent, but included African Americans, Hispanic and Asians (Suk *et al.*, 2005)
rs1800947	↔	↑ (Schumacher *et al.*, 2009); ↓ French elderly men (Ancelin *et al.*, 2015)	---	↔ Mainly white but included Mulatto/black and Asian individuals from Brazil (Araujo *et al.*, 2004) The CG genotype in the Mexican American population was associated with decreased CRP but not in non-Hispanic blacks (Crawford *et al.*, 2006)
rs1130864	↔	↓ (Lawlor *et al.*, 2008); ↓ French elderly men (Ancelin *et al.*, 2015)	---	---
rs1205ϕ	↓	↓ French elderly women (Ancelin *et al.*, 2015)	---	↓ non-Hispanic American black and Mexican American populations (Crawford *et al.*, 2006)
rs3093068	↑	↑ (Hage & Szalai, 2007)	---	---
rs2808630	↔	---	---	↓ non-Hispanic American population (Crawford *et al.*, 2006)
rs2794520ϕ	↓	---	---	↓ Filipinos (Rhodes *et al.*, 2008)

Changes in CRP in the PURE study determined by Nienaber-Rousseau *et al.* (2014). These SNPs were selected based upon their observational frequency in 30 randomly selected individuals. *CRP = C-reactive protein* gene, rs = accession number for SNPs. ↓ reduction in circulating CRP concentrations observed. ↑ increase in circulating CRP concentrations observed. ↔ no significant difference observed in circulating CRP concentration.

As mentioned earlier, approximately 60–65% of circulating CRP concentrations cannot be explained by genetics alone and other factors should, therefore, also be investigated in any study aiming to determine increased CRP concentrations at population level. Consequently, the role of environmental and behavioural factors cannot be excluded in any elucidation of the

possible factors contributing to higher circulating CRP levels in sub-Saharan Africans. As indicated above, environmental and behavioural factors, such as nutritional intake (Nienaber-Rousseau *et al.*, 2014), can also have a major modulating effect on the genetic associations with CRP concentrations.

2.3.4 Other factors influencing CRP concentrations

Earlier in this chapter, we discussed the effects that achieving the "25x25" WHO targets would have on circulating CRP concentrations. Consequently, the focus of this review will be placed upon other factors known to affect CRP concentrations.

Not only ethnicity but also age and gender affect the risk of presenting with elevated CRP. Both IL-6 and CRP concentrations were reported to increase in an age-dependent manner in a group of Eastern Europeans. This was found in the absence of any NCD, including CVD, type II diabetes, or cancer (Puzianowska-Kuźnicka *et al.*, 2016). Reports have also indicated that women tend to present with higher CRP concentrations when compared with men, even after adjusting for various confounding factors (Khor *et al.*, 2004; Lakoski *et al.*, 2006; Ridker, 2016b; Wener *et al.*, 2000). The elevated nature of CRP concentrations in ageing women, compared with age-matched men, seems to exist irrespective of ethnicity, although older black women tend to have higher CRP concentrations than white women matched in age and smoking status (Wener *et al.*, 2000). Increased adiposity in post-menopausal women also increases the likelihood of presenting with elevated CRP concentrations (Ebong *et al.*, 2016), which may be offset by following a healthy diet and maintaining adequate levels physical activity (Fedewa, 2017). Diet, however, is highly reliant on socio-economic factors, and even more so in South Africa (Micklesfield *et al.*, 2013).

The effects of the living environment and SES can be a key contributing factor to poorer health (O'Neill *et al.*, 2007). A meta-analysis conducted recently concluded that 25% higher CRP concentrations were observed in adults who had a low SES during their formative years (Liu *et al.*, 2017). CVD risk can also be offset by improving SES, with low levels of SES also being associated with an increased incidence of CVD (Egbujie *et al.*, 2016; Min *et al.*, 2017). SES, however, is multi-factorial, constituting various aspects of the individual's immediate living environment. Using proxy measures of pathogen exposure, including water source quality, exposure to faeces, modes of waste disposal and household cleanliness, McDade *et al.* (2008) determined increased odds ratios for these factors with regard to elevated CRP.

Vitamin D has been implicated as having a preventative effect on a range of chronic maladies, including cancer, diabetes mellitus, hypertension and CVD (Rosen *et al.*, 2012). A meta-analysis (Brondum-Jacobsen *et al.*, 2012) observed a 39% (25–54%) increase in the risk of

developing ischaemic heart disease for the lowest *vs* the highest quartile of 25-hydroxyvitamin D or 25(OH)D levels. Similar results were found in an original study, substantiated by the meta-analysis conducted by Brondum-Jacobsen *et al.* (2013), where a stepwise increase in the risk of developing symptomatic ischaemic stroke was observed with decreasing 25(OH)D concentrations. Chowdhury *et al.* (2014) included a total of 880,128 individuals from 73 cohort and 22 randomised controlled studies in a meta-analysis, and concluded that an inverse association exists between 25(OH)D and the risks associated with all-cause mortality.

The possible preventative effects of 25(OH)D on adverse vascular conditions resulted in the hypothesis that 25(OH)D may influence CRP, which is associated with these conditions. A recent meta-analysis by Chen *et al.* (2014) showed that in 924 participants, vitamin D supplementation decreased circulating CRP by 1.08 $mg.L^{-1}$ (95% confidence interval or CI, –2.13, –0.03), while individuals with baseline CRP levels of >5 $mg.L^{-1}$ had a higher reduction of CRP i.e. 2.21 $mg.L^{-1}$ (95% CI, –3.5, –0.92) when being supplemented.

Various diseases associated with high levels of circulating CRP have dietary origins in common (Ma *et al.*, 2006). Interactions have been reported on the elemental as well as micronutrient and macronutrient level. Dibaba *et al.* (2014) reported that in 32,918 participants included in their meta-analysis, magnesium intake was inversely associated with CRP concentrations. Schwingshackl and Hoffmann (2013) determined, when including 15 randomised control trials, that protein intake did not affect CRP. The influence of diet, and the diet x *CRP*-SNP interaction, was also extensively reported in Nienaber-Rousseau *et al.* (2014) for the PURE study. Furthermore, a recent meta-analysis conducted by Neale *et al.* (2016) concluded that a healthy diet was associated with reductions in CRP (weighted mean difference: –0.75, $p < 0.0003$). The collective results synthesised from fourteen randomised controlled trials in obese individuals found that increasing dietary fibre or fibre-rich foods leads to a significant, albeit slight, reduction in circulating CRP (Jiao *et al.*, 2015). Increased dietary fibre intake results in changes in the composition of the gut microbiome, for which the maintenance of a mutualistic relationship may pose many health benefits (Kaczmarczyk *et al.*, 2012), including prevention of infections such as tuberculosis (Luo *et al.*, 2017).

As CRP is an acute-phase protein, infection with several pathogens will increase circulating CRP concentrations as well (Pepys & Hirschfield, 2003). South Africa is at the epicentre of the HIV epidemic, with a co-burden of dual infection with TB-causing pathogens, which are often resistant to current antibiotic therapies. South Africa is ranked 6th globally in having the largest number of new TB infections for the year 2014 (Khodaee *et al.*, 2016). Lau *et al.* (2006) reported increased CRP in HIV-infected individuals, with increases in CRP also being observed as the disease progressed to AIDS. Similarly, in an age, sex, BMI and locality

matched case-control study of the population under investigation in this thesis, elevated CRP concentrations, coinciding with dyslipidemia, were reported in HIV-1 subtype C-infected black individuals (Fourie, 2010). CRP levels have been used in the determination and prognosis of TB since the 1960s (Haghighi & Doust, 1966), where a negative CRP test was indicative of a treatment that worked. Individuals who are co-infected with both HIV- and TB-causing agents have higher CRP concentrations than those that are only HIV positive (Wilson *et al.*, 2011).

Circulating CRP concentrations above 10 $mg.L^{-1}$ are usually associated with acute infection (Ridker, 2003) and, therefore, merit exclusion in longitudinal studies. However, Nienaber-Rousseau *et al.* (Unpublished) observed that individuals with CRP concentrations above that which is regarded as indicating acute infection had genotype distributions that were significantly different from those of individuals with CRP concentrations lower than 10 $mg.L^{-1}$, thus leading them to believe that extreme CRP concentrations might result from carrying certain *CRP SNPs*. Obese women are also reported to present repeatedly with CRP concentrations above 10 $mg.L^{-1}$ (Ishii *et al.*, 2012). The impact of dysbiosis within the gut microbiome can also affect circulating concentrations of CRP. However, much more data on this subject is needed (Graessler *et al.*, 2013). Therefore, exclusion of these individuals, especially of black individuals known to present with elevated CRP, may result in biased reporting.

2.4 Socio-economic, phenotypical and cultural factors specific to black individuals that may explain the origin of elevated CRP concentrations

Research on several factors that may influence circulating CRP concentrations has been quite ambiguous and underrepresented in African populations. In searching for reasons why black individuals may be more prone to presenting with elevated CRP, the PhD candidate investigated several key differences, supported by scientific evidence, that are common differentiators between black individuals and other ethnicities. Black ethnicities have a long history of oppression, both globally and in South Africa. Specific to South Africa, racial segregation under apartheid rule resulted in large discrepancies in SES of the black population when compared with other ethnicities. This resulted in black individuals being compromised in terms of access to healthcare, earning capacity and education (Micklesfield *et al.*, 2013). The age of the cohort included in the PURE study meant that volunteers had lived under the apartheid regime before its fall in the 1990s. Racial segregation was based on the colour of skin, a major phenotypical difference between black individuals and those of other ethnic backgrounds.

In order to see whether skin colour affects CRP concentrations, links between factors influenced by skin colour and CRP were investigated in the literature. A negative association

between 25(OH)D and CRP has been described (Liefaard *et al.*, 2015). Furthermore, black individuals in the PURE-SA study were prone to presenting with deficient or insufficient 25(OH)D concentrations, the latter affecting blood pressure, a risk factor in the aetiology of CVD (Kruger *et al.*, 2013a). Therefore, we chose to explore the relationship between CRP and vitamin D status in our cohort.

Lastly, cultural differences were also investigated that could contribute to elevated CRP. As reported in an extensive review by Micklesfield *et al.* (2013), various socio-cultural, behavioural and environmental determinants exist which result in black women being overweight or obese. As results from Nienaber-Rousseau *et al.* (2014) indicated that interactions exist between anthropometric markers and *CRP* SNPs, we extended their research by including the adipocytokine IL-6, a metabolic determinant known to affect CRP concentrations which is elevated with increasing adiposity, as a potential contributing factor towards CRP status in black South Africans.

2.4.1 Socio-economic factors influencing CRP concentrations

In South Africa, the legacy of a colonial and apartheid past resulted in ongoing poverty and inequality (Mathee, 2011). According to a WHO meta-synthesis, 109,100 deaths for the year 2002 were directly associated with factors pertaining to water, sanitation, hygiene and pollution in South Africa (Prüss-Üstün *et al.*, 2008). The PURE study's population is quite unique, with major differences in SES. Pisa *et al.* (2012) found that, in rural individuals, those with low education levels and who were unemployed were more likely to present with increased markers of CVD. CRP concentrations decreased with increasing levels of education in the PURE population (Pisa *et al.*, 2012), which was reiterated and the effect quantified in our results (Chapter 3). The impact of environmental and socio-economic factors on health, therefore, needs to be investigated. The literature on how different SNPs interact with different environmental conditions also remains, at least to our knowledge, limited.

2.4.2 Skin colour and environment

Darker skin colour is a photoprotective mechanism, which originated in individuals living in areas with high UVR such as the equator (Jablonski & Chaplin, 2010). A photoprotective effect such as darker skin results in reduced vitamin D production by the skin. This may have led to the evolutionary reduction of pigmentation as a result of migration of humans to higher latitudes, where UVR is lower, which may have occurred to counteract the negative impacts of vitamin D deficiency (Juzeniene *et al.*, 2009; Yuen & Jablonski, 2010).

Luxwolda *et al.* (2012) reported that, in the traditional populations of East Africa (the Maasai and Hadzabe) who were living traditional lifestyles close to the equator, the mean serum

25(OH)D levels were 115 nmol.L^{-1}, which is equivalent[2] to 46 ng.mL^{-1} (range 23.2–68.4 ng.mL^{-1}; 58–171 nmol.L^{-1}). Therefore, 25(OH)D concentrations were above the sufficient threshold of 30 ng.mL^{-1}. Unfortunately, no data are currently available for CRP concentrations in either of the two populations previously mentioned. Abhimanyu *et al.* (2015), in a review of 25(OH)D data in South Africa, indicated a large seasonal variation within the black population, with summer measurements just above the sufficient cut-off limit of 30 ng.mL^{-1} (75 nmol.L^{-1}). This review included data from the PURE population described in section 3.2, where urban females had lower 25(OH)D levels compared with their rural counterparts and the whole female population had a marginally insufficient 25(OH)D status (Kruger *et al.*, 2011). Nienaber-Rousseau *et al.* (2013) reported that the study population had a high mean CRP level of 8.5 ± 12.4 mg.L^{-1} (mean ± SD; 8.67 ± 11.9 mg.L^{-1} for women).

Currently, and to our knowledge, no interaction studies have been published on the interactions of 25(OH)D and SNPs present in the *CRP*-gene. The combination of a possible association between vitamin D status and circulating levels of CRP, as well as the data published on the population under investigation, however, indicate that further investigation into the association between these biochemical markers is merited. Our work will be the first report of whether 25(OH)D interacts with *CRP* SNPs to influence circulating CRP concentrations.

2.4.3 Adiposity and its potential role on inflammation in the black South African population

Adipose tissue, especially visceral adipose tissue, is a major source of the pleiotropic cytokine IL-6 (Crichton *et al.*, 1996; Fontana *et al.*, 2007; Ganter *et al.*, 1989; Memoli *et al.*, 2007; Pini *et al.*, 2012). In measuring visceral obesity, WC is a valuable resource in the absence of more specialised equipment and can be used as an indicator of cardiometabolic risk (Klein *et al.*, 2007). However, the use of WC thresholds developed for western populations to measure cardiometabolic risk in Africans is being challenged (Ekoru *et al.*, 2017). Substantially lower WC is proposed for black men, whereas similar thresholds are proposed for women (Ekoru *et al.*, 2017).

Culturally, in South Africa, black women are seen as healthy and well-looked after if they have excessive body weight, which may be a contributing factor towards the high levels of obesity seen in the country (Micklesfield *et al.*, 2013). The effects of obesity on the inflammatory profile was described earlier; however, of particular interest in obesity-related inflammation is

[2] The original manuscript reported the values in nmol.L^{-1}, however 25(OH)D levels are generally reported in ng.mL^{-1}. For this study ng.ml^{-1} will be used. The conversion factor is 1 nmol.L^{-1} = 0.4 ng.mL^{-1}.

the pleiotropic cytokine, IL-6, which is produced in adipose tissue (Amaral *et al.*, 2015; Crichton *et al.*, 1996; Pini *et al.*, 2012). Not only does IL-6 play a significant role in the inflammatory response, but it is also a key mediator in metabolic control and in bone metabolism (Scheller *et al.*, 2011). In apparently healthy individuals, circulating IL-6 can be found in the 1 pg.mL^{-1} range (D'Auria *et al.*, 1997) and in slightly elevated concentrations during menstruation (Angstwurm *et al.*, 1997) while large elevations and variations (29.8–1400.8 pg.mL^{-1}) have been observed after surgery (Sakamoto *et al.*, 1994).

Visser *et al.* (2002) reported that elderly (between the ages of 70–79 years) African American individuals, when compared with European Americans, had elevated IL-6 concentrations, which were associated with decreased muscle mass (white males: 1.84 pg.mL^{-1}; black males 2.06 pg.mL^{-1}, white females 1.63 pg.mL^{-1}; black females 1.92 pg.mL^{-1}). Chapman *et al.* (2009) also reported that African Americans had elevated IL-6 levels as opposed to their European counterparts, although their report did not include any anthropometric data. In South Africa, Kruger *et al.* (2010) reported that boys had higher (although not statistically significant) serum levels of IL-6 compared with girls. In a population of South African men (n=600), median IL-6 concentrations were 4.2 pg.mL^{-1}, with differences observed based on HIV status, where HIV-positive individuals tested with higher concentrations (Fourie *et al.*, 2010). Botha *et al.* (2015) reported on the PURE population, with individuals who survived between 2005 and 2010 having mean IL-6 concentrations of 2.71 pg.mL^{-1}. Phulukdaree *et al.* (2013) reported an IL-6 concentration of 6.62 ± 0.63 pg.mL^{-1} in 87 black individuals. The large variation in reported values necessitates further investigation into this inflammatory marker in a South African context. From these reports, it is indicated that IL-6 concentrations were elevated above the reference value in the black South African population, although more information is still required. As IL-6 is a major determinant of CRP concentrations, and CRP has been shown to be elevated in individuals with increased body weight, the combined effects could be a sizable contributing factor to CRP status in black South Africans. The collected information necessitates the need for further investigation into the interplay between IL-6, anthropometry and CRP, which may further be altered in differences in *CRP*-genotypes.

2.5 Summary of the literature

All indications are that specific factors exist which contribute towards the elevated CRP concentrations observed in black individuals. Although some factors are already well described, most of what we know regarding CRP originated from non-African populations, resulting in a major void in our current understanding of CRP. In the following three chapters, the studies we conducted will be presented to contribute to the field and extend our current understanding. First, socio-economic determinants will be investigated in an attempt to define

those specific factors that result in elevated CRP concentrations. Next, we investigate whether the previously described negative association between vitamin D status and CRP exists in a group of black volunteers, whether having low 25(OH)D and high CRP affected cardiovascular markers, and how this differed between *CRP* genotypes (Myburgh *et al.*, 2018). Lastly, we investigate how increased anthropometric markers, an aspect which is culturally preferred in the black tribes of South Africa, affect both IL-6 and CRP in different *CRP* genotypes. We also compare how different WC thresholds stratified the population, especially in terms of their inflammatory profiles. By identifying these specific factors, intervention efforts could focus on making a sustainable impact. The prognostic value of CRP in indicating future CVD risk makes measuring this protein a prominent tool in the arsenal of healthcare providers. Therefore, understanding ethnic differences and the factors contributing towards elevated CRP will enable us to recommend possible areas for future interventions.

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CHAPTER 3: MANUSCRIPT ONE

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Aims and scope:

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EDUCATION, SMOKING AND *CRP* GENETICS IN RELATION TO CRP CONCENTRATIONS IN A GROUP OF BLACK SOUTH AFRICANS

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3.1 Abstract:

Introduction:

Elevated concentrations of C-reactive protein (CRP), prominent in black individuals, have been implicated in predicting future risk of developing cardiovascular disease (CVD), with socio-economic status (SES) also implicated as a determinant in the etiology of CVD.

Objectives:

We investigated whether specific SES factors associates with elevated CRP concentrations and how these factors may result in altered CRP phenotypes based on polymorphisms in the *CRP* gene.

Methods:

1 569 black South Africans were included, for which CRP concentrations and twelve *CRP* single nucleotide polymorphisms (SNPs) were determined, together with markers of cardiovascular health and self-reported factors of SES.

Results:

None of the investigated SES factors was found to associate with CRP concentrations when measured individually; however, in adjusted analyses, attaining twelve or more years of formal education could hypothetically result in a predicted 18.9% lower CRP concentration. We also present the first evidence that active smokers with a C-allele at rs3093068 are at an increased risk of presenting with elevated CRP.

Conclusions:

Although SES affects the general well-being of the individual, we found insufficient evidence that specific SES factors associated with inflammation.

Keywords: CVD, education, inflammation, SES, smoking, socio-economic status

3.2 Introduction:

Non-communicable diseases (NCDs) accounted for 71.3% of global deaths between 2005 and 2015 (Wang *et al.*, 2016). Of these NCDs, cardiovascular disease (CVD) took the greatest toll in developing nations such as South Africa (Nyirenda, 2016). Several CVDs share an inflammatory origin, which is regulated by numerous factors, including anthropometry, level of physical activity and the genetic background of an individual (Mangge *et al.*, 2014). One such marker of inflammation, which has been determined to predict future CVD risk, is the cytokine, C-reactive protein (CRP), elevated levels of which i.e. >3 mg/L are predictive of a future CVD diagnosis (Shrivastava *et al.*, 2015; Strang & Schunkert, 2014). CRP is generally elevated in black individuals in comparison with other ethnicities, coinciding with notably stronger inflammatory responses as well as increased CVD risk (Fonseca & de Oliveira Izar, 2016; Min *et al.*, 2017; Nédélec *et al.*, 2016; Shah *et al.*, 2010). Other factors besides ethnicity, such as age and gender, are also determinants of CRP concentrations, with indications that differing CRP concentrations could be attributed to socio-economic status (SES; Liu et al. 2017).

SES is, however, multi-layered, reflecting educational-employment factors, access to infrastructure (providing water, sanitation and electricity, for example) and quality of housing Socio-economic status (SES) has an effect on the inflammatory state as well as the CVD risk of an individual. Various studies reported that lower SES co-presented with elevated CRP concentrations (Kershaw *et al.*, 2010; Koster *et al.*, 2006), while low SES in childhood was reported by a recent meta-analysis to result in 25% higher CRP concentrations during adulthood (Liu *et al.*, 2017). Recently, Egbujie *et al.* (2016) linked SES to CVD risk in black South Africans, while another report indicated that the higher risk among black Americans of developing CVD can be offset by improved SES (Min *et al.*, 2017). In South Africa, it was reported that a change in CVD risk factors among our study population was observed between 1996 and 2005, with CVD risk shifting from individuals with a high SES to those with lower SES (Pisa *et al.*, 2012).

The mechanisms by which SES affects CRP concentrations are still being debated, with varying hypotheses currently under investigation. Lower SES is usually associated with poor diet, low levels of physical activity and higher incidence of tobacco and alcohol use (Kershaw *et al.*, 2010). Kershaw *et al.* (2010) report that poverty and low levels of education were the main determinants in observing elevated CRP concentrations, with 87.9% of CRP variation attributed to education level being primarily explained by an increased prevalence of smoking, lower dietary quality and reduced levels of exercise.

Specific phenotypes, such as elevated CRP, are influenced not only by environmental factors, but also by clinical and genetic factors. Data from twin and family studies attribute up to 40% of heritability in CRP concentrations to heritable factors such as genetics; however, little is known about the genetics underpinning CRP in African populations (Pankow *et al.*, 2001; Reiner *et al.*, 2012). Expression of a gene can be modulated by the environment and behavior of an individual, which may alter the phenotype observed (Nienaber-Rousseau *et al.*, 2014). To our knowledge, nothing has been reported on how different *CRP* single nucleotide polymorphisms (SNPs) interact with markers of SES and how this interaction could modulate CRP concentrations. We, therefore, investigated whether specific socio-economic factors associates with CRP concentrations, as a proxy for inflammation, and whether these factors mitigated the associations determined between specific *CRP* polymorphisms known to be associated with alterations in CRP concentrations. The interplay between SES, inflammation and the etiology of CVD could, therefore, be used to mitigate CVD risk by reducing inflammation through improving SES factors. Knowledge regarding the impact of SES on the genetic expression of CRP concentrations may help direct tailored preventive efforts for diseases contingent on inflammation.

3.3 Methods:

This cross-sectional, observational study was nested within the South African arm of the Prospective Urban and Rural Epidemiology (PURE) study, with details of the sampling strategy described by Pisa *et al.* (2012). In total, 2 010 apparently healthy adults over the age of 30 years, from both rural and urban communities, were included at the baseline in 2005. Individuals with a measured fever (tympanic temperature >38.0˚C), were excluded. Further exclusions were that participants could not be on any known prescribed chronic medication, have pre-existing lifestyle diseases, be pregnant or lactating or have a chronic infection, including the human immune deficiency virus (HIV) and/or tuberculosis at the time of sampling.

3.3.1 Biochemical measurements

Fasting blood samples were collected by registered nurses. High-sensitivity CRP concentrations were measured on a Sequential Multiple Analyzer Computer (SMAC), using a particle-enhanced immunoturbidometric assay (Konelab™ auto analyzer, Thermo Fisher Scientific Oy, Vantaa, Finland). Quantitative determination of high-density lipoprotein cholesterol (HDL-c), triglycerides and total cholesterol in the sera of participants was done on a Konelab™ 20i auto analyzer (Thermo Fisher Scientific). Low-density lipoprotein concentrations (LDL-c) were calculated using the Friedewald equation for those with triglycerides below 400 mg/dL. Nurses trained in voluntary counseling and HIV testing performed HIV testing in accordance with prevailing governmental and WHO guidelines. Pre-

test counseling was provided in group format, after which signed informed consent was obtained individually. Those testing positive for HIV on a rapid First Response HIV1-2.O card test (Transnational Technologies Inc. PMC Medical, Nani Daman, India), were retested using a second card test developed by Pareeshak (BHAT Bio-tech, Bangalore, India) to ensure diagnostic accuracy. All participants, irrespective of HIV status, received individual post-test counseling. Whole EDTA blood was used for measuring glycated hemoglobin (HbA_{1C}) values from fasting participants, with a D-10 Hemoglobin testing system (Bio-Rad Laboratories, Hercules, CA, USA).

3.3.2 Anthropometric and physiological measurements and lifestyle questionnaires

The participant's body weight was measured in minimal clothing with arms hanging freely at the side. Weight was measured in duplicate, with the mean recorded. Each participant's height was measured in duplicate with a stadiometer, with the head in the Frankfort plane in a fully erect state while the subject inhaled. The mean was then calculated and recorded in meters. Body mass index (BMI) was calculated using the standard formula and reported as kg/m^2. Waist circumference (WC) and hip circumference were measured using unstretchable metal tape in accordance with the recommendations of the International Society for the Advancement of Kinanthropometry. An Omron automatic digital blood pressure monitor (Omron HEM-757) was used to measure right brachial artery blood pressure in the sitting position. Participants were not allowed to smoke, exercise or eat 30 minutes beforehand, and had to be rested and calm for five minutes prior to measurement. Volunteers responded to an interviewer-administered questionnaire in their language of choice, in which various socio-demographic variables [age, gender, medical history (stroke and diabetes incidence), tobacco use, alcohol usage and SES factors (*i.e.* roof type, access to electricity, primary cooking fuel, primary heat source, water source, and education)] were collected. Water sources were grouped into sourced water (i.e. from wells, rivers, boreholes etc.) or municipal water sources. Food portion books were specifically designed and standardized for the South African PURE population. Validated, interviewer-based quantitative food frequency questionnaires or qFFQs (MacIntyre *et al.*, 2001) were completed to determine dietary intakes. The data obtained from qFFQs were entered into the Foodfinder3 program (Medical Research Council, Tygerberg, 2007) and sent to the Medical Research Council of South Africa for nutrient analyses.

3.3.3 Genetic analyses

In total, 12 *CRP* SNPs were determined through BeadXpress analysis in 1,587 participants of the PURE 2005 cohort. The *CRP* SNPs included in our analyses were previously reported by Nienaber-Rousseau *et al.* (2014). The BeadXpress analysis was performed by the National Health Laboratory Service (NHLS) at the University of the Witwatersrand, Johannesburg.

3.3.4 Statistical analyses

A total of 1,569 individuals, for whom we had both CRP concentrations and all the genetic information regarding the SNPs investigated in the *CRP* gene, were included in our analyses. Statistical analyses were conducted using R (R Core Team, 2017). Continuous variables were inspected for normality using histograms and measures of skewness. Variables with a skewed distribution were natural log-transformed and reported as median and interquartile ranges. Based on global recommendations for CRP cut-off values, data subsets were created i.e. ≤3 mg/L; >3 mg/L (Strang & Schunkert, 2014). The compareGroups library was used to construct bivariate tables comparing our constructed cohorts, using non-parametric methods for both continuous and categorical data. Spearman correlations were computed, testing for correlation of continuous values, while median values and interquartile ranges for each categorical variable were reported. Significance testing was conducted using the independent two-group Mann–Whitney U test or the Kruskal–Wallis One-Way ANOVA by Ranks Test. A backward stepwise linear regression was conducted using the stepAIC function within the MASS library. Models were evaluated based on the Akaike information criterion (AIC) obtained. The final variables obtained were evaluated for co-linearity. Association analyses for SNP X environment interaction were then performed, using the SNPSassoc library, including the co-variates obtained from the linear regression model, and included based on lowest scoring AIC value. This was done for each SNP in combination with each demographic and SES factor. Where applicable, p-values were adjusted using the methods suggested by Bonferroni.

3.4 Results:

3.4.1 Demographics and anthropometrics of the study population and their CVD-risk factors stratified to at-risk CRP phenotypes

Women were more likely to present with elevated CRP concentrations (median unadjusted value of 3.58 mg/L). Post-menopausal women (self-reported with amenorrhea), had significantly higher median CRP concentrations (4.31 [1.72; 11.9] mg/L) than men (2.42 [0.72; 7.87] mg/L) and pre-menopausal women (3.05 [0.82; 9.00] mg/L; $p < 0.0001$). Individuals with elevated CRP concentrations were also physically larger than those with normal CRP, as indicated by higher BMI and other anthropometric markers, even though similar daily dietary

intakes were noted. Post-menopausal women also had significantly larger WC (median: 82.4 cm) than pre-menopausal women (median: 79.0 cm) and males (median: 74.2 cm). After adjusting for WC, which differed between the genders ($p < 0.0001$), the difference in CRP concentrations observed between men and women, as well as pre- and post-menopausal women, disappeared. Those with elevated CRP were also significantly older, although age was only weakly, but significantly, associated with CRP ($\rho = 0.12$). Median CRP concentrations were similar irrespective of HIV status, tobacco and alcohol use. Smokers had a lower median WC (74 cm) as opposed to grouped individuals who had never smoked or were former smokers (81.4 cm, $p < 10^{-12}$).

Median CRP concentrations were similar ($p > 0.05$) in rural and urban participants (Table 3.1), with similar proportions of individuals being classified as having normal or elevated CRP concentrations observed in these two areas. Factors pertaining to SES differed between the two localities (data not shown). Rural participants were more likely to be married and had lower education levels than urbanites, thereby pointing toward a lower SES level for rural dwellers. Ruralists were also more likely to access public water systems such as communal wells, to use wood as a primary heating and cooking fuel source, and have roofs constructed of corrugated iron sheeting with no insulation.

Table 3.1: Demographic characteristics of the study population

Variables indicated as n (%) or median [IQR]	Normal CRP (<3 mg/l) n = 751	Elevated CRP (>3 mg/l) n = 818	p-value¤	Statistic‡	p-value∞
Men/women	317 (42.2)/434 (57.8)	270 (33.0)/548 (67.0)	<0.001	2.42 [0.72; 7.87]/3.58 [1.24; 10.2]	<0.0002
Menorrhea/amenorrhea	229 (54.7)/190 (45.3)	236 (43.6)/305 (56.4)	0.001	3.05 [0.82; 9.00]/4.31 [1.72; 11.9]	<0.0001
Age (years)*	46.0 [41.0; 53.0]	49.0 [42.0; 58.0]	<0.001	ρ = 0.12	<0.05
HIV-positive/negative	128 (17.1)/620 (82.9)	131 (16.1)/683 (83.9)	*NS*	3.11 [0.93; 12.0]/3.25 [0.96; 9.14]	*NS*
Tobacco use:					
Formerly	25 (3.36)	33 (4.04)	*NS*	3.98 [1.29; 18.3]	*NS*
Currently	403 (54.1)	413 (50.6)		3.05 [0.89; 9.18]	
Never	317 (42.6)	370 (45.3)		3.44 [1.04; 9.34]	
Alcohol consumption:					
Formerly	29 (3.90)	38 (4.68)	*NS*	3.74 [1.31; 14.1]	*NS*
Currently	321 (43.1)	305 (37.6)		2.78 [0.85; 9.28]	
Never	394 (53.0)	469 (57.8)		3.53 [1.05; 9.20]	
Body mass index (kg/m^2)	21.6 [18.9;24.9]	25.6 [19.7;32.2]		ρ = 0.24	
Underweight	152 (20.4)	134 (16.5)		2.51 [0.64-11.5]	
Healthy	407 (54.6)	253 (31.1)	<0.001	1.89 [0.60-5.67]	<0.0001
Overweight	127 (17.0)	145 (17.8)		3.25 [1.30-7.46]	
Obese	59 (7.92)	282 (34.6)		8.24 [3.74-15.9]	
Waist circumference (cm)*	74.3 [68.5;81.2]	82.4 [72.2;92.9]	<0.001	ρ = 0.27	<0.0001
Hip circumference (cm)*	90.0 [83.8;98.4]	98.2 [85.5;112]	<0.001	ρ = 0.21	<0.0001
Dietary intake (kJ)*	6996 [5265;9719]	7284 [5259;10025]	*NS*	ρ = 0.03	>0.05
Urban/rural	388 (51.7)/363 (48.3)	420 (51.3)/398 (48.7)	*NS*	3.20 [1.06; 9.83]/3.26 [0.86; 8.75]	*NS*

*Continuous variables presented as median and interquartile [25th; 75th percentile] ranges. ‡ Median CRP concentrations [interquartile ranges] calculated per grouping category or Spearman's rho (ρ) for continuous variables. All other variables presented as the number of individuals (%) and median values. ¤p- value for the difference in distribution of categorical variables of normal and elevated groups, or difference in continuous variables. ∞P-values for difference in CRP concentrations within grouping variable between categorical variables or p-value for Spearman's rho. Abbreviations: CRP, C-reactive protein; HIV, human immunodeficiency virus; IQR, interquartile range (25th and 75th percentile); kJ, kilojoule; n, number of individuals; *NS*, not significant (p >0.05).

Next, we stratified factors pertaining to SES according to CRP risk values (Table 3.2). Except for marital status, similar distributions and median CRP concentrations were observed for all investigated SES factors. Individuals presenting with normal CRP concentrations were more likely to identify as never being married; however, when adjusting for age and WC, similar CRP concentrations were observed across all marital status categories. Smokers had significantly lower formal educational attainment than non-smokers (data not shown).

Table 3.2: Factors of SES stratified according to baseline CRP cut-off values for elevated CVD risk

Variables indicated as n (%) or median [IQR]		Normal CRP (<3 mg/L) n = 751	Elevated CRP (>3 mg/L) n = 818	p-value¤	Statistic‡	p-value∞
Education level	None	251 (34.1)	286 (36.3)	*NS*	3.33 [0.93; 9.91]	*NS*
	Primary	304 (41.4)	334 (42.4)		3.25 [1.01; 9.17]	
	Secondary	180 (24.5)	167 (21.3)		2.80 [0.83; 9.00]	
Marital status	Never married	282 (39.1)	268 (33.5)	0.04	2.86 [0.88; 8.69]	*NS*
	Partnered	357 (49.4)	405 (50.7)		3.36 [0.91; 9.34]	
	Separated	30 (4.16)	45 (5.63)		3.73 [1.21; 11.6]	
	Widowed	53 (7.34)	81 (10.1)		4.41 [1.65; 9.84]	
Time to nearest grocery store (minutes)*		30.0 [20.0; 60.0]	30.0 [20.0; 60.0]	*NS*	ρ = –0.015	*NS*
Time to nearest bank facility (minutes)*		30.0 [20.0; 60.0]	40.0 [20.0; 60.0]	*NS*	ρ = 0.018	*NS*
Access/no access to electricity		639 (87.2) / 94 (12.8)	714 (88.8) / 90 (11.2)	*NS*	3.29 [0.97; 9.49]/ 2.91 [0.83; 7.76]	*NS*
Heat source	Coal open fire	92 (12.6)	97 (12.2)	*NS*	3.25 [0.97; 9.87]	*NS*
	Wood open fire	343 (47.1)	342 (42.9)		2.97 [0.84; 8.45]	
	Portable heater	28 (3.85)	38 (4.76)		4.17 [1.05; 15.3]	
	None	122 (16.8)	129 (16.2)		3.17 [0.92; 8.98]	
	Electricity	94 (12.9)	131 (16.4)		3.86 [1.42; 11.2]	
	Other	49 (6.73)	61 (7.64)		4.02 [1.25; 16.0]	
Water source	Sourced water	418 (57.1)	440 (55.1)	*NS*	3.25 [0.86; 9.05]	*NS*
	Municipal water	314 (42.9)	359 (44.9)		3.22 [1.13; 9.47]	
Roof structure	Galvanized iron sheets	601 (82.1)	641 (79.7)	*NS*	3.19 [0.90; 9.13]	*NS*
	Asbestos sheets	86 (11.7)	112 (13.9)		3.66 [1.21; 13.1]	
	Other	45 (6.20)	51 (6.40)		3.22 [1.10; 9.04]	
Cooking fuel	Electricity	275 (37.6)	352 (43.8)	*NS*	3.58 [0.99; 9.87]	*NS*
	Kerosene	224 (30.6)	222 (27.6)		2.96 [0.99; 9.28]	
	Gas	32 (4.38)	35 (4.36)		3.26 [1.05; 9.21]	
	Wood	188 (25.7)	177 (22.0)		2.87 [0.82; 8.86]	
	Other	45 (6.20)	51 (6.40)		3.22 [1.10; 9.04]	

*Continuous variables presented as median and interquartile [25th; 75th percentile] ranges. ǂ Median CRP concentrations [interquartile ranges] calculated per grouping category or Spearman's rho (ρ) for continuous variables. All other variables presented as the number of individuals (%) and median values. ¤p- value for the difference in distribution of categorical variables of normal and elevated groups, or difference in continuous variables. ∞P-values for difference in CRP concentration within grouping variable between categorical variables or p-value for Spearman's rho. Abbreviations: CRP, C-reactive protein; IQR, interquartile range (25th and 75th percentile); n, number of individuals; NS, not significant (p >0.05)

Individuals with elevated CRP concentrations presented with significantly poorer markers of CVD risk than those with normal CRP concentrations (Table 3.3). Cases of elevated CRP were prone to co-present with increased blood pressure, increased heart rate and a poorer lipid profile. Median glycated hemoglobin concentrations were also significantly increased in individuals with elevated CRP concentrations.

Table 3.3: Physiological and biochemical markers of increased CVD risk

Variables indicated as n (%) or median [IQR]	Normal CRP (<3 mg/L) n = 751	Elevated CRP (>3 mg/L) n = 818	p-value¤	Statistic‡	p-value∞
Systolic blood pressure (mmHg)*	127 [114; 144]	131 [117; 147]	0.002	ρ = 0.06	*NS*
Diastolic blood pressure (mmHg)*	85.0 [76.0; 94.0]	88.0 [79.0; 97.0]	<0.001	ρ = 0.08	*NS*
Heart rate (BPM)*	70.0 [61.0; 81.0]	73.0 [64.0; 87.0]	<0.001	ρ = 0.17	<0.0001
Hypertensive / normotensive*	176 (23.6) / 569 (76.4)	228 (28.0) / 586 (72.0)	*NS*	3.63 [1.27; 8.88] / 3.11 [0.84; 9.32]	*NS*
Total cholesterol (mmol/L)*	4.76 [4.02;5.79]	4.95 [4.03;6.01]	0.035	ρ = 0.04	>0.05
HDL-c (mmol/L)*	1.48 [1.14;1.98]	1.34 [1.02;1.80]	<0.001	ρ = -0.15	<0.0001
LDL-c (mmol/L)*	3.01 [2.32;3.77]	3.23 [2.44;4.14]	<0.001	ρ = 0.1	<0.0001
Triglycerides (mmol/L)*	1.01 [0.76;1.41]	1.14 [0.85;1.65]	<0.001	ρ = 0.144	<0.0001
HbA_{1c} (%)*	5.40 [5.20;5.70]	5.60 [5.30;5.90]	<0.001	ρ = 0.23	<0.0001

*Continuous variables presented as median and interquartile [25th; 75th percentile] ranges. ǂ Median CRP concentrations [interquartile ranges] calculated per grouping category or Spearman's rho (ρ) for continuous variables. All other variables presented as the number of individuals (%) and median values. ¤p- value for the difference in distribution of categorical variables of normal and elevated groups, or difference in continuous variables. ∞P-values for difference in CRP concentration within grouping variable between categorical variables or p-value for Spearman's rho. Abbreviations: BPM, beats per minute; CRP, C-reactive protein; HbA_{1C}, glycated hemoglobin; HDL-c, high-density lipoprotein cholesterol; IQR, interquartile range (25th and 75th percentile); kJ, kilojoule; LDL-c, low-density lipoprotein cholesterol; mmHg, millimeters of mercury; n, number of individuals; *NS*, not significant (p >0.05).

To describe CRP concentrations and the interactions of modulators thereof on a physiological scale, natural log-transformed CRP (lnCRP) concentrations were modeled using a stepwise, backward linear regression approach. Eight statistically significant predictors were identified from the measured variables, including clinical, demographic and socio-economic factors (Table 3.4). The model presented accounted for 14.3% of the variation observed in CRP concentrations of our black population. A 22.0% predicted reduction in CRP concentration was observed in response to an increase of 1 mmol/L in HDL-c. All SES factors investigated in this study failed to predict CRP concentrations, except for whether an individual had attained 12 or more years of formal education, which resulted in a predicted reduction of 18.9% in CRP concentrations.

Table 3.4: Natural log-transformed CRP concentrations as a function of covariates

Variable	Estimate β coefficients	Standard error	Change (%)*	p-value
Intercept	-3.387	0.388		<0.0001
Age	0.014	0.004	1.41	0.0002
Heart rate	0.020	0.002	2.21	<0.0001
WC	0.027	0.003	3.10	<0.0001
HDL-C	-0.249	0.059	-22.0	<0.0001
HbA_{1c}	0.117	0.043	12.4	0.006
Completed at least seven years of formal education	-0.090	0.085	-8.60	0.292
Twelve or more years of formal education	-0.209	0.104	-18.9	0.044

*% change in CRP calculated for a 1 unit change in covariate [(e^{β}–1)*100)]. Abbreviations: HbA1c, glycated hemoglobin; HDL-c, high-density lipoprotein cholesterol; WC, waist circumference.

3.4.2 Effects of SES factors on association between different *CRP* genotypes and CRP concentrations

The odds of presenting with elevated CRP concentrations were independently investigated for each demographic or SES factor included in this study in combination with each of the twelve *CRP* genotypes. The only significant interaction observed in our population was that of smoking status in individuals of differing rs3093068 genotypes. Individuals indicating that they were former smokers were included in our association analysis as abstainers to enable sufficient statistical power. Smokers had lower median WC (74 cm) as opposed to individuals who had never smoked or were former smokers (81.4 cm, $p < 10^{-12}$). In contrast, current smokers presented with higher median daily dietary intake (7306 kJ) than current abstainers (7037 kJ). The odds of presenting with elevated CRP concentrations were 71% higher for those homozygous for the minor allele (C/C) than non-smokers (Figure 3.1). Individuals with the wild-type had similar odds of presenting with elevated CRP concentrations, irrespective of their smoking status.

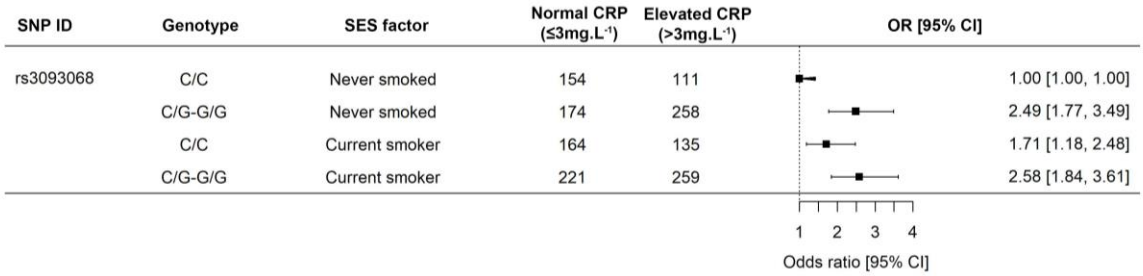

Figure 3.1: Interaction between tobacco smoke and rs3093068 in the Prospective Urban/Rural Epidemiology study – South African arm.
The minor allele is associated with increased CRP concentrations, which are further increased in smokers. Homozygous smokers for the minor allele had a 71% increased risk of presenting with elevated CRP concentrations. Abbreviations: CRP, C-reactive protein; C, cytosine; CI, 95% confidence interval; G, guanine.

3.5 Discussion:

Little evidence exists on whether — and indeed, if — individual SES factors that constitute an individual's immediate living environment affect their inflammatory status. In this study, we failed to find sufficient evidence that the investigated SES factors acted individually as impetus for elevated CRP concentrations, the exception being that lower CRP concentrations were predicted from adjusted analyses in individuals completing at least 12 years of formal education. Our evidence, however, highlights the fact that the inflammatory phenotype observed in black populations is the result of a combination of various factors, including, but not limited to, the combined effects of genetics with certain lifestyle choices such as smoking. Moreover, our results indicated that black individuals with CRP concentrations above 3 mg/L have a higher prevalence of CVD risk factors.

Several epidemiological studies exclude individuals with CRP concentrations above 10 mg/L, which is seen as the clinical cut-off point for acute infections. However, Ishii *et al.* (2012) reported that certain individuals, especially obese women, had repeatedly presented with CRP concentrations above 10 mg/L without any indication of acute infection. In our study, all individuals examined had normal body temperatures, reducing the likelihood of acute infection as a cause of excessively elevated CRP concentrations in the 363 (23.1%) individuals presenting with CRP concentrations above 10 mg/L. Nienaber-Rousseau et al. (unpublished) proved statistically that excluding participants within our population with CRP concentrations higher than 10 mg/L leads to the exclusion of certain *CRP* genotypes, which results in a biased representation of the actual drivers of increased CRP concentrations observed in black populations. Furthermore, we included these individuals as excluding them would have decreased the statistical power when stratifying within the different SES factors, which may have resulted in inappropriate conclusions. We also included individuals who were seropositive for HIV, as median CRP values were similar regardless of HIV status. Infection

rates are also higher among individuals with low SES, which could result in the introduction of bias should HIV-positive individuals be excluded (Bunyasi & Coetzee, 2017).

Elevated CRP concentrations (above 3 mg/L) were regularly observed in the women included in our study. Cushman *et al.* (2009) reported that black women were more likely to have CRP concentrations above 3 mg/L and that elevated CRP was more frequently observed in post-menopausal women, although it was strongly correlated with abdominal obesity. Likewise, the gender and menstrual biases dissipated when we corrected for WC in our study, implicating WC as a major contributing factor to the development of an elevated CRP phenotype. Anthropometric markers such as waist and hip circumferences, weight and BMI had significant positive correlations with CRP concentration (Table 3.2, ρ = 0.27, 0.21, 0.22 and 0.24, respectively). Various other reports record the influence of adiposity on the inflammatory state of the individual (Greenberg & Obin, 2006; Timpson *et al.*, 2011). The association between BMI and CRP, irrespective of ethnicity, was reported in another study (Wee *et al.*, 2008), and elevated CRP concentrations, as well as increased CVD risk, are often the result of increased adiposity (Timpson *et al.*, 2011).

Using CRP as a prognostic marker for future CVD risk appears to be independent of ethnic or geographical factors (Fonseca & de Oliveira Izar, 2016). Factors pertaining to CVD risk were observed as being elevated in individuals harboring elevated CRP concentrations in our population. Similar to our findings, a multi-ethnic study reports increased resting heart rate to be associated with increased concentrations of inflammatory markers, including CRP (Whelton *et al.*, 2014). Inflammation markers, and especially CRP, are also linked to vascular stiffness, atherosclerosis and the development of end-organ damage, characteristics of a long-term hypertensive state combined with hyperlipidemia (Hage, 2014). African Americans are also reported to be more likely to exhibit elevated HbA_{1c} concentrations, with CRP highly correlated with HbA_{1C} levels (Liu *et al.*, 2015). Excessive weight, hyperlipoproteinemia, and decreased insulin sensitivity are traits associated with the metabolic syndrome or MetS (Carr, 2003). Combined with the elevated inflammation levels, MetS was, therefore, prominent in the group of volunteers studied and even more so in post-menopausal women, regardless of their SES status.

SES factors differed significantly between urban and rural participants; however, CRP concentrations were similar regardless of where individuals resided. The lack of any impact exerted on CRP concentrations by SES factors (Table 3.2) further strengthens our observation that individual SES factors are not the main causative effect of elevated CRP concentrations in this population. The detected similarity in CRP concentrations between different levels of

urbanization with varying markers of SES is in contrast to observations made in an Asian population, where urbanized individuals had significantly higher CRP concentrations (Ye *et al.*, 2007). The years following the fall of apartheid in South Africa were marked by unprecedented rates of urbanization, which improved economic activity and increased rural-to-urban migrations (Pisa *et al.*, 2012). Furthermore, improved access to basic utilities resulted from governmental efforts, even in the rural areas included in this study (Department of Cooporative Governance and Traditional Affairs, 2012). It may, therefore, be argued that the definition of what constitutes a rural area differed between our two studies, which may have resulted in this discrepancy. Off all the included SES factors, only education was determined to be an influencer of CRP concentration, and only when controlling for other confounding variables.

Although some of the values in Table 3.4 suggest substantial changes in CRP for a single unit change in a specific variable, the interpretation should consider the physiological changes of such alterations. Age-dependent increases in CRP were associated with elevated adiposity due to changes in hormonal balances, as reported in previous studies (Puzianowska-Kuźnicka *et al.*, 2016) similar to our study. Substantial reductions in CRP were predicted with a 1 mmol/L change in HDL-c; however, eliciting this response may prove difficult in a resource-poor environment. These covariates, however, do predict possible routes of intervention, whereby proper nutrition (focusing on weight management, treatment of hyperlipidemia, and glycemic control), as well as increased physical activity (to improve resting heart rate) and increasing education levels, can reduce inflammation in populations (Galland, 2010; Medenwald *et al.*, 2015; Nimmo *et al.*, 2013). Completing 12 or more years of formal education was associated with reduced CRP concentrations (Table 3.1, unadjusted), although this reduction was found to be non-significant. In our multivariate model, completing secondary school or tertiary education corresponded to a significant 18.9% reduction in predicted CRP concentration. Kershaw *et al.* (2010) estimate that 87.9% of CRP variation attributed to education level could be primarily explained by the higher number of smokers, the lower dietary quality and reduced levels of exercise in lower educated individuals. Similarly, it was reported for our study cohort that education levels were associated, in both men and women, with lower BMIs (Pisa *et al.*, 2012).

Various other studies have also failed to find significant differences in the CRP concentrations of smokers versus non-smokers, although smoking is reported to affect CVD risk (Aksu *et al.*, 2013; Aldaham *et al.*, 2015). Smokers in our study had lower median WC, with higher daily dietary intakes compared with non-smokers. Previously, African American smokers were reported to have lower levels of weight gain than white Americans (Chiolero *et al.*, 2008).

Nicotine does, however, increase energy expenditure (Chiolero *et al.*, 2008), which may have resulted in the smaller WC observed in active tobacco users in our study. To our knowledge, we present the first indication that smoking status results in increased CRP concentrations in individuals harboring the minor allele of rs3093068, of which the major allele is associated with increased CRP concentrations (Nienaber-Rousseau *et al.*, 2014). Smokers with the minor allele had odds of presenting with elevated CRP concentrations statistically similar to those with the wild-type, negating the CRP-lowering effects of the minor allele.

3.5.1 Limitations:

Even though we did not have access to a standardized SES-index, we overcame this by focusing on individual factors that constitute an individual's living environment, to identify factors for which mitigation efforts could be instituted in an attempt to lower CRP concentrations. By using this approach, we found that individual factors did not affect the inflammatory state of the individual, although increasing education levels may have mitigated the risk associated with developing an inflammatory state, probably through an increased awareness of what constitutes healthy living. This study was also limited regarding the genetic markers available for analysis; however, the lack of association between individual SES factors and CRP concentrations might be indicative of the absence of SES X gene interactions. Our data were also cross-sectional in nature, and, therefore, do not account for changes in SES factors for which future elevated CRP concentrations were yet to be moderated by improvements in these SES factors. Future studies measuring SES factors should, consequently, also include questions regarding the period for which the individual had access to improved standards of living.

3.5.2 Implications:

Despite these limitations, our main findings strongly suggest that CRP concentrations in black South Africans are not associated with individual SES factors. Even though the SES factors included are not primarily responsible for the elevated CRP concentrations observed, improving the general SES of individuals commonly results in better health outcomes, and, therefore, there should be collective efforts to improve the general socio-economic standing of the people of the Republic of South Africa. Health promotion efforts should focus on reducing the individual symptoms that constitute MetS, with public health promotion efforts especially focused on individuals with lower education levels. Here we also presented the first evidence that smoking status increases CRP concentrations in individuals who are homozygous for the minor allele of rs3093068, although more evidence is needed from other ethnicities.

3.6 Notes:

3.6.1 Compliance with ethical standards

The authors and study coordinators complied with all ethical standards. The PURE-SA (North West province) study was approved by the Health Research Ethics Committee of the Faculty of Health Sciences, North-West University (NWU), in accordance with the ethical principles outlined by the Declaration of Helsinki with approval numbers 04M10, for the larger study, and NWU-00004-17-S1 for our affiliated study. Goodwill permission was granted by household heads and community leaders (mayors and traditional leaders), as well as the Department of Health of South Africa. Signed informed consent was given by each participant after being apprised of the aims of the study. Sufficient time for reflection was given, and subjects could withdraw at any time, or withhold whatever information they were not willing to share, without reprisal.

3.6.2 Conflict of interest

The authors declare that they have no conflict of interest.

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CHAPTER 4: MANUSCRIPT TWO

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Article

CRP Genotypes Predict Increased Risk to Co-Present with Low Vitamin D and Elevated CRP in a Group of Healthy Black South African Women

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Abstract: Low 25-hydroxyvitamin D (25(OH)D) and elevated C-reactive protein (CRP) concentrations are independently associated with adverse health outcomes, including cardiovascular disease (CVD). Although an inverse association between these factors has been described, the underlying mechanisms remain unknown. We postulate that environment–gene interactions, through which 25(OH)D interacts with single nucleotide polymorphisms (SNPs) within the *CRP* gene, modulate CRP; that certain *CRP* genotypes predispose individuals to a co-phenotype of low 25(OH)D and elevated CRP concentrations; and that this co-phenotype is associated with higher CVD risk. Twelve *CRP* SNPs were genotyped, and both 25(OH)D and CRP were quantified, in 505 black South African women. Alarmingly, 66% and 60% of the women presented with deficient/insufficient 25(OH)D and elevated CRP concentrations, respectively. CRP concentrations were higher in individuals with lower 25(OH)D concentrations. However, no 25(OH)D–*CRP* genotype interactions were evident. Several genotypes were associated with an altered risk of presenting with the co-phenotype, indicating a genetic predisposition. Women presenting with this co-phenotype had higher blood pressure and increased anthropometric measures, which may predispose them to develop CVD. We recommend increasing vitamin D fortification and supplementation efforts to reduce inflammation among black women with vitamin D deficiency, thereby possibly curbing diseases contingent on the co-phenotype described here.

Keywords: 25-hydroxyvitamin D; 25(OH)D; calcidiol; calciferol; C-reactive protein; nutrigenetics; single nucleotide polymorphisms; SNPs; Tswana

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CRP GENOTYPES PREDICT INCREASED RISK TO CO-PRESENT WITH LOW VITAMIN D AND ELEVATED CRP IN A GROUP OF HEALTHY BLACK SOUTH AFRICAN WOMEN

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4.2 Introduction

Cardiovascular disease (CVD) is the leading cause of mortality, with 80% of the global burden carried by developing countries (Nyirenda, 2016). In South Africa, at the time of this study, one third of all deaths were attributable to CVD (Sliwa *et al.*, 2008). Since then, an increase in CVD-related deaths has been reported (Statistics South Africa, 2017). The role of chronic inflammation in the etiology of CVDs has recently come to the fore. Although the inflammatory response is regarded as crucial for survival, dysregulation of this process has detrimental effects, including the development of CVDs (Ruiz-Núñez *et al.*, 2013; Shrivastava *et al.*, 2015).

Several biochemical markers of inflammation have been identified, including tumor necrosis factor-α, interleukin 1 (IL-1) and 6 (IL-6), and C-reactive protein (CRP). CRP is by far the most widely investigated biomarker of low-grade systemic inflammation, as it has a stable half-life with well-established associations with disease, and has known cut-off values (Ridker, 2016). People with a basal CRP concentration greater than 3 mg/L are at higher risk of experiencing cardiovascular events (Strang & Schunkert, 2014). Circulating CRP concentrations can be influenced by demographic factors (age, sex, and ethnicity) as well as environmental and behavioral factors (alcohol intake, diet, socio-economic status, and tobacco use) (Albert *et al.*, 2003; Deverts *et al.*, 2012; King, 2013; Shen & Ordovas, 2009; Wener *et al.*, 2000). Among dietary factors, an inverse association between vitamin D and CRP has been established (Carlson *et al.*, 2013; Liefaard *et al.*, 2015). A recent meta-analysis (Chen *et al.*, 2014) indicated that vitamin D supplementation decreased CRP concentrations by 1.08 mg/L (95% confidence interval (CI), −2.13, −0.03), while those with baseline CRP levels of >5 mg/L registered a more significant reduction of 2.21 mg/L (95% CI, −3.5, −0.92). Various genetic factors have been reported to affect CRP concentrations, with twin and family studies indicating substantial heritability of between 35% and 40% (Pankow *et al.*, 2001). Furthermore, interactions have been observed between CRP single nucleotide polymorphisms (SNPs) and dietary intake influencing circulating CRP concentrations (Nienaber-Rousseau *et al.*, 2014). In contrast, infection with the human immune deficiency virus (HIV) is not associated with CRP (Bipath *et al.*, 2012), an observation re-established by the present study.

Black individuals tend to have higher circulating CRP concentrations than those from other groups (Nazmi & Victora, 2007). African Americans, included in a meta-analysis, presented with CRP concentrations of 2.6 mg/L compared to 2.51 mg/L in Hispanics, 2.03 mg/L in white Americans, and 1.01 mg/L in East Asians (Shah *et al.*, 2010). Elevated CRP concentrations are also typical in black South Africans (Nienaber-Rousseau *et al.*, 2014); comparisons show

them to be higher (3.61 mg/L) than those of their white (1.13 mg/L) compatriots (Mokhaneli *et al.*, 2016).

Ethnicity also affects vitamin D status. Darker skin (type V and VI skin on the Fitzpatrick scale (Fitzpatrick, 1988)), as observed in African individuals, seems to have originated in persons living in areas with high ultra-violet (UV) radiation (UVR) (Jablonski & Chaplin, 2010). Even though it is a photoprotective mechanism, darker skin reduces the synthesis of vitamin D (Harris, 2006) as measured by circulating 25-hydroxyvitamin D (25(OH)D), or calcidiol, concentrations. Darker-skinned humans require approximately six times more UVR exposure than their fairer-skinned counterparts to produce similar amounts of vitamin D (Wright *et al.*, 2012). South Africa, ranging between the ~22° and 34° southern latitudes, experiences relatively intense UVR, although not as extreme as at the equator (Wright *et al.*, 2012). Black South Africans present with lower 25(OH)D concentrations than dark-skinned people living closer to the equator (O'Connor *et al.*, 2013). The rapid urbanization observed in most African countries, resulting in reduced exposure to UVR, has been proposed as a major contributing factor to the low 25(OH)D status observed in black Africans (Norval *et al.*, 2016). Other risk factors associated with low 25(OH)D status include age, obesity, HIV infection, and smoking (Je & Js, 2011; Kassi *et al.*, 2015; Tsiaras & Weinstock, 2011).

Apart from influencing inflammation, vitamin D itself is related to disease risk. 25(OH)D has preventive effects on a range of chronic maladies, including CVD (Rosen *et al.*, 2012). Two meta-analyses found an increase in the risk of developing ischemic heart disease, as well as an augmented risk of symptomatic ischemic stroke for the participants in the lowest quartiles of 25(OH)D concentrations (Brondum-Jacobsen *et al.*, 2012; Brondum-Jacobsen *et al.*, 2013). Another meta-analysis reported an inverse association between 25(OH)D concentrations and the risks associated with all-cause mortality (relative risk (RR) of 1.35 (1.22–1.49)) and CVD (RR: 1.35 (1.13–1.61)) (Chowdhury *et al.*, 2014).

As elevated concentrations of CRP are associated with increased CVD risk, whereas CVD risk is reduced with elevated levels of 25(OH)D, a hypothesis has been proposed that 25(OH)D might influence CRP [35]. However, there is no evidence of a direct pathway by which 25(OH)D or its metabolized product, 1,25-dihydroxyvitamin D (or calcitriol), affects the expression of CRP (or vice versa) (Liefaard *et al.*, 2015). Vitamin D-mediated mechanisms for a reduction in vascular damage have been proven experimentally, with the inhibition of cholesterol uptake by macrophages and the suppression of the renin gene (Carlson *et al.*, 2013). In vitro studies have also described the diminished production of IL-6 in monocytes treated with vitamin D3 (cholecalciferol, or calciol) compared to untreated cells (Dickie *et al.*,

2010). IL-6, synthesized by macrophages, is transported to the liver, where the transcriptional activation of CRP is mediated via Signal Transducer and Activator of Transcription factor 3 (STAT3) (Bode *et al.*, 2012; Zhang *et al.*, 1996). These physiological mechanisms might, therefore, act as potential indirect pathways by which vitamin D and its metabolites could influence CRP concentrations. Thus, having elevated CRP as well as low 25(OH)D concentrations, which are both independently associated with increased CVD risk, may exacerbate disease development.

Because black individuals tend to be predisposed to lower 25(OH)D and higher CRP concentrations, investigations that involve this particular population will increase the chance of observing sufficient numbers of the phenotypes of interest. An inverse association between 25(OH)D and inflammation has been described, but the underlying mechanisms remain unknown (Liefaard *et al.*, 2015). Moreover, it has to be established whether vitamin D status influences CRP differently in individuals harboring specific CRP genotypes in modulating CRP concentrations. We determined whether vitamin D status possibly interacts with these CRP genotypes to affect the CRP concentrations. In addition, we tested whether CRP SNPs affected 25(OH)D concentrations. Studying environment–gene interactions is important, as identifying these interactions and modifying behaviors accordingly could improve health outcomes. Furthermore, we determined whether specific CRP genotypes predispose individuals to a co-phenotype of low 25(OH)D and elevated CRP, as well as whether this co-phenotype is associated with a higher CVD risk in black South African women. This research is important, because unraveling possible mechanisms for the observed relationship between vitamin D and CRP leads to a better understanding of the foundation of this relationship and paves the way for designing targeted approaches to treat the corresponding elevated CRP concentrations and low 25(OH)D concentrations in black individuals. This research might also indicate whether efforts to increase responsible sunlight exposure and include more vitamin-D-rich foods in their diet are, and/or whether supplementation with vitamin D is, desirable for black South African women.

4.3 Materials and Methods

4.3.1 Ethical Considerations

For this cross-sectional investigation, we used data collected for the South African arm of the Prospective Urban and Rural Epidemiology (PURE-SA) study, at baseline (2005). Ethical approval, in accordance with the Declaration of Helsinki as revised in 2004 (Carlson *et al.*, 2004), was obtained for the larger study from the Health Research Ethics Committee of the Faculty of Health Sciences, North-West University (NWU–HREC, ethics number: 04M10). Ethical approval was also granted for this affiliated study (ethics number: NWU-00004-17-A1).

Goodwill permission was granted to the PURE study by mayors, household heads, community leaders of the communities included, and tribal chiefs before the research started. Participants were well-advised about the research project and were asked to sign an informed consent form, after sufficient time for reflection, to indicate their agreement to take part in the study. Subjects could withdraw at any time, or withhold any information they were not comfortable sharing.

4.3.2 Research Design and Study Population

The PURE-SA study aims to investigate the development of chronic lifestyle diseases, with a focus on CVDs, by stratifying populations at different levels of urbanization (Teo *et al.*, 2009). Four communities were selected in 2005 in South Africa, based on their degree of urbanization, and grouped into either being urban (Location A) or rural (Location B). The initial sampling strategy is explained elsewhere (Vorster *et al.*, 2014).

Eligible participants were all apparently healthy adults who were older than 30 years. On the day of enrollment, individuals with elevated body temperatures (above 38 °C) were excluded to reduce the number of volunteers with acute infections. Further exclusion criteria were that potential volunteers were not allowed to use chronic medication, to have any known lifestyle disease, be pregnant or lactating, or to have a known infection, such as tuberculosis-causing agents and/or the human immune deficiency virus (HIV) (details in (Vorster *et al.*, 2014)). Sampling was conducted between August and November 2005, which is late winter to late spring in the southern hemisphere. Of 6000 individuals screened, 2010 were included at baseline. The 25(OH)D status of a subset of 660 randomly selected women was determined, because of constrained budgets and the fact that women are more likely to develop skeletal disorders associated with low 25(OH)D status.

4.3.3 Biochemical and Blood Pressure Measurements

Fasting participants, defined as *sans* food and beverages (water permitted) from the evening before enrollment, arrived at the study site, upon which professional nurses obtained blood samples. Blood tubes were centrifuged at 2000× *g* for 15 min at 10 °C. Plasma, serum, and buffy-coat were aliquoted and snap-frozen on dry-ice pellets before storage at −70 °C. Serum high-sensitivity CRP concentrations were measured on a Sequential Multiple Analyzer Computer using a particle-enhanced immunoturbidometric assay (Konelab TM auto analyzer, Thermo Fisher Scientific, Vantaa, Finland). Total 25(OH)D (sum of D_2 and D_3) in serum was quantified using a Roche Elecsys 2010 COBAS system (functional sensitivity: 10.0 nmol/L; Roche Diagnostics, Indianapolis, IN, USA). Lipograms, including high-density lipoprotein cholesterol, triglycerides, and total cholesterol, were performed using a Konelab 20i auto analyzer (Thermo Fisher Scientific, Vantaa, Finland). The Friedewald equation was used to

calculate the low-density lipoprotein cholesterol (LDL-c) in those with triglyceride concentrations below 400 mg/dL (Friedewald *et al.*, 1972). Research nurses—trained in voluntary counseling and the testing of HIV, adhering to the UNAIDS/WHO policy statement on HIV testing as well as the protocols set by the National Department of Health of South Africa—gave all participants pre-test counseling. Volunteers could then decide whether they wanted to be tested, with specific signed informed consent obtained for HIV testing after pre-test counseling. HIV determination was conducted using a rapid First Response HIV 1-2.O card test (Transnational Technologies Inc., PMC Medical, Nani Daman, India). Persons testing positive were re-tested using a second card test, developed by Pareeshak (BHAT Bio-Tech, Bangalore, India) to affirm HIV status. All participants, irrespective of their HIV status, were given post-test counseling individually. Blood pressure was measured in duplicate with an Omron automatic digital blood pressure monitor (Omron HEM-757) after 5 min of sitting in a calm environment.

4.3.4 Anthropometric Measurements

Body weights (kg) were measured twice on calibrated and tared scales, with the mean recorded, while participants were lightly clothed and their arms hanging freely at their sides. Heights (cm), with volunteers' heads in the Frankfort plane, bodies fully extended while inhaling, were measured twice to the nearest 10 mm, using stadiometers, and the mean was reported in meters. Body mass index was computed as kg/m^2.

4.3.5 Factors Pertaining to Lifestyle

Participants responded to various interviewer-administered questionnaires in a language of their choice. These test instruments included questions on medical history and tobacco use. Nutritional information from the previous 30 days was obtained using validated, interviewer-based quantitative food frequency questionnaires (qFFQs) and employing food portion books standardized for the population under investigation (MacIntyre *et al.*, 2001). qFFQs' data were entered into FoodFinder 3 (Medical Research Council, Tygerberg, South Africa) and analyzed by the Medical Research Council of South Africa for nutrient content.

4.3.6 Genetic Analyses

Determination of the genotypes via a BeadXpress analysis was performed by the National Health Laboratory Service located at the University of the Witwatersrand, Johannesburg. For details on the genetic analyses, please refer to Nienaber-Rousseau et al. (Nienaber-Rousseau *et al.*, 2014).

4.3.7 Environmental Data

Locations A and B were compared using the means of data from 1 August (late winter) to 1 December 2005 (late spring). Environmental factors were investigated using satellite data obtained from an online repository, Giovanni (Acker & Leptoukh, 2007). Average mean temperature, UV index, erythemal dose rate, and total ozone column were downloaded as Google Earth data files (.kmz files).

4.3.8 Statistical Analyses

As previously mentioned, 25(OH)D concentrations were available for 660 randomly selected women. Only individuals for whom 25(OH)D concentrations, CRP concentrations, *CRP* genetic data, and all anthropometric markers were available were included in our statistical analyses (n = 534). Furthermore, women with 25(OH)D or natural log-transformed (ln)CRP concentrations greater than 5 standard deviations were excluded as outliers. The final number of participants was 505. Statistical analyses were conducted in R (R Core Team, 2017).

Numeric variables were visually inspected for normality as well as measures of skewness. Non-parametric variables (CRP) were log-transformed, yet still reported as median and interquartile ranges. Women were grouped as two phenotypes: the case phenotype including individuals with deficient/insufficient 25(OH)D (<75 nmol/L) and elevated CRP (>3 mg/L), and a control phenotype consisting of the remaining volunteers. Pairwise comparisons using the Wilcoxon ranked-sum test were performed to identify significant differences in stratified continuous variables. Comparative tables were created with the compare Groups library in R (Subirana *et al.*, 2014) using non-parametric methods. Spearman correlations were used for testing associations between numeric variables. Multivariate linear models predicting lnCRP concentrations from continuous 25(OH)D values were constructed using backward step-wise linear regressions and evaluated based on the Akaike Information Criterion (AIC). Adherence to Hardy–Weinberg equilibrium (HWE) was tested by a chi-squared (χ^2) test using SNPassoc, and linkage disequilibrium (LD) was calculated using the LDheatmap library of the R package.

Variables identified in regression analyses were evaluated for co-linearity. Possible environment–SNPs interaction was determined using SNPassoc (Gonzalez *et al.*, 2007) while including covariates obtained from the linear regression model. To determine whether *CRP* SNPs influence vitamin D status, 25(OH)D was used as the dependent variable. To evaluate the risk associated with certain *CRP* SNPs to present with the phenotype of low 25(OH)D combined with elevated CRP concentrations, the case and control phenotypes were entered as dependent variables.

Where applicable, *p*-values were adjusted using the methods suggested by Bonferroni. Significance was defined as an α level of 0.05.

4.4 Results

4.4.1 Association of 25(OH)D Concentrations/Status with Circulating CRP Concentrations

Median concentrations for 25(OH)D and CRP were 68.2 nmol/L and 4.13 mg/L, respectively, indicating that the 25(OH)D status of the women in our cohort was insufficient, while they also presented with elevated inflammation based on CRP. CRP concentrations decreased across increasing 25(OH)D categories, with the median CRP concentration being significantly lower in the sufficient 25(OH)D group compared to both the deficient and insufficient subdivisions (Figure 4.1). The largest variability in CRP concentrations was observed for those in the 25(OH)D-deficient category, with decreasing variability in the insufficient and sufficient groups. In the population investigated, 42% (*n* = 216) of individuals presented with both 25(OH)D concentrations lower than 75 nmol/L and elevated CRP concentrations above 3 mg/L (case phenotype).

In Table 4.1, we have summarized the demographic characteristics of the case and control phenotypes: 25(OH)D concentrations decreased with age. The distribution between rural and urban cases and controls was similar. In addition, the environmental exposure that could have influenced vitamin D status did not differ between the rural or urban areas. Individuals representing the case phenotype were significantly older. Similar distributions were also observed in respect of smoking and HIV status. The median dietary intake of vitamin D sources did not differ for the two groups either.

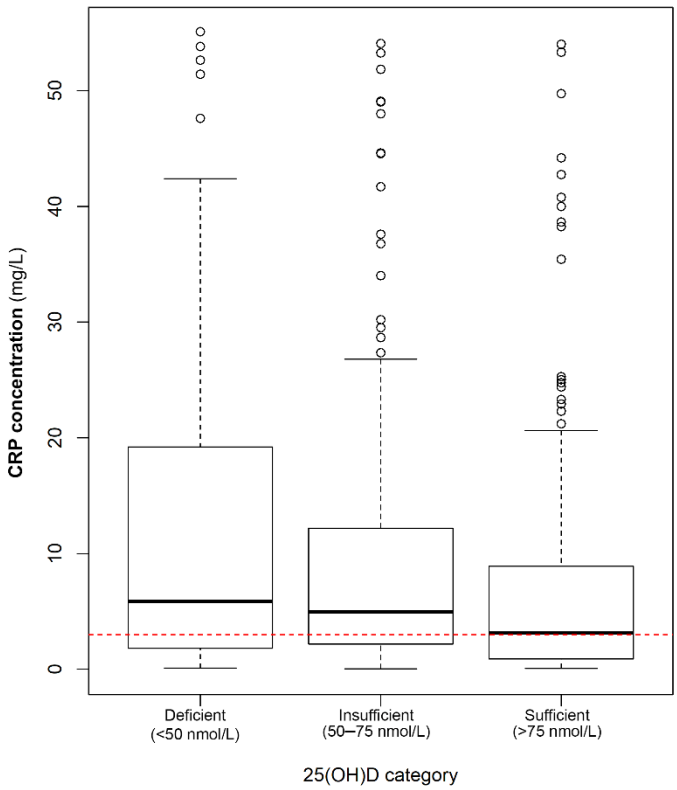

Figure 4.1: Median C-reactive protein (CRP) concentrations across different categories of 25(OH)D status (*p* = 0.001).
Pairwise Wilcoxon ranked-sum test with Bonferroni adjustment revealed that women with sufficient 25(OH)D concentrations had significantly lower CRP concentrations than those with deficient or insufficient 25(OH)D. Outliers depicted as open circles. The red dashed line indicates the cut-off value for CRP concentrations with elevated CRP being greater than 3 mg/L.

Table 4.1: Comparisons of demographical and biochemical factors in the cohort stratified by control and case phenotypes.

Variable	Controls (*n* = 289, 57.2%)	Cases (*n* = 216, 42.8%)	*p*-Value
Urban/Rural	144 (49.8%)/145 (50.2%)	126 (58.3%)/90 (41.7%)	*NS*
Age (years)	53.0 (49.0; 59.0)	56.0 (51.0; 63.0)	<0.001
Smoking status: Former/Current/Abstainer	6 (2.10%)/138 (48.3%)/142 (49.7%)	7 (3.24%)/96 (44.4%)/113 (52.3%)	*NS*
HIV positive/negative	26 (9.03%)/262 (91.0%)	15 (6.98%)/200 (93.0%)	*NS*
Vitamin D intake (µg/day)	2.00 (1.02; 3.30)	2.05 (1.02; 3.66)	*NS*
Menorhea/Amenorhea	64 (23.0%)/214 (77.0%)	37 (17.2%)/178 (82.8%)	*NS*

Data presented as median (25th and 75th percentiles) for continuous data and number of observations (percentage) for categorical data. Abbreviations: 25(OH)D, 25-hydroxyvitamin D; HIV, human immune deficiency virus; *NS*, not significant ($p > 0.05$).

Differences in CVD risk markers between the two phenotypes are presented in Table 4.2. Those representing the control phenotype had lower blood pressure. However, heart rate was similar in the two groups. Anthropometric markers indicated that cases had significantly higher body weight and increased waist circumference. More obese individuals were also observed in the case phenotype. HDL-c was significantly higher in the control group; however, after adjusting for age, waist circumference, and LDL-c, the significance was eliminated.

Furthermore, no statistical differences were observed for LDL-c, triglycerides, or energy intake, although energy intake was lower for the controls than for the case phenotypes.

Table 4.2: Markers of cardiovascular disease (CVD) risk among the control and case phenotypes

Variable		Controls (*n* = 289, 57.2%)	Cases (*n* = 216, 42.8%)	*p*-Value	Adjusted *p*-Value
Systolic blood pressure (mmHg)		133 (118; 148)	138 (124; 159)	<0.001	<0.01
Diastolic blood pressure (mmHg)		87.0 (78.0; 96.0)	91.0 (83.8; 101)	<0.001	<0.01
Heart rate (BPM)		73.0 (65.0; 83.0)	72.0 (63.0; 85.0)	*NS*	*NS*
Waist circumference (cm)		79.3 (70.8; 87.7)	86.4 (74.8; 95.5)	<0.001	<0.001
Hip circumference (cm)		98.0 (89.5; 106)	106 (94.1; 119)	<0.001	0.01
WHR		0.81 (0.76–0.87)	0.81 (0.76–0.86)	*NS*	*NS*
Weight (kg)		60.2 (52.1; 72.8)	72.0 (56.5; 85.5)	<0.001	<0.01
BMI (kg/m^2)		24.7 (21.3; 29.0)	29.9 (23.3; 35.2)	<0.001	<0.001
BMI category	*Underweight*	29 (10.0%)	22 (10.2%)	<0.001	<0.001
	Healthy	122 (42.2%)	45 (20.8%)		
	Overweight	80 (27.7%)	42 (19.4%)		
	Obese	58 (20.1%)	107 (49.5%)		
Total cholesterol (mmol/L)		5.21 (4.38; 6.33)	5.30 (4.52; 6.20)	*NS*	*NS*
High-density lipoprotein cholesterol (mmol/L)		1.48 (1.14; 1.92)	1.36 (1.07; 1.76)	0.03	*NS*
Low-density lipoprotein cholesterol (mmol/L)		3.34 (2.64; 4.23)	3.58 (2.69; 4.41)	*NS*	*NS*
Triglycerides (mmol/L)		1.22 (0.90; 1.79)	1.32 (0.92; 1.78)	*NS*	*NS*
Dietary intake (kJ)		6620 (5056; 9265)	7432 (5294; 9283)	*NS*	*NS*

Adjusted for age, waist circumference, and LDL-c. Abbreviations: BMI, body mass index; BPM, beats per minute; CRP, C-reactive protein; mmHg, millimeters of mercury; WHR, waist hip ratio. Data presented as median (25th and 75th percentiles) for continuous data and number of observations (percentage) for categorical data. BMI categories' cut-off values: Underweight <18.5 kg/m^2; healthy 18.5–24.9 kg/m^2; overweight 24.9–29.9 kg/m^2; obese >29.9 kg/m^2. Values presented in accordance with the International System of Units: to convert kJ to Cal multiply by 0.24.

4.4.2 Quantification of the Associations of 25(OH)D with CRP Concentrations

Spearman correlation analyses (results not shown) revealed that 25(OH)D was inversely, albeit weakly ($\rho > -0.20$; $p \leq 0.05$), associated with age. No other factors were associated with 25(OH)D with a correlation greater than 0.20, so that these are not reported here except for the correlation with CRP presented later. CRP was moderately associated with anthropometric markers (all $\rho > 0.30$; $p < 0.05$) and lipid profile markers of which LDL-c ($\rho = 0.13$; $p < 0.05$) presented with the strongest correlation. Similar to vitamin D status, other variables did not correlate strongly with CRP even though these correlations were statistically significant.

A weak, yet significant, negative correlation was observed between 25(OH)D and CRP ($\rho = -0.15$; $p < 0.05$). Converting 25(OH)D from nmol/L to mg/L, using a conversion factor of 0.0004 (Equation (1)), indicated that a one-unit increase in 25(OH)D was associated with a 0.15 mg/L decrease in CRP concentration. Vitamin D intake did not correlate with 25(OH)D or CRP concentrations.

$$1\ \text{nmol/L}\ 25(\text{OH})\text{D} = 0.4\ \text{ng/mL}\ 25(\text{OH})\text{D} = 0.0004\ \text{mg/L}\ 25(\text{OH})\text{D}$$
$$0.0004\ \text{mg/L}\ 25(\text{OH})\text{D} \equiv -0.15\ (\text{CRP})\ \text{mg/L} \quad (1)$$

The linear relationship between 25(OH)D as a factor influencing lnCRP concentrations was modelled in two ways: for the first model, we adjusted for age; and in the second, we adjusted for age, anthropometrical marker (see discussion below), and LDL-c. These covariates were chosen owing to the likelihood of these variables influencing the model based on their previous association with CRP concentrations, as well as having the lowest AIC score. As there is a

large degree of co-linearity between anthropometric markers, each marker (i.e., BMI, waist and hip circumference, and weight) was entered into the model separately, and models were evaluated based on their resulting AIC value. The lowest AIC value was observed for waist circumference and LDL-c; therefore, these markers were used as a proxy for all other anthropometric and lipid profile markers, respectively. Dietary sources of vitamin D did not affect the model (p >0.05). The unadjusted model (Model 1) accounted for 2.1% of the variance of lnCRP (calculated from adjusted R^2 values; p = 0.001). For Model 1, a 1.1% reduction in CRP concentration (converting lnCRP to CRP by using $(e^{\beta} - 1) \times 100$)) for each 1 nmol/L increase in 25(OH)D was observed. In Model 2, when adjusting for age, waist circumference, and LDL-c, 18.3% of the lnCRP variation could be explained (p <0.00001). The inverse relationship between vitamin D status and CRP was slightly intensified when controlling for the covariates. Here, for each 1 nmol/L increase in 25(OH)D, CRP decreased by 1.1%. Excluding individuals with CRP concentrations above 10 mg/L—the clinical cut-off point for acute inflammation—resulted in similar trends being observed (0.71% and 0.82% reduction per 1 nmol/L increase of 25(OH)D for unadjusted and adjusted models, respectively; results not shown).

4.4.3 SNP Interaction

All genotyped SNP frequencies reflected the assumptions of what would be expected under Hardy–Weinberg equilibrium. Previously, in our population, LD was reported between rs2027471 with rs1341665 and rs3093058 with rs3093062 (Nienaber-Rousseau *et al.*, 2014). In the subset of women studied here, the same LD pattern (Figure 4.2) was observed. Here, linkage was also detected for a haplogroup linking rs7553007, rs1341665, rs2027471, rs1205, and rs2794520 (Figure 4.2).

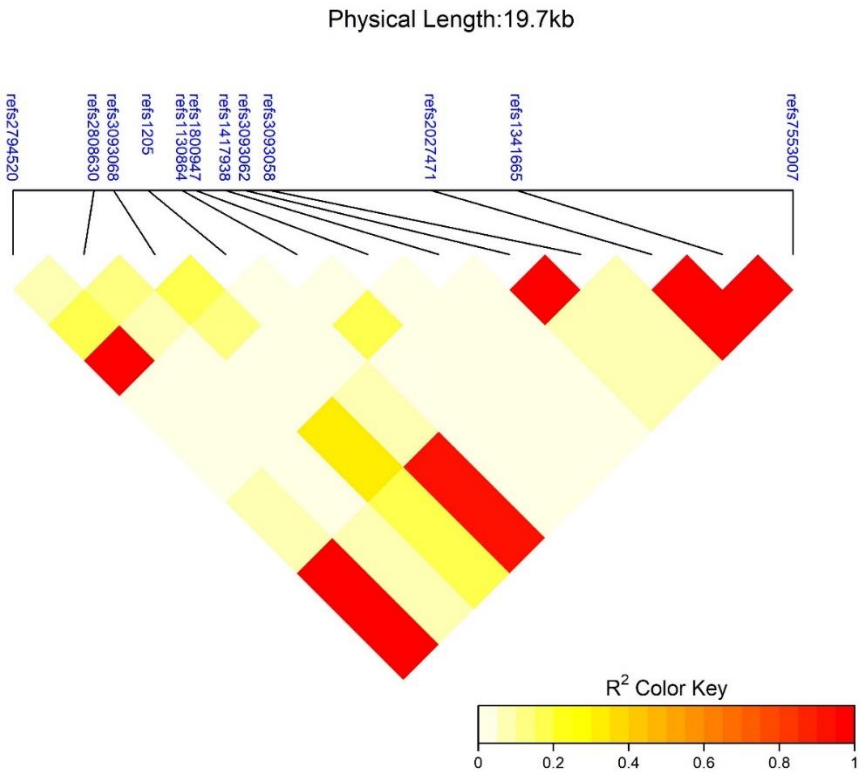

Figure 4.2: Linkage disequilibrium heatmap indicating linkage between 12 single nucleotide polymorphisms (SNPs) on the CRP gene.

Using the association analyses provided by the SNPassoc package in R, trends were investigated based on models of interaction between 25(OH)D concentrations with *CRP* SNPs on lnCRP concentrations. Associations were investigated under co-dominant, dominant, additive, and recessive genetic models, and the genetic model with the lowest AIC values was included (Supplementary Table 4.1). The minor alleles of the five SNPs (i.e., rs7553007, rs1341665, rs2027471, rs1205, and rs2794520) in high linkage were previously reported to be associated with decreases in circulating CRP (Nienaber-Rousseau *et al.*, 2014), which was echoed by our results. Three SNPs were also significantly associated with increased CRP concentrations: rs3093058, rs3093062, and rs3093068.

To quantify whether 25(OH)D concentrations interacted with any of the 12 *CRP* SNPs to affect lnCRP concentrations, factorial analyses of co-variance with age, LDL-c, and waist circumference as covariates were performed. In the 12 SNPs investigated, 96.0% of the genetic variation could be grouped into six haplotypes. No interactions were observed between 25(OH)D concentrations and the identified haplotypes to affect lnCRP concentrations (p for trend = 0.68). No significant associations between *CRP* SNPs and 25(OH)D were found under either the co-dominant or dominant model.

To investigate whether a genotype was associated with an increased risk of presenting with either deficient or insufficient 25(OH)D and elevated CRP concentrations, odds ratios (OR)

were calculated using the SNPassoc library in R for each of the 12 SNPs while adjusting for age, LDL-c, and waist circumference (Table 4.3). The minor alleles of SNPs previously associated with significant increases in CRP concentrations were found to be at higher odds of co-presenting with insufficient/deficient 25(OH)D concentrations and vice versa. The minor alleles of the five SNPs in LD were associated with a reduced risk of presenting with the phenotype of inadequate 25(OH)D combined with elevated CRP concentrations (cases) compared to the phenotype presenting with either sufficient 25(OH)D or normal (<3 mg/L) CRP concentrations. Of these, rs3093068, rs3093062, and rs3093058 presented with increased odds (1.54, 1.64, and 1.67, respectively), while reduced odds were observed in rs2794520 and rs7553007 (0.65 and 0.67, respectively) for individuals harboring the minor alleles. A trend towards significance for ORs for carriage of the minor alleles at two *CRP* SNPs (rs2027471 and rs1341665) to present with the co-phenotype was observed (OR: 0.05; p >0.05).

Table 4.3: Genetic predisposition to develop insufficient/deficient 25(OH)D combined with elevated CRP concentrations adjusting for age, low-density lipoprotein cholesterol (LDL-c), and waist circumference.

SNP ID	Allele	Control Phenotypes	%	Case Phenotypes	%	Odds Ratio (95% CI)	*p*-Value
rs2794520	C/C	166	57.4	141	66.8		0.03
	C/T–T/T	123	42.6	70	33.2	0.65 (0.44–0.95)	
rs2808630	T/T	198	68.5	154	71.6		*NS*
	C/T–C/C	91	31.5	61	28.4	0.79 (0.53–1.18)	
rs3093068	C/C	123	42.9	72	33.5		0.03
	C/G–G/G	164	57.1	143	66.5	1.54 (1.05–2.26)	
rs1205	C/C	171	59.2	142	66		*NS*
	C/T–T/T	118	40.8	73	34	0.72 (0.49–1.05)	
rs1130864	C/C	216	74.7	170	79.1		*NS*
	C/T–T/T	73	25.3	45	20.9	0.86 (0.56–1.34)	
rs1800947	C/C	289	100	213	99.1		*NS*
	C/G	0	0	2	0.9	0	
rs1417943	A/A	277	95.8	203	95.3		*NS*
	A/T	12	4.2	10	4.7	1.7 (0.70–4.13)	
rs3093062	G/G	214	74.3	137	63.7		0.02
	A/G–A/A	74	25.7	78	36.3	1.64 (1.10–2.45)	
rs3093058	A/A	215	74.4	135	63.4		0.01
	A/T–T/T	74	25.6	78	36.6	1.67 (1.12–2.50)	
rs2027471	T/T	167	57.8	142	66		0.05
	A/T–A/A	122	42.2	73	34	0.68 (0.46–1.00)	
rs1341665	G/G	167	57.8	142	66		0.05
	A/G–A/A	122	42.2	73	34	0.68 (0.46–1.00)	
rs7553007	G/G	164	56.7	141	65.6		0.04
	A/G–A/A	125	43.3	74	34.4	0.67 (0.46–0.98)	

Cases are those presenting with the phenotype of deficient or insufficient 25(OH)D together with elevated (>3 mg/L) CRP concentrations. Controls were individuals with normal CRP and/or sufficient 25(OH)D concentrations. The reference group comprised those homozygotes for the wild-type allele. Abbreviations: 25(OH)D, 25-hydroxyvitamin D; A, adenine; C, cytosine; CI, confidence interval; G, guanine; rs, reference SNP cluster ID; T, thymine.

4.4.4 Discussion

In this research, we confirmed the existence of an inverse relationship between CRP and vitamin D and attempted to unravel the mechanisms involved. From this study, we know that vitamin D status does not directly modulate CRP via *CRP* SNPs nor do *CRP* SNPs influence vitamin D, even though these SNPs influence CRP concentrations (data presented elsewhere). Since we found that the double phenotype of high CRP and low vitamin D was

associated with particular *CRP* SNPs, we hypothesized that negative feedback mechanisms were at play (will be described later). In cases where these genotypes were co-observed with low 25(OH)D concentrations, poorer CVD markers were also observed, such as elevated blood pressure.

The majority (65.5%) of the South African women in our cohort had 25(OH)D concentrations lower than the recommended 75 nmol/L. Moreover, 42.8% of them presented with the co-burden of low vitamin D status combined with elevated CRP concentrations. Having deficient or insufficient 25(OH)D and elevated CRP concentrations was previously shown to increase the risk of developing various chronic non-communicable conditions, such as CVD (Chowdhury *et al.*, 2014; Rosen *et al.*, 2012; Shrivastava *et al.*, 2015). In our study, volunteers classified as cases (those presenting with both low vitamin D status and high CRP concentrations) had significantly higher blood pressure and anthropometrical markers (even when adjusting for waist circumference), precursors in the etiology of CVD, than controls. We established that vitamin D correlated inversely with CRP, which is in accordance with a previous report (Liefaard *et al.*, 2015). Moreover, we found that CRP decreased within increasing strata of 25(OH)D categories as recommended by the Endocrine Society (Holick *et al.*, 2011) and confirmed this inverse association with our regression models. Furthermore, we proposed that 25(OH)D might interact with SNPs located on the *CRP* gene, thereby influencing CRP concentrations. Contrary to our hypothesis, none of the *CRP* SNPs investigated showed any interactions with circulating 25(OH)D; thus, vitamin D does not modulate *CRP* genotypes to influence CRP concentrations. The inverse association between vitamin D and CRP is therefore not due to nutrigenetic effects. A recent Mendelian association study also found a lack of association between 25(OH)D and genetic markers influencing CRP concentrations (Liefaard *et al.*, 2015) even though they detected a negative correlation between 25(OH)D and CRP concentrations.

We also investigated whether *CRP* SNPs influenced 25(OH)D concentrations directly to learn whether a possible unknown backward feedback mechanism might exist or whether certain *CRP* SNPs predispose individuals to having heightened inflammation, with concurring maladies, leading to reduced UV exposure resulting in altered vitamin D status, but found no associations. We then determined whether harboring CRP genotypes could increase the odds of co-presenting with both high CRP and low vitamin D status. Carriage of the minor allele at three SNPs (rs3093068, rs3093058, and rs3093062) was associated with increased odds, while harboring the variant allele at two SNPs (rs2794520 and rs7553007) resulted in lower odds to present with the phenotype of insufficient/deficient 25(OH)D (<75 nmol/L) and

elevated CRP concentrations (>3 mg/L). This is, to our knowledge, a novel addition to the existing literature, as these genetic effectors were not previously reported in this context.

How blood pressure is influenced by vitamin D status remains inconclusive (Tamez *et al.*, 2013), although there are suggestions of causative pathways (Sluyter *et al.*, 2017). Li (2003) hypothesized that vitamin D could have an influence on the renin–angiotensin system (RAS), which was substantiated by a study that reported how low concentrations of 25(OH)D upregulated the RAS (Tomaschitz *et al.*, 2010). In our population, 25(OH)D was reported to be associated with carotid wall thickening and arterial stiffness (Gafane *et al.*, 2015), both being attributes observed in individuals with increased CRP concentrations. This is a possible mechanism whereby low 25(OH)D could result in increased arterial stiffness, and in turn result in increased blood pressure (Kruger *et al.*, 2013), leading to elevated CRP concentration by negative feedback mechanisms. Individuals with the case phenotype were also investigated in another population, where cases had worse pro-inflammatory marker panels than controls (Azizieh *et al.*, 2016). However, with increasing CRP concentrations, it was reported that the anti-inflammatory effects of vitamin D decreased substantially and most of the other pro-inflammatory markers were upregulated (Azizieh *et al.*, 2016). These risk factors are further exacerbated by the presence of abdominal adiposity observed in our population (Nienaber-Rousseau *et al.*, 2017), thereby further increasing the risk of developing CVD by means of increased pro-inflammatory factors (such as IL-6) released by adipose tissue. Another possibility is that low vitamin D status has harmful side effects that are pro-inflammatory themselves; alternatively, synergistic effects between these two factors may exist, explaining the co-existence of low vitamin D with high CRP, as was the case in Kuwaiti women (Azizieh *et al.*, 2016). Low concentrations of 25(OH)D have been linked to an increased risk of developing CVD (Muscogiuri *et al.*, 2017), although definite conclusions remain ambiguous (Al Mheid & Quyyumi, 2017). Similarly, CRP has been strongly associated with increased CVD risk (Ridker, 2016). Future studies should explore possible mechanisms for the inverse association between 25(OH)D and CRP further, as well as whether the presence of both low vitamin D status and inflammation might heighten disease risk.

Differences in 25(OH)D were observed among individuals residing in the two different locations (A and B) that were investigated here (Supplementary Table 4.2). Although 25(OH)D and CRP values were closer to the recommended concentrations in rural participants, neither median 25(OH)D nor CRP concentrations met the recommended guidelines in both population subdivisions. Rural-urban differences in 25(OH)D disappeared when adjustments for age were made. Although age was reported as a non-significant contributor to our linear models, it was included in analyses as a possible covariate based on four previously reported reasons.

First, aging results in decreased concentrations of 7-dehydrocholesterol in the epidermis, which in turn reduces the response to UV light and subsequently results in decreased formation of pre-vitamin D_3 (Gallagher, 2013; MacLaughlin & Holick, 1985). Second, a decline in absorption, transport, or liver hydroxylation of orally ingested vitamin D sources was reported in older individuals (Harris *et al.*, 1999). Increased frailty with advancing age may also result in individuals spending less time outdoors, affecting their exposure to UV sources. Lastly, age, sex, and ethnicity are recommended factors to adjust for when conducting predictive analyses for CRP (Wener *et al.*, 2000), with sex and ethnicity controlled for in our black, female population. It could, however, be argued that environmental and climate differences, such as reduced UVR, could have affected 25(OH)D concentrations between individuals located in the two different locations of our study. These places, A and B, whence we drew our samples, were on similar latitudes; when measured using the equator as a reference the difference between them was 14 km. In terms of elevation above sea level, they differed by less than 80 m. The likely differences in UV radiation were therefore small. Data pertaining to environmental factors influencing vitamin D synthesis in individuals were not available from ground observations in these localities and, therefore, satellite observations were used. Similar mean average temperatures near the earth's surface, UV indices, erythemal dose rates, and total ozone columns were observed between the two areas from which we recruited volunteers. Another factor that could have affected 25(OH)D concentrations in rural participants is differences in lifestyle, as observed in Asian (Anwar *et al.*, 2016), other African (Wakayo *et al.*, 2015), and European (Manicourt & Devogelaer, 2008) populations. As no data were available pertaining to sun exposure in our study, no inference about differences in lifestyle was possible. Low 25(OH)D concentrations have also been linked to an increased risk of obesity (Rosen *et al.*, 2012); however, for the population investigated similar anthropometric markers were observed across differing 25(OH)D status. The lack of association between BMI and 25(OH)D concentrations was reported in another South African study, where recruitment was done in a province that neighbors the one from which we selected our volunteers (Lategan *et al.*, 2016). This may indicate that the correlation between anthropometric markers and 25(OH)D status does not apply to black individuals living in South Africa, which necessitates further investigation.

Nutritional intake of vitamin D in our population did not differ between the three 25(OH)D categories and was well below the recommended 15 µg/day (Norval *et al.*, 2016). Dietary vitamin D also failed to influence the linear regression modeling of lnCRP concentrations, which might be due to the extremely low dietary intake observed. A low intake of dietary vitamin D is common in African populations, with only margarine being fortified with vitamin D in South Africa (Norval *et al.*, 2016). Dietary sources of vitamin D did not significantly contribute

to vitamin D status (Holick & Chen, 2008), as ingested vitamin D is more readily excreted (Haddad *et al.*, 1993). This result aligns with the fact that the primary factor contributing to 25(OH)D concentration is exposure to UV light (Harris, 2006), resulting in vitamin D_3 (cholecalciferol or calcidiol) synthesis from 7-dehydrocholesterol (a precursor of cholesterol); after hydroxylation by the liver and kidneys, vitamin D_3 becomes 25(OH)D and then 1,25-dihydroxyvitamin D. That said, a study controlling caloric intake of obese women showed that replenishment of 25(OH)D by supplementation with vitamin D at 2000 IU per day led to participants in that group losing more weight, having smaller waist circumferences, and a 46% higher reduction in CRP concentrations compared to those in a placebo group (Mason *et al.*, 2014). Because a placebo group was included in this other study, the reduction in CRP was attributed to 25(OH)D values stabilizing at sufficient levels (Mason *et al.*, 2014), not simply a reduction of body composition markers. We attributed the elevated CRP concentrations observed in our urban population to increased abdominal adiposity, as urban individuals had increased waist circumferences. In their review, Brooks *et al.* (2010) reported that the association between inflammation, as measured by CRP, and abdominal adiposity is highly correlated, even when correcting for BMI. Women with increased waist circumferences were previously reported to be at greater risk of co-presenting with elevated CRP concentrations (Rexrode *et al.*, 2003), with our results indicating that waist circumference was also the largest effector contributing to CRP concentrations. Reductions in abdominal adiposity in response to dietary interventions were previously reported to reduce CRP concentrations (Su *et al.*, 2015). These dietary interventions included supplementation with fish oil tablets, and although not stated in the original work, fish oil naturally contains bioavailable vitamin D, which could have contributed to the reduction in CRP concentrations (Grossmann & Tangpricha, 2010). Here, we report a predicted 0.15 mg/L reduction in CRP concentration with a 1 nmol/L increase in 25(OH)D concentrations as determined from Spearman correlation. Recommendations intended to achieve sufficient 25(OH)D status should, therefore, aim to include responsible guidelines for exposure to sunlight, as well as an increase in the intake of good dietary sources of vitamin D to improve health in a country such as South Africa, which has a history of health policies failing its citizens.

At the time of our study, South Africa was rife with HIV denialism by government and stigmatization of seropositive individuals, which resulted in a large segment of the population not knowing their HIV status. Overall, 8.1% of our study population was first diagnosed as being HIV-positive during our investigation. Median values, when excluding HIV-positive individuals, were similar, in terms of both 25(OH)D and CRP concentrations, to those of the whole group. Both 25(OH)D (Havers *et al.*, 2014) and CRP (Shivakoti *et al.*, 2016) concentrations can be affected by the use of anti-retroviral (ARV) treatments; however, as

these individuals were first diagnosed during this study, they were not receiving ARVs and were therefore not excluded.

Our study was not without limitations. Because gender is a factor that contributes to both 25(OH)D and CRP concentrations, including men in our sample would have made the study more informative. Future studies should aim to explore the relationship between vitamin D and inflammation in men as well as women. Furthermore, no data were available on sun exposure time, which could have contributed to explaining the variance in 25(OH)D concentrations. However, measuring 25(OH)D concentrations is a strength of our study as it avoids the necessity for UVR, sun exposure time, and even dietary vitamin D intake data. The cohort was also randomly selected, without prior genetic screening. Future studies, including more extensive numbers of minor allele carriers, could establish associations between SNPs where our population had too few genotypes, resulting in lowered statistical power. Because 1,25(OH)2D is epigenetically active (Bahrami *et al.*, 2017), future investigations to explore a possible mechanism to explain the anti-inflammatory effects of vitamin D could also incorporate epigenetics.

Our study cohort was very well-described, with data available on daily vitamin D intake, and was particularly well-characterized in terms of demographic, genetic, and biochemical factors that could address the variance in circulating CRP concentrations. The unique population investigated here was ideal, as individuals presented with both low 25(OH)D and elevated CRP concentrations. Including both CRP concentrations, known to fluctuate quite extensively, and genetic constants with known effects on CRP phenotype further strengthens the data presented here.

4.4.5 Conclusions

Disturbingly, 43% of our female cohort presented with both elevated CRP concentrations and deficient/insufficient 25(OH)D levels; because the combination is linked to CVD, this may compound their disease risk and predispose them to future disease. A negative association was identified between 25(OH)D and circulating CRP concentrations, but no vitamin D–gene interactions were observed between common SNPs on the *CRP* gene and 25(OH)D in this study. Moreover, *CRP* SNPs did not influence vitamin D status. Several genotypes were, however, associated with an altered risk of presenting with the co-phenotype of insufficient/deficient 25(OH)D and elevated CRP, indicating that a genetic predisposition exists. The present research extends past work by demonstrating that the link between 25(OH)D and CRP is not associated with vitamin D–*CRP* gene interactions and that other pathways need to be investigated. This finding is important, as it offers a starting point for unraveling the possible mechanisms for the previously reported inverse relationship between

25(OH)D and CRP, which in turn may ultimately result in therapeutic and policy recommendations to combat CVD.

This paper highlights the necessity of public health efforts in South Africa to assist women to achieve sufficient vitamin D status through responsible exposure to sunlight, increased intake of natural and fortified dietary sources of vitamin D, and for those who require it, vitamin D supplementation. Improved vitamin D status would reduce inflammation and thus possibly curb diseases contingent on both low vitamin D and elevated CRP: the co-phenotype described here.

4.5 Supplementary Materials:

The following are available online at www.mdpi.com/link. Table S1. Environment-SNP associations with CRP concentrations based on different genetic models of inheritance. Table S2. Demographic markers associated with differing 25(OH)D status;

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4.7 Author Contributions:

Pieter H. Myburgh concept, statistical analyses, and writing and critical review of manuscript; Cornelie Nienaber-Rousseau refining concept of study, isolated DNA, and writing and critical review of manuscript; Iolanthé M. Kruger PI of PURE SA–NW, funding and management of anthropometrical, nutritional, and biochemical measures, and critical review of manuscript; G. Wayne Towers refining concept, funding and management of genetic analyses, and critical review and writing of manuscript.

4.8 Conflicts of Interest:

The authors declare no conflict of interest.

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4.10 Supplementary data

Supplementary Table 4.1. Environment-SNP associations with CRP concentrations based on different genetic models of inheritance.

SNP ID	Allele	Genetic model	n	Mean adjusted CRP mg/L	Standard Error	Association with CRP	p-value	AIC
rs2794520	C/C	Co-dominant	307	4.48	1.08	Lowered CRP	0.03	1665
	C/T		162	3.25	1.12			
	T/T		31	2.94	1.25			
	C/C	Dominant	307	4.48	1.08		0.01	1663
	C/T—T/T		193	3.20	1.11			
rs2808630	T/T	Dominant	352	4.02	1.07		0.62	
	C/T—C/C		152	3.82	1.13			
rs3093068	C/C	Co-dominant	195	3.34	1.11	Increased CRP	0.04	1670
	C/G		243	4.31	1.09			
	G/G		64	5.04	1.19			
	C/C	Dominant	195	3.34	1.11		0.02	1668
	C/G—G/G		307	4.45	1.08			
rs1205	C/C	Dominant	313	4.43	1.08	Lowered CRP	0.02	1676
	C/T—T/T		191	3.22	1.11			
	C/C—T/T	Over-dominant	343	4.26	1.08		0.04	1677
	C/T		161	3.38	1.12			
rs1130864	C/C	Dominant	386	4.22	1.07		0.25	
	C/T—T/T		118	3.21	1.13			
rs1800947	C/C	Co-dominant	502	3.95	1.06		0.68	
	C/G		2	8.03	1.62			
rs1417938	A/A	Co-dominant	480	4.01	1.07		0.94	
	A/T		22	2.97	1.40			
rs3093062	G/G	Dominant	351	3.43	1.08	Increased CRP	0	
	A/G—A/A		152	5.64	1.11			
rs3093058	A/A	Dominant	350	3.38	1.08	Increased CRP	0	
	A/T—T/T		152	5.64	1.11			
rs2027471	T/T	Dominant	309	4.49	1.08	Lowered CRP	0.01	
	A/T—A/A		195	3.24	1.11			
rs1341665	G/G	Dominant	309	4.49	1.08	Lowered CRP	0.01	
	A/G—A/A		195	3.24	1.11			
rs7553007	G/G	Dominant	305	4.53	1.08	Lowered CRP	0.01	
	A/G—A/A		199	3.22	1.11			

Abbreviation: A, adenine; AIC, Akaike Information Criterion; CRP, C-reactive protein; C, cytosine; G, guanine; SNP, single nucleotide polymorphism; rs, reference SNP cluster ID; T, thymine

Supplementary Table 4.2

Although rural (Location B) and urban (Location A) participants had similar distributions stratified to 25(OH)D status, rural dwellers had higher median concentrations of 25(OH)D (69.4 nmol.L^{-1} compared to 66.6 nmol.L^{-1}, $p < 0.05$) and lower median CRP concentrations (3.58 mg.L^{-1} vs 4.78 mg.L^{-1}, $p < 0.05$) compared to their urban counterparts. Rural women were, however, significantly younger (median age 53 compared to 56 years), with significantly lower waist circumferences (81 cm compared to 83.3 cm). Correcting for the effects of age on 25(OH)D

concentration, and waist circumference on lnCRP concentration, resulted in the rural-urban differences falling away. All HIV-positive individuals (n = 41) were first diagnosed during this study, and both 25(OH)D and CRP concentrations were similar between HIV-positive and -negative women (whole group medians excluding HIV-positive individuals 25(OH)D: 68.2 nmol.L^{-1}; CRP: 4.27 mg.L^{-1}; $p > 0.05$ observed for both 25(OH)D and CRP concentrations when comparing the whole cohort and the cohort excluding HIV-positive individuals). HIV-positive individuals were, therefore, not excluded from further analyses. Smokers had significantly lower 25(OH)D concentrations. However, smoking was found not to be a significant predictor of 25(OH)D concentrations when modeled via linear regression analysis. Vitamin D intake from nutritional sources was low across all three categories of 25(OH)D status, with none of the participants ingesting the recommended 15 µg.day^{-1}.

Supplementary Table 4.2. Demographic markers associated with differing 25(OH)D status.

Variable	Deficient <50 nmol.L^{-1} (n = 81; 16.0%)	Insufficient 50—75 nmol.L^{-1} (n = 250; 49.5%)	Sufficient >75 nmol.L^{-1} (n = 174; 34.5%)	p-value
Urban / Rural	51 (63.0%) / 30 (37.0%)	132 (52.8%) / 118 (47.2%)	87 (50.0%) / 87 (50.0%)	*NS*
Age	57.0 [50.0;61.0][a]	55.0 [50.0;62.0][a]	52.5 [49.0;58.0][b]	0.001
Smoking status: Former / Current / Abstainer	2 (2.47%) / 53 (65.4%) / 26 (32.1%)	8 (3.21%) / 103 (41.4%) / 138 (55.4%)	3 (1.74%) / 78 (45.3%) / 91 (52.9%)	0.003
HIV–positive/negative	5 (6.25%) / 75 (93.8%)	19 (7.63%) / 230 (92.4%)	17 (9.77%) / 157 (90.2%)	*NS*
Vitamin D intake (µg.day^{-1})	2.12 [1.17;3.49]	1.98 [0.98;3.57]	2.03 [0.99;3.35]	*NS*

Data presented as median [25th and 75th percentile] for continuous data and number of observations (percentage) for categorical data. Abbreviations: 25(OH)D, 25-hydroxyvitamin D; HIV, human immune deficiency virus; NS, not significant ($p > 0.05$).

CHAPTER 5: MANUSCRIPT THREE

Manuscript three will be submitted to the journal, *Cytokine.*

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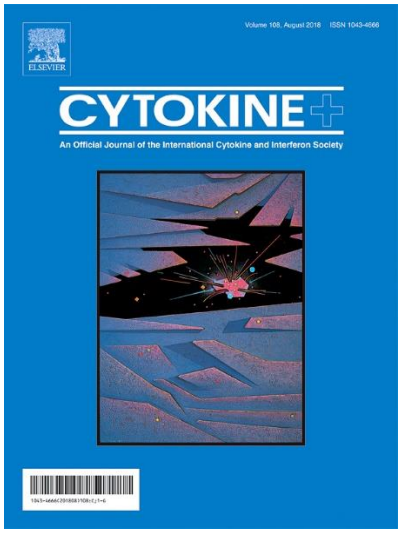

Aims and scope:

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LOW-GRADE INFLAMMATORY MARKERS IN BLACK SOUTH AFRICANS: INTERPLAY BETWEEN ANTHROPOMETRY, IL-6 AND *CRP* POLYMORPHISMS IN RELATION TO CRP

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HM: Conceptualized the paper, conducted the statistical analyses, wrote and critically revised the manuscript, final approval of manuscript.

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IMK: PI for PURE study, data collection, revised the manuscript, final approval of manuscript.

HSK: supervision and quality control of anthropometry data, revised the manuscript, final approval of the manuscript.

GWT: Managed and obtained funding for the isolation of the DNA (second round) as well as the genotyping and quality control of the *CRP* single nucleotide polymorphisms, refined the conceptualization of the paper and revision of the manuscript, final approval of manuscript.

5.1 Abstract

The inflammatory origins of cardiovascular diseases (CVD) are increasingly being investigated, especially with regard to the cytokine, C-reactive protein (CRP). The CRP phenotype is controlled by various factors, among which we investigate the interplay between anthropometry, genetics and the metabolic determinant of CRP, interleukin-6 (IL-6), in a group of black individuals prone to presenting with elevated CRP. Very few reports combine these factors. We hypothesized that this integrative approach would enable us to understand possible reasons why black individuals present with elevated CRP. Our aim was to determine whether IL-6 and CRP were associated with anthropometric markers, especially waist circumference (WC), and whether these differed between several *CRP* genotypes. Two proposed WC cut-off values, from the harmonized joint interim statement (JIS) and those recently recommended by Ekoru *et al.* (2017), for cardiometabolic risk, were also evaluated by stratifying according to the cut-offs as well as CRP genotypes and then determining whether the inflammatory markers, among other CVD risk factors, differed. The main distinction in the cut-off values is lower WC values proposed for men by Ekoru *et al.* (2017) than by the JIS. In this cross-sectional analysis, data collected from 1 327 men and women taking part in the South African arm of the Prospective Urban and Rural Epidemiology (PURE) study, were analyzed. CRP did not mimic IL-6 across the twelve *CRP* genotypes included in this study even though both these low-grade inflammatory markers were positively associated with visceral adiposity. As IL-6 concentrations did not explain CRP, with both of these factors associated with adiposity, other adipocytokines may result in altered CRP. When stratifying using the WC values of Ekoru *et al.* (2017), we failed to differentiate between IL-6 concentrations for men that are deemed at risk compared with low-risk men. We believe that the elevated CRP concentrations observed in black individuals are more likely to be associated with anthropometric and related determinants as based on the results of our regression analysis. It is important to manage WC so that it is within the normal range, and, in doing so, alleviate inflammation and possibly reduce the risk of developing inflammation-induced diseases.

Keywords: abdominal obesity, CRP, C-reactive protein, IL-6, interleukin-6, waist circumference

Highlights:

- Concentrations of both IL-6 and CRP are positively associated with visceral adiposity in black South Africans
- Although both IL-6 concentrations and *CRP* SNPs were associated with CRP concentrations, no significant interactions were observed
- We recommend a reduction in WC, in those with WC above the healthy ranges, to possibly reduce inflammation

5.2 Introduction

The role of inflammation in the development of cardiovascular disease (CVD) is increasingly being investigated (Ruiz-Nunez *et al.*, 2013). Reports indicate that inflammatory causes, rather than atherosclerosis, are more prominent in the etiology of CVD in sub-Saharan Africans (Moran *et al.*, 2013). CVD is becoming a global pandemic, with as much as 80% of the global burden of CVD occurring in low- and middle-income countries and its prevalence increasing in sub-Saharan Africa (Gaziano, 2007; Mathers & Loncar, 2006; Nyirenda, 2016). Various markers of inflammation are responsible for regulating the inflammatory response; however, one of the most widely researched markers is the cytokine, C-reactive protein or CRP (Ridker, 2014; Ridker, 2016).

A meta-analysis has concluded that individuals with a baseline CRP concentration of more than 3 mg/L are at a greater risk of developing events related to CVD (Strang & Schunkert, 2014). Most black Africans (Nazmi & Victora, 2007), including South Africans (Kruger *et al.*, 2013), tend to have elevated circulating CRP concentrations, previously determined as most probably attributable to adiposity, genetics and gene–environment interactions (Myburgh *et al.*, 2018; Nienaber-Rousseau *et al.*, 2014).

Apart from the factors described above, CRP's transcription is activated and regulated by other cytokines i.e. interleukin-6 (IL-6) together with IL-1$_\beta$ (Hage & Szalai, 2007; Hage & Szalai, 2009). IL-6 upregulates the expression of *CRP* by increasing the binding activity of human antigen R to *CRP* mRNA, which increases *CRP* mRNA stability and promotes CRP translation (Kim *et al.*, 2015). A major source of both IL-6 and IL-1$_\beta$ is adipose tissue, especially in viscerally obese individuals (Crichton *et al.*, 1996; Fontana *et al.*, 2007; Ganter *et al.*, 1989; Memoli *et al.*, 2007; Pini *et al.*, 2012). Evidence from previous studies indicates that individuals are at an increased risk of developing cardiometabolic conditions when central obesity is present (Abbasi *et al.*, 2013; Czernichow *et al.*, 2011; Farzad *et al.*, 2012). WC, as a proxy for central obesity, is a major determinant of increased CRP concentrations (Nakamura *et al.*, 2008). The large burden of obesity in South Africa, especially among women (National Department of Health, 2017; Shisana *et al.*, 2013), may contribute to elevated cytokine concentrations in the black population. It is critical, therefore, to investigate whether central obesity has an aggravating effect on the association between the genetic variation in the *CRP* gene and variable CRP concentrations or whether the increased concentrations of CRP in black South Africans are due to the high prevalence of obesity in this population. Recently, Ekoru *et al.* (2017) proposed new WC cut-off values of <81.2 cm for men and <81.0 cm for women to predict cardiometabolic risk in sub-Saharan Africans. For

men, this is substantially lower than the recommended cut-off point of <94 cm proposed in the harmonized joint interim statement (JIS; Alberti et al., 2009) , whereas for women it is similar.

Our aim was to determine whether IL-6 and CRP were associated with anthropometric markers, and whether these differed between certain *CRP* genotypes. Using both the recommendations of the JIS (Alberti *et al.*, 2009) and Ekoru *et al.* (2017) for WC cut-off values predictive of cardiometabolic risk, we investigated the interplay between increased central adiposity, inflammatory markers and genetic variation in the *CRP* gene. If the lifetime exposure to elevated CRP is important in terms of CVD development, then the identification of genetic factors that are associated not only with steady-state CRP, but also with fluctuations in CRP due to changes in IL-6 concentrations or anthropometric measures, might help to refine assessments of susceptibility to CVD. Data obtained from this study will not only be an important addition to our understanding of the etiology of elevated CRP in black individuals, but also extend the knowledge base of *CRP* genetics in African populations.

5.3 Materials and Methods

5.3.1 Ethical considerations

This is a cross-sectional investigation affiliated to the 12-year Prospective Urban and Rural Epidemiology South Africa (PURE-SA) study, based on samples and data collected in 2005 in the North West province of South Africa. Ethical considerations were reviewed and approval (ethics numbers 04M10 for 2005 and NWU-00004-17-S1 for this study) was acquired from the Health Research Ethics Committee of the North-West University, in accordance with the ethical principles of the Declaration of Helsinki as revised in 2004. Written informed consent was obtained from all participating individuals after the study methodology and purpose were explained in a language of their choosing and sufficient time was provided for reflection to decide whether to participate in the research. All individuals were given the option to withdraw from the study or refuse any of the tests described below.

5.3.2 Study population

Four communities were selected based on their degree of urbanization and grouped into whether they were from an urban or rural environment. Goodwill permission to conduct the study was sought from tribal chiefs, community leaders, household heads, and mayors of the included communities. The initial sampling strategy is explained elsewhere (Vorster *et al.*, 2014). In total, 6000 individuals were screened, of which 2010 were included in the data collection phase of the study. Samples were collected during a 12-week period. Participants were ostensibly healthy adults older than 30 years. Exclusions on the day of enrollment were elevated tympanic temperatures (above 38˚C), any known lifestyle disease, pregnant or

lactating women, or a known infection with the human immune virus (HIV) or tuberculosis-causing agents.

5.3.3 Blood collection and biochemical analysis

Participants were asked to be in a fasting state for at least 10 hours prior to study enrollment. Professional nurses performed venipuncture on consenting candidates in a relaxed environment. These samples were centrifuged within 20 minutes for 15 minutes at 2 000 x gravitational force and a constant temperature of 10˚C. Serum, plasma and buffy coat were aliquoted and frozen on dry-ice pellets prior to storage at –70˚C. IL-6 was measured in sera samples using a Cobas Elecsys 2010 (Cobas, Basel, Switzerland) apparatus, with a limit of detection of 1.5 pg/mL. A Sequential Multiple Analyzer Computer (SMAC), using a particle-enhanced immunoturbidometric assay (Konelab TM auto analyzer, Thermo Fisher Scientific, Vantaa, Finland) was used to quantify high-sensitivity CRP concentrations. The Friedewald-equation was used to calculate low-density lipoprotein cholesterol (LDL-c) in those with triglyceride concentrations below 400 mg/dL (Friedewald *et al.*, 1972), following lipograms performed on a Konelab 20i auto analyzer (Thermo Fisher Scientific, Vantaa, Finland). Glycated hemoglobin was measured on a D-10 Hemoglobin System (Bio-Rad, Hercules, CA).

HIV determination was performed by research nurses trained in Voluntary Counseling and Testing of HIV, adhering to the UNAIDS/WHO policy statement on HIV testing as well as the protocols set forth by the National Department of Health of South Africa. All participants received pre-test group counseling and were then given the opportunity to consent to the test. Individual post-test counseling was provided for all volunteers. HIV determination was conducted using a rapid First Response HIV 1-2.O card test (Transnational Technologies Inc., PMC Medical, Nani Daman, India). Individuals testing positive were retested using a second card test developed by Pareeshak (BHAT Bio-Tech, Bangalore, India) to confirm HIV status.

Blood pressure was measured with an Omron automatic digital blood pressure monitor (Omron HEM-757) after participants had been seated in a calm environment for five minutes.

5.3.4 Anthropometric measurements

Lightly clothed participants, with arms hanging freely at the side, were weighed on tared and calibrated scales in duplicate. Duplicate lateral heights to the nearest 1 mm, with the participant's head in the Frankfort plane, inhaling and fully extended, were measured using stadiometers. Body mass index was computed as the mean weight per square mean body height (kg/m^2). WC and hip circumference were measured using an unstretchable metal tape (Lufkin, Cooper Tools, Apex, NC, USA) in accordance with the recommendations of the International Society for the Advancement of Kinanthropometry. WC was stratified according

to the recommendations of the JIS: normal WC <80 cm for women and <94 cm for men (Alberti *et al.*, 2009), and compared with the newly established WC for sub-Saharan individuals: 81 cm for women and 81.2 cm for men (Ekoru *et al.*, 2017).

5.3.5 Factors pertaining to lifestyle

Several interviewer-administered questionnaires were completed in our study, with volunteers responding to questions in their home language. Questionnaires included questions regarding substance abuse (tobacco and alcohol) and medical history.

5.3.6 Genetic analyses

An in-depth discussion by Swanepoel (2013) and Nienaber-Rousseau *et al.* (2014) describe how the twelve SNPs were chosen. The twelve SNPs were investigated, using polymerase chain reaction (PCR) amplification with primers designed specifically for the *CRP* gene. Amplicons of the regions of interest were subjected to BeadXpress analysis, performed by the South African National Health Laboratory Service (NHLS), located at the University of Witwatersrand (Johannesburg, South Africa).

5.3.7 Statistical analysis

Statistical analyses were performed using R (R Core Team, 2017). Outliers were defined as upper values exceeding four times the standard deviation (SD) and applied to data obtained for IL-6 and hs-CRP. Values were investigated for normality by means of visual inspection (QQ-plots and histograms). In total, 385 (29.0%) individuals had IL-6 concentrations below the 1.5 pg/mL limit of detection (LOD). To reduce the effects of left censoring observed, LOD values were included as divided by $\sqrt{2}$, i.e. 1.08 pg/mL, as simulation studies showed sufficient evidence that this is a valid substitution (Tekindal *et al.*, 2017). Both IL-6 and CRP concentrations were skewed right and were subsequently natural log transformed (hereafter referred to as lnIL-6 and lnCRP), which improved normality. Our results of analyses using natural log-transformed data were back-transformed for ease of interpretation.

Significance of differences between grouped continuous variables was tested using the Wilcoxon rank-sum test and, where more than two groups were tested, p-values were adjusted using the methods of Holm. Correlation between variables was tested using the Spearman rank correlation coefficient. Genotype-IL-6 interactions were assessed using two-way ANOVA/ANCOVA (factorial), which allowed for the assessment of any interaction effect over and above the main effects of the independent predictors in the model. Where applicable, Bonferroni correction was used for p-values. Hardy-Weinberg equilibrium and SNP associations were performed using the SNPassoc library (Gonzalez *et al.*, 2007). Linkage was determined using the LDHeatmap library (Shin *et al.*, 2006). A linear model predicting

lnCRP and lnIL-6 concentrations was also constructed using backward stepwise methods. The lowest Akaike information criterion (AIC) was used as measure of comparison between models, including genetic models. Only interactions that remained after adjusting for multiple testing and after excluding possible statistical outliers were reported as being significant. Significance was set at $\alpha = 0.05$.

5.4 Results

Previously identified modulating factors pertaining to the demographic composition of the 1 327 volunteers, of which the majority (63.1%) were women, included in this study are presented in Table 5.1. Values were reported as median and interquartile range in subsequent summaries.

CRP concentrations were higher in women than men; however, after adjusting for WC (median WC: 80.3 *vs.* 74.5 cm for women and men, respectively) the difference was no longer significant. IL-6 concentrations were higher in urban than rural dwellers, with similar BMI and WC observed between these groups ($p > 0.05$, data not shown). Urbanites were significantly older than rural volunteers ($p < 0.001$) and, upon adjusting for age, the difference in IL-6 concentrations between the localities was no longer significant. In total, 206 (15.5%) individuals were diagnosed as being HIV-positive during this study, with no history of active antiretroviral treatment. Both median IL-6 and CRP concentrations were similar, however, regardless of HIV-status ($p > 0.05$). Sero-positive individuals had smaller median WC values (73.5 cm) than HIV-negative individuals (78.4 cm). Both IL-6 and CRP concentrations were similar among tobacco use categories.

Table 5.1: Modulating factors for IL-6 and CRP

Modulating factor	Number of individuals (%)	IL-6 (pg/mL)	CRP (mg/L)
Sex			
Men	490 (36.9)	2.67 [1.06 – 5.39]	2.13 [0.48 – 6.81]
Women	837 (63.1)	2.71 [1.06 – 4.95]	3.43 [1.10 – 9.09]
p-value	*NA*	*NS*	<0.0001*
Location			
Rural	694 (52.3)	2.39 [1.06 – 5.20]	3.09 [0.82 – 8.06]
Urban	633 (47.7)	2.97 [1.52 – 4.97]	2.79 [0.96 – 8.68]
p-value	*NA*	0.01	*NS*
HIV status			
Positive	211 (15.9)	2.69 [1.06 – 5.27]	2.64 [0.86 – 7.72]
Negative	1109 (83.6)	2.69 [1.06 – 5.00]	3.05 [0.89 – 8.34]
Never tested	7 (0.50)	4.15 [2.40 – 5.72]	3.48 [0.99 – 9.53]
p-value	*NA*	*NS*	*NS*
Tobacco use			
Formerly	42 (3.20)	3.40 [2.09 – 5.90]	2.48 [1.02 – 9.65]
Currently	689 (51.9)	2.78 [1.06 – 5.33]	2.76 [0.83 – 7.67]
Never	588 (44.3)	2.57 [1.06 – 4.63]	3.28 [0.91 – 8.61]
p-value	*NA*	*NS*	*NS*
Missing		*8 (0.60)*	

Data presented as numbers (%) or medians [25th – 75th percentiles]. Significance tested using Wilcoxon rank-sum test and the *post-hoc* test of Holm, where applicable. Abbreviations: CRP, C-reactive protein; HIV, human immunodeficiency virus; IL-6, interleukin-6; *NA,* not applicable; *NS,* not significant. *After adjusting for WC, CRP did not differ between the sexes.

As both BMI and WC were previously indicated as risk factors for increased IL-6 and CRP concentrations (Todendi *et al.*, 2016), these markers of inflammation were compared between different BMI (Figure 5.1) and WC categories (Figure 5.2). IL-6 concentrations were higher for underweight (3.02 pg/mL) and obese (3.62 pg/mL) individuals than for those who were of normal weight (2.24 pg/mL) or overweight (2.38 pg/mL; Figure 5.1). Similar CRP concentrations were observed in underweight and normal weight participants, with higher concentrations observed in overweight participants and even higher concentrations in the obese.

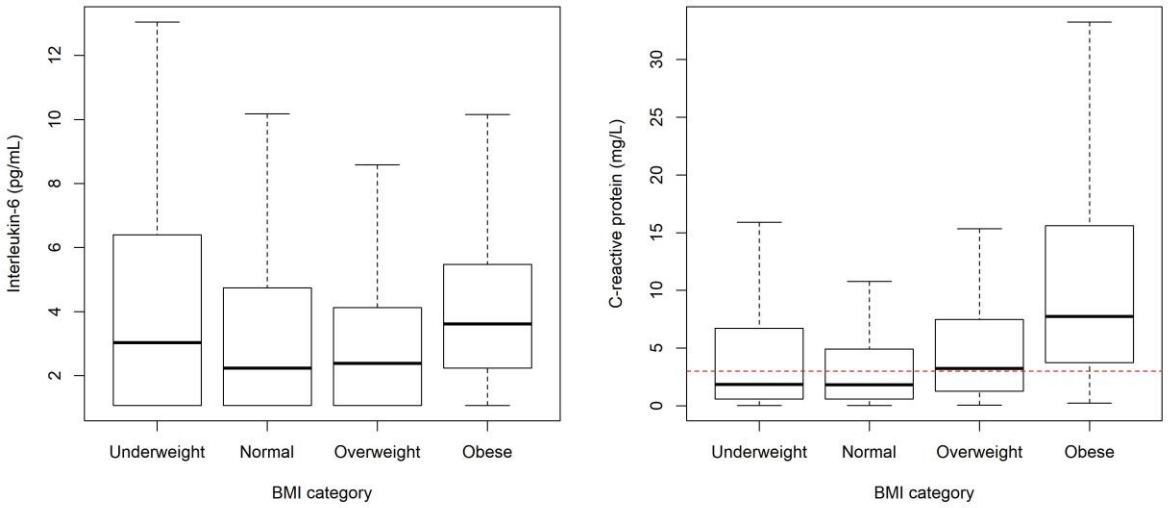

Figure 5.1: IL-6 and CRP concentrations stratified to WHO BMI categories
IL-6 was significantly elevated in underweight and obese individuals compared with normal and overweight individuals. CRP differed significantly between BMI categories; however, similar concentrations were observed for underweight and normal individuals. Dotted line indicative of 3 mg/L cut-off value for elevated CRP. Abbreviations: BMI, body mass index.

In Supplementary Table 5.1, we stratified the population based on the JIS and newly proposed WC cut-off points for increased CVD risk in sub-Saharan individuals (Ekoru *et al.*, 2017) to compare IL-6 and CRP concentrations between certain anthropometric subsets. Individuals identified with an at-risk WC according to the JIS (Alberti, 2009) presented with higher IL-6 (2.34 *vs.* 3.11 pg/mL; p <0.0001) and CRP concentrations (1.88 *vs.* 5.91 mg/L; p <0.0001; Figure 5.2) than low-risk individuals (Figure 5.2). Similarly, higher IL-6 (2.35 *vs.* 2.99 pg/mL; p <0.0001) and CRP (1.82 *vs.* 5.47 mg/L; p <0.0001) concentrations were also observed based on the recommended optimal *vs.* at-risk thresholds for WC as determined by Ekoru *et al.* (2017). Although IL-6 and CRP concentrations for individuals exceeding the JIS WC recommendations were higher than for those exceeding the newly established recommendations for black individuals, concentrations of these cytokines were similar when comparing normal and elevated WC groups according to these two classification systems. All other subsets had statistically similar IL-6 and CRP concentrations when comparing the groups generated by applying the two different sets of WC recommendations.

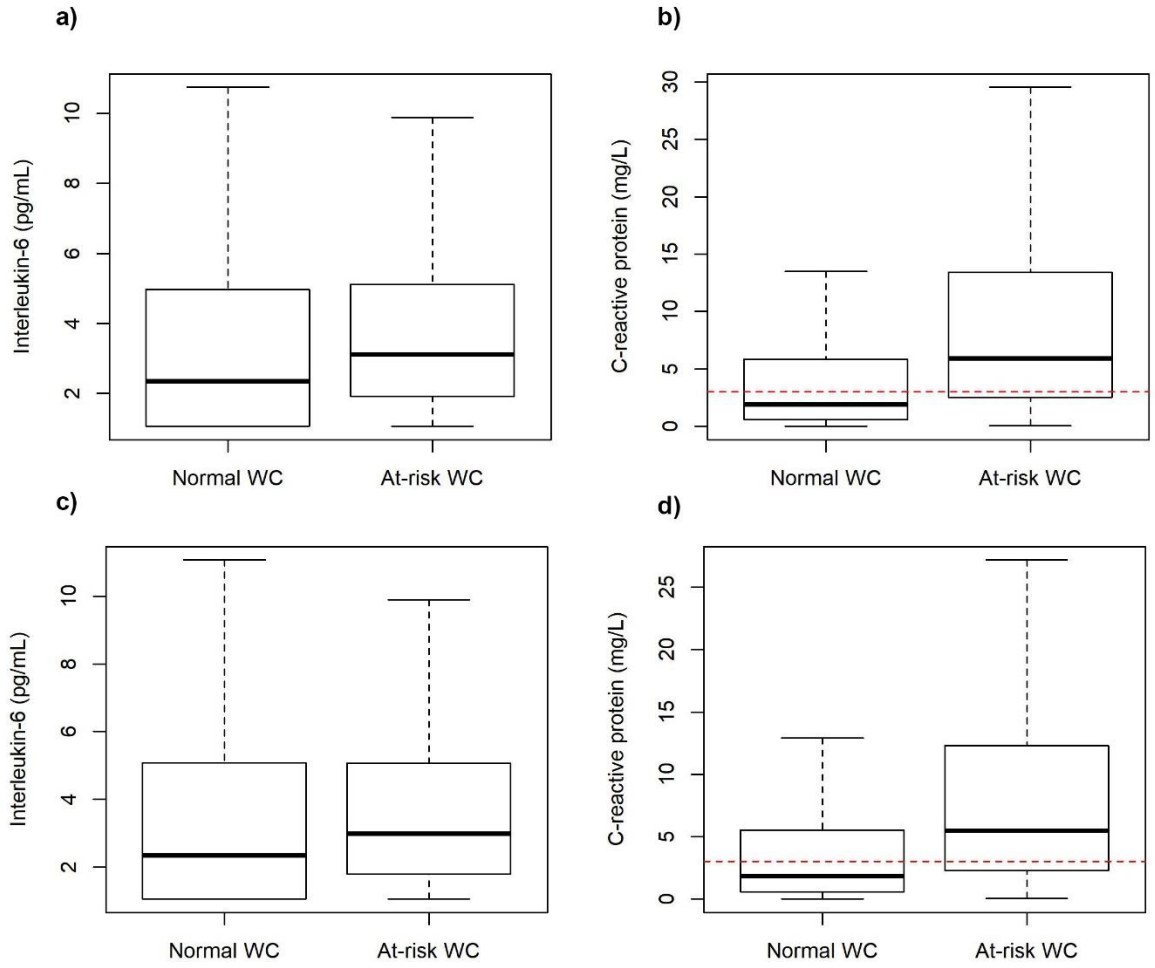

Figure 5.2: Baseline differences in IL-6 and CRP concentrations between the WC categories

a) IL-6 and b) CRP concentrations compared across JIS WC recommendations; c) IL-6 and d) CRP concentrations for newly established WC cut-off values for black Africans (Ekoru *et al.*, 2017). Both IL-6 and CRP concentrations were elevated in the increased WC groups. No significant difference in either IL-6 or CRP concentrations was observed between at-risk and normal WC groups based on either the JIS criteria or Ekoru *et al.* (2017) WC cut-off values. Red, dotted line indicates the cut-off value for normal CRP concentrations (3 mg/L). Abbreviations: WC, waist circumference.

Cardiovascular and anthropometric markers are presented in Table 5.2, with associations between these markers with both IL-6 and CRP concentrations indicated. All measured CVD risk factors differed significantly between normal and at-risk WC groups, except for heart rate between the WC groups as defined by the recommendations of Ekoru *et al.* (2017). In comparing the subsets resulting from the two WC thresholds (Alberti *et al.*, 2009; Ekoru *et al.*, 2017) in terms of CVD risk, we determined that only anthropometric markers differed between similar WC risk groups. IL-6 and CRP concentrations were moderately correlated ($\rho = 0.45$; $p < 0.001$) with each other, whereas various weak yet significant correlations were observed between anthropometric markers and CRP concentrations.

Table 5.2: Markers of cardiovascular health and association with IL-6 and CRP

Variable	All	JIS WC Recommendations			Ekoru *et al.* (2017)			Correlation (Spearman's rho)	
		Normal WC	At risk WC	p-value◊	Normal WC	At risk WC	p-value◊	IL-6	CRP
Age (years)	48.0 [41.0 – 55.0]	47.0 [41.0 – 55.0]	49.0 [42.0 – 57.0]	0.003	46.5 [40.0 – 54.0]	49.0 [43.0 – 57.0]	<0.001	0.18*	0.13*
Systolic blood pressure (mmHg)	129 [115 – 146]	128 [114 – 144]	133 [118 – 150]	0.001	126 [113 – 142]	134 [119 – 152]	<0.001	0.15*	0.10*
Diastolic blood pressure (mmHg)	86.0 [77.0 – 95.0]	84.0 [76.0 – 94.0]	89.0 [82.0 – 98.0]	<0.001	84.0 [75.0 – 93.0]	89.0 [81.0 – 99.0]	<0.001	0.13*	0.12*
Heart rate (BPM)	71.0 [62.0 – 83.0]	70.0 [61.0 – 83.0]	73.0 [66.0 – 84.0]	0.001	71.0 [62.0 – 83.0]	72.0 [63.0 – 83.0]	0.313	0.19*	0.14*
Waist circumference (cm)	77.2 [70.2 – 87.3]	72.7× [67.6 – 77.0]	91.0 [84.6 – 99.3]	<0.001	71.9× [67.2 – 75.8]	90.1 [84.7 – 97.8]	<0.001	0.13*	0.33*
Hip circumference (cm)	92.8 [85.0 – 105.6]	87.4 [82.5 – 93.7]	110× [102 – 119]	<0.001	86.8 [82.0 – 92.7]	107× [98.9 – 117]	<0.001	0.03	0.27*
Weight (kg)	59.7 [51.5 – 72.7]	54.0× [48.1 – 60.7]	77.3 [67.2 – 88.6]	<0.001	53.0× [47.8 – 58.7]	76.0 [67.2 – 86.5]	<0.001	0.04	0.28*
Body mass index (kg/m^2)	22.8 [19.4 – 28.7]	20.3 [18.4 – 22.9]	31.4× [27.8 – 34.9]	<0.001	20.0 [18.2 – 22.5]	30.2× [26.3 – 34.1]	<0.001	0.05	0.30*
Total cholesterol (mmol/L)	4.86 [4.05 – 5.92]	4.75 [3.95 – 5.73]	5.18 [4.27 – 6.18]	<0.001	4.74 [3.94 – 5.67]	5.14 [4.26 – 6.26]	<0.001	–0.02	0.09*
HDL-c (mmol/L)	1.44 [1.09 – 1.9]	1.54 [1.16 – 2.05]	1.28 [1.00 – 1.60]	<0.001	1.57 [1.20 – 2.08]	1.25 [0.98 – 1.58]	<0.001	–0.03	–0.14*
LDL-c (mmol/L)	3.13 [2.38 – 4.01]	2.95 [2.24 – 3.72]	3.59 [2.76 – 4.42]	<0.001	2.93 [2.21 – 3.66]	3.57 [2.74 – 4.40]	<0.001	–0.04	0.15*
Triglycerides (mmol/L)	1.06 [0.81 – 1.53]	0.97 [0.75 – 1.36]	1.29 [0.91 – 1.92]	<0.001	0.95 [0.73 – 1.29]	1.32 [0.92 – 1.92]	<0.001	0.05	0.17*
Glycated hemoglobin (HbA_{1c}, %)	5.50 [5.20–5.80]	5.40 [5.20 – 5.70]	5.70 [5.40 – 6.10]	<0.001	5.40 [5.20 – 5.70]	5.70 [5.40 – 6.00]	<0.001	0.09*	0.23*

Concentrations reported as median [25th – 75th percentile]. ◊p-value between WC categories. × significant ($p < 0.05$) difference between JIS and Ekoru thresholds. *significant ($p < 0.05$) Spearman Rho values. Abbreviations: HDL-c, high-density lipoprotein cholesterol; CRP, C-reactive protein; IL-6, interleukin-6; LDL-c, low-density lipoprotein; mmHg, millimeters mercury.

Twelve SNPs located within the *CRP* gene were investigated in this study. A five-SNP group consisting of rs2794520, rs1205, rs1341665, rs2027471 and rs7553007 was determined as being in linkage disequilibrium (LD) (Supplemental Figure 5.1). The genotypic proportions for the remaining SNPs were in Hardy–Weinberg equilibrium (data not shown). Subsequently, rs1205 was used as a tag-SNP for rs2794520, rs1341665, rs2027471 and rs7553007, while rs3093062 tagged rs3093058.

Next, we investigated whether harboring a specific genotype may have resulted in pre-existing differences in cardiovascular health markers or anthropometric measurements, which could be associated with either IL-6 or CRP concentrations. For each genotype (under the dominant model), the variables listed in Table 5.2 were compared by means of the Wilcoxon rank-sum test to identify potential significant differences. Heart rate (median: 72 *vs.* 67 bpm), BMI (median: 22.9 *vs.* 21.2 kg/m^2) and WC (median: 77.7 *vs.* 75.0 cm) were slightly higher ($p < 0.05$) in homozygotes for the T-allele of rs1417938 compared with heterozygotes, with no homozygotes for the A-allele included in our study. HDL-c was higher (median 1.52 *vs.* 1.43 mmol/L, $p < 0.05$) in homozygotes for the minor (G) allele in rs3093068. Otherwise, all other variables were similar between the genotypes.

Owing to left censoring observed in IL-6 concentrations, Tobit models were used to identify possible predictor variables for lnIL-6 concentrations in a backward stepwise manner, including the variables included in Tables 5.1 and 5.2. lnIL-6 concentrations were significantly predicted by WC, age, LDL-c and heart rate. Each of the *CRP* SNPs was then also added to these models, with none reaching significance. A linear model to account for lnCRP concentrations was also constructed using backward stepwise methods, including the same aforementioned variables. Based on significance and AIC values, a model explaining 28.9% of CRP variance was obtained including covariates age, lnIL-6, HbA_{1c}, LDL-c and WC. For each 1 cm increase in WC, CRP concentrations were 2.63% higher. A 1% increase in IL-6 concentration predicted a 0.73% rise in CRP concentrations (data not shown).

Median lnIL-6 and lnCRP, and Pearson correlation between stratified values, were calculated for each genotype (Table 5.3). Moderate associations (r: 0.40 – 0.55) were observed for all genotypes ($p < 0.05$), apart from the genotype homozygous for the minor allele of rs1800947, where correlation was not significant. Unadjusted median lnIL-6 values (exponentiated to ease interpretation) did not differ between *CRP* SNP genotypes. Median CRP concentrations did, however, differ between individuals harboring certain genotypes. Homozygotes for the major allele of rs2794520, rs2808630, rs1205, rs2027471, rs1341665 and rs7553007 presented with significantly higher median CRP concentrations compared with heterozygotes and homozygotes of the minor allele, with similar median IL-6 concentrations observed. Three SNPs (rs3093068, rs3093062 and rs3093058) had increased CRP concentrations with each addition of the minor allele ($p < 0.05$).

To quantify the difference that each genotype triggers in terms of lnCRP concentrations, the association analysis provided by the SNPassoc library in R (Gonzalez *et al.*, 2007) was used (Table 5.3). Models were adjusted for lnIL-6, age, WC, HbA_{1c} and LDL-c (identified from the previously conducted, backward stepwise regression). Genetic model selection was based upon lowest scoring AIC value, as well as whether the minor genotype had more than 30 individuals (Table 5.3). A ~31% lower adjusted geometric mean CRP concentrations were observed for the heterozygotes of the five-SNP group (rs7553007, rs1341665, rs2027471, rs1205, and rs2794520). Homozygotes for the minor allele of the five-SNP group presented with ~51% lower CRP concentrations. The CRP concentrations were 11.5% lower in those harboring the minor allele at the rs2808630 locus, under a dominant model of genetic inheritance ($p < 0.0001$). Three SNPs (rs3093068, rs3093058 and rs3093062), however, did present with higher CRP concentrations with the addition of the minor allele, with the homozygotes for the minor allele of rs3093068 having double the concentrations of CRP compared with the homozygotes for the major allele.

Table 5.3: Sera concentrations of IL-6 and CRP in relation to the different *CRP* genotypes as well as the correlation between IL-6 and CRP concentrations stratified to *CRP* genotypes

CRP SNP (Alleles)	MAF (%)	Genetic model	Genotype	Pearson's r	IL-6 (pg/mL)	CRP (mg/L)	% change in geometric mean CRP‡	Significance of change in geometric mean‡
rs2808630 (T/C)	14.2	Dominant	T/T	0.44	2.69 [1.06 – 5.05]	3.19 [1.00 – 8.58][a]		<0.0001
			C/T–C/C	0.43	2.75 [1.06 – 5.01]	2.44 [0.60 – 7.46][b]	–11.5	
rs3093068 (C/G)	37.6	Codominant	C/C	0.55	2.80 [1.06 – 5.37]	2.23 [0.59 – 6.23][a]		<0.0001
			C/G	0.42	2.69 [1.06 – 4.81]	3.46 [1.12 – 8.67][b]	53.9	
			G/G	0.45	2.46 [1.06 – 5.10]	4.31 [1.45 – 12.5][b]	102.8	
rs1205 (C/T)	22.9	Codominant	C/C	0.44	2.72 [1.06 – 5.21]	3.49 [1.16 – 9.39][a]		<0.0001
			C/T	0.45	2.66 [1.06 – 4.90]	2.25 [0.63 – 6.42][b]	–31.0	
			T/T	0.49	2.94 [1.06 – 4.85]	1.60 [0.73 – 4.01][b]	–50.7	
rs1130864 (C/T)	13.2	Dominant	C/C	0.44	2.77 [1.06 – 5.16]	2.89 [0.89 – 8.08][a]		*0.2753*
			C/T–T/T	0.45	2.61 [1.06 – 4.95]	3.13 [1.00 – 8.67][a]	8.99	
rs1800947 (C/G)	0.3	Codominant	C/C	0.44	2.72 [1.06 – 5.10]	2.94 [0.90 – 8.33][a]		*NS*
			C/G	*0.75	2.01 [1.35 – 3.22]	3.56 [0.37 – 5.58][a]	–54.9	
rs1417938 (T/A)	2.5	Codominant	T/T	0.44	2.69 [1.06 – 5.05]	2.92 [0.89 – 8.41][a]		*NS*
			T/A	0.50	3.25 [1.06 – 5.53]	3.49 [0.84 – 6.82][a]	–1.18	
rs3093062 (G/A)	16.4	Dominant	G/G	0.45	2.77 [1.06 – 5.26]	2.53 [0.69 – 7.32][a]		NS
			A/G–A/A	0.43	2.66 [1.06 – 4.62]	4.10 [1.42 – 12.2][b]	68.4	

Concentrations represented as median [25th – 75th percentiles]. ǂ Models adjusted for lnIL-6, age, HbA1c, WC and LDL-cholesterol. a, b Indicates where significant differences in CRP concentration among genotypes within each SNP were observed; similar lettering indicates similar concentrations. Genetic models were chosen based on the lowest AIC values. Median values were calculated on log transformed variables and exponentiated for interpretation. rs1205 represents a 5-SNP group, including rs2794520, rs1341665, rs2027471 and rs7553007; rs3093062 also represents rs3093058. *All Pearson correlations were significant, except for the minor allele of rs1800947. Abbreviations: A, adenine; C, cytosine; G, guanine; CRP, high-sensitivity C-reactive protein; IL-6, Interleukin-6; MAF, minor allele frequency; NS, not significant (p >0.05); rs, reference SNP cluster ID; T, thymine.

Next, we compared the sera concentrations of both IL-6 and CRP across the different *CRP* genotypes stratified according to WC risk (Table 5.4). Because of the stratification process, the SNPs were evaluated under dominant models of genetic inheritance to maintain sufficient statistical power. The addition of a minor allele in rs1130864 was associated with higher IL-6 concentrations in individuals with elevated WC, whereas lower ($p < 0.05$) IL-6 concentrations were observed in individuals with a minor allele and a normal WC. The major alleles of rs2808630, rs1205, rs1130864, rs1800947, rs1417938 and rs3093062 presented with significantly higher IL-6 concentrations in individuals that fell into the at-risk group according to their WC. CRP concentrations were significantly higher in all individuals with elevated WC.

Table 5.4: Median IL-6 and CRP concentrations stratified to JIS WC categories and *CRP* SNPs

SNP	Genotype	Normal WC		Elevated WC			Normal WC		Elevated WC		
		n	IL-6 [IQR]	n	IL-6 [IQR]	p-value	n	CRP [IQR]	n	CRP [IQR]	p-value
rs2808630	T/T	656	2.34 [1.06 – 4.85]	320	3.11 [1.94 – 5.28]	<0.0001	655	2.19 [0.70 – 6.08]	320	5.97 [2.62 – 13.43]	<0.0001
	C/T–C/C	223	2.36 [1.06 – 5.34]	128	3.10 [1.79 – 4.86]	*NS*	223	1.16 [0.50 – 4.22]	128	4.85 [2.43 – 12.54]	<0.0001
	p-value	---	*NS*	---	*NS*	---	---	<0.001	---	*NS*	---
rs3093068	C/C	336	2.56 [1.06 – 5.57]	173	3.06 [1.94 – 5.13]	*NS*	336	1.44 [0.50 – 4.36]	173	3.74 [1.84 – 9.20]	<0.0001
	C/G–G/G	542	2.21 [1.06 – 5.13]	274	3.14 [1.86 – 5.07]	<0.0001	541	2.31 [0.72 – 6.65]	274	7.10 [3.32 – 14.74]	<0.0001
	p-value	---	*NS*	---	*NS*	---	---	<.0001	---	<0.0001	---
rs1205	C/C	512	2.21 [1.06 – 4.91]	271	3.49 [2.00 – 5.29]	<0.0001	511	2.36 [0.71 – 7.04]	271	7.16 [3.21 – 14.9]	<0.0001
	C/T–T/T	367	2.49 [1.06 – 5.00]	177	2.99 [1.81 – 4.90]	*NS*	367	1.46 [0.50 – 4.40]	177	3.87 [1.92 – 9.34]	<0.0001
	p-value		*NS*		*NS*			<0.0001		<0.0001	
rs1130864	C/C	653	2.45 [1.06 – 5.22]	344	3.06 [1.86 – 4.93]	0.007	653	1.82 [0.59 – 5.80]	344	5.88 [2.32 – 13.6]	<0.0001
	C/T–T/T	226	2.07 [1.06 – 4.48]	104	3.49 [2.42 – 6.23]	<0.0001	225	1.99 [0.61 – 5.89]	104	6.07 [2.99 – 12.2]	<0.0001
	p-value		0.047		0.035			*NS*		*NS*	
rs1800947	C/C	875	2.34 [1.06 – 5.00]	445	3.11 [1.91 – 5.10]	<0.0001	874	1.89 [0.60 – 5.83]	445	5.89 [2.53 – 13.4]	<0.0001
	C/G	4	1.54 [1.06 – 2.48]	3	2.67 [2.19 – 4.87]	0.37	4	0.37 [0.33 – 1.17]	3	6.31 [5.64 – 9.64]	0.05
	p-value		*NS*		*NS*			*NS*		*NS*	
rs1417938	T/T	822	2.25 [1.06 – 4.85]	435	3.11 [1.88 – 5.09]	<0.0001	822	1.82 [0.59 – 5.79]	435	5.97 [2.52 – 13.4]	<0.0001
	T/A	52	3.25 [1.06 – 5.12]	13	3.44 [2.48 – 6.76]	*NS*	51	2.69 [0.69 – 6.00]	13	4.61 [3.17 – 9.33]	0.048
	p-value		*NS*		*NS*			*NS*		*NS*	
rs3093062	G/G	611	2.35 [1.06 – 5.17]	304	3.23 [2.05 – 5.31]	<0.0001	611	1.56 [0.53 – 5.10]	304	4.60 [2.20 – 9.90]	<0.0001
	A/G–A/A	266	2.24 [1.06 – 4.63]	143	2.97 [1.73 – 4.62]	0.068	265	2.71 [0.98 – 7.18]	143	8.54 [3.62 – 17.1]	<0.0001
	p-value		*NS*		*NS*			<0.0001		<0.0001	

Abbreviations: A, adenine; C, cytosine; G, guanine; CRP, C-reactive protein; IL-6, Interleukin-6; MAF, minor allele frequency; NS, not significant (p >0.05); rs, reference SNP cluster ID; SNP, single nucleotide polymorphism; T, thymine; WC, waist circumference.

At rs2808630 and rs1205 (representing 5-SNP group), similar IL-6 concentrations were observed whereas the CRP concentrations were significantly lower. At rs3093068 and rs3093062 (tagging rs3093058), IL-6 concentrations were similar with the addition of the minor allele, whereas CRP increased. At rs1130864, IL-6 differed across genotypes whereas CRP did not differ.

5.5 Discussion

This is the first time a study of this magnitude, integrating anthropometry, IL-6, genetics and other related factors, has been conducted to shed light on CRP concentrations in black South Africans, known to present with higher CRP than other ethnicities. Here we determined the association of WC, as a proxy for abdominal obesity, with specific *CRP* genetic markers on IL-6 and CRP concentrations in a cohort of 1,327 black South Africans. Among our study

population women presented with elevated CRP as a result of larger WC than men. Additionally, we compared groups defined by the JIS (Alberti *et al.*, 2009) and those for sub-Saharan Africans (Ekoru *et al.*, 2017) according to WC and also stratified according to genotype in terms of well described CVD risk factors. We found that both sets of cut-off values were able to group participants so that CVD risk factors (blood pressure and heart rate, lipogram and HbA1c) were significantly worse in those considered to have a WC placing them at risk for cardiometabolic conditions. CRP is produced under regulatory control from IL-6, especially, among other cytokines (Ridker, 2016), providing physiological evidence for the association observed in our study. Intriguingly, CRP did not mimic IL-6 across the twelve CRP genotypes included in this study. We found that volunteers harboring different genotypes at the rs1417938 and rs3093068 loci presented with differing cardiovascular markers. In our black South African cohort, the variance in IL-6 was determined by age, heart rate, LDL-c and WC whereas CRP was determined by age, HbA_{1c}, IL-6, LDL-c and WC. Furthermore, we indicated that participants with an at-risk WC presented with significantly higher IL-6 and CRP concentrations, regardless of *CRP* genotype, compared with those with normal WC, thus increasing their risk of developing complications in which inflammation plays a role.

We determined that women had both higher CRP concentrations and WC compared with men, similar to previous observations in Americans (Khera *et al.*, 2005) and Latin Americans (Todendi *et al.*, 2016). The sex-related WC differences in our study could be attributed to physiological factors, cultural factors or a combination of these. Owing to hormonal changes, women experiencing menopause are more likely to experience an increase in abdominal fat deposition, which persists into the postmenopausal period (Davis *et al.*, 2012). Increases in BMI, and particularly, in WC, are associated with larger increases in CRP concentrations in women when compared with men (Khera *et al.*, 2005). In the African culture, the perception also exists that overweight or obese women are happier and better cared for by their partners and that obesity is a sign of beauty (Micklesfield *et al.*, 2013). After adjustment for either BMI or WC, the differences observed in CRP concentrations between the sexes were no longer significant, indicating that these anthropometric markers are associated with increased inflammation irrespective of sex. The literature on whether IL-6 differs among sexes is ambiguous (Chapman *et al.*, 2009), but in this study, we did not observe any differences between IL-6 concentrations in men and women. IL-6 differed between urban and rural localities, a result which was attributed to age differences between the two cohorts. Both IL-6 and CRP have previously been shown to increase in an age-dependent manner (Puzianowska-Kuźnicka *et al.*, 2016), with weak yet significant correlation between age and these markers also being observed in our cohort.

In total, 216 newly diagnosed cases of HIV infection were observed in our study. Although the differences were not significant, HIV-positive individuals had lower median IL-6 and CRP concentrations, which was most likely because of smaller median WC. Treatment with anti-retroviral agents (ARVs) had previously been reported to increase IL-6 (Borges *et al.*, 2015) and CRP (Guimaraes *et al.*, 2008) concentrations; however, as these individuals were first diagnosed during this study, none was prescribed ARV treatment. We observed associations between WC and both IL-6 and CRP, with the correlation being stronger between WC and CRP.

A third of the population under investigation here presented with WC above cut-off points, placing them at risk of developing metabolic complications. These individuals presented with elevated IL-6 and CRP concentrations among other CVD risk factors, indicative of the presence of chronic inflammation. The association between increased waist girth and inflammation has been reported in various populations, including Asians (Uemura *et al.*, 2017), and individuals of European descent (Piché *et al.*, 2005), Hispanics (Todendi *et al.*, 2016) and African-Americans (Effoe *et al.*, 2015). WC is considered the more sensitive anthropometric marker when compared with BMI in predicting cardiometabolic risk (Lee *et al.*, 2008; World Health Organisation, 2008), reiterated in our results by the greater contribution of WC in models predicting IL-6 and CRP concentrations cross-sectionally. Similar to our findings, Stepanikova *et al.* (2017) also reported that WC was a better predictor than BMI of both CRP and IL-6, irrespective of ethnicity. We predicted a 2.63% increase in CRP concentrations with the addition of 1 cm to WC, which corresponds to the 2 – 3% increase per cm previously observed in a Brazilian population (Nazmi & Victora, 2007). It has been suggested that WC risk categories in black, sub-Saharan individuals should differ from the recommendations of the JIS (Kalk *et al.*, 2011; World Health Organisation, 2008). Our results, however, indicate that individuals classified with an at-risk WC based on the JIS recommendations predict significantly elevated IL-6 and CRP concentrations (Figure 5.2). When stratifying according to the newly proposed cut-off points of Ekoru *et al.* (2017), cardiometabolic at-risk men were found to have higher CRP concentrations; however, similar IL-6 concentrations were reported (Supplementary Table 5.1). Therefore, the WC cut-off points recently proposed by Ekoru *et al.* (2017) were sensitive enough to differentiate between individuals according to CRP concentrations, which could better predict future cardiometabolic risk, but failed to stratify men with elevated IL-6 concentrations. Both the proposed WC thresholds can be used to screen individuals that might be high risk in terms of presenting with low-grade inflammation and also other risk factors known to be involved in development of NCDs.

Utilizing backward stepwise linear regression models, IL-6 concentrations were explained by WC, age, LDL-c and heart rate, while covariates in models predicting CRP were IL-6, HbA_{1c}, LDL-c and WC. LDL-c was a covariate in both models, even though Spearman ρ between LDL-c and IL-6 was insignificant. LDL-c is a well-established risk factor in the development of coronary heart disease, with evidence for an elevation in LDL-c with increased visceral obesity still lacking (Ridker, 2016; Van Gaal *et al.*, 2006). Statins, the most widely used lipid-lowering therapy, have been shown in previous studies to reduce not only LDL-c levels, but also IL-6 and CRP concentrations, indicative of the link between LDL-c and inflammation (Loppnow *et al.*, 2011; Ridker, 2016; Ridker *et al.*, 1998). Previously, a 14% reduction in risk for myocardial infarctions has been reported with a 1% reduction in HbA_{1c} (Stratton *et al.*, 2000), with significant correlations between CRP and HbA_{1c} also observed in another study (Vinitha *et al.*, 2015).

Our study predicted a 7.3% increase in CRP concentrations when IL-6 concentrations were increased by 10%. Although other factors are known to influence CRP concentrations, between 35 and 40% of CRP variation has been shown to be a result of genetic factors in other populations (Pankow *et al.*, 2001). Even though IL-6 is synthesized upstream from CRP in the inflammation cycle, we investigated whether CRP genotypes associated with IL-6 to detect possible negative feedback responses or pleiotropy. Based upon similar median IL-6 concentrations (Table 5.3), we found no evidence that *CRP* SNPs were associated with IL-6 concentrations. Reports on these SNPs are limited in terms of their association with IL-6 concentrations. In a Brazilian cohort comprising children and adolescents, rs1205 (which can be used as surrogate for the five-SNP group in LD described earlier), was also not associated with IL-6 concentrations, irrespective of WC (Todendi *et al.*, 2016). Genome-wide association studies also failed to implicate *CRP* polymorphisms as influencers of IL-6 concentrations (Suhre *et al.*, 2017; Veenstra *et al.*, 2017).

Nienaber-Rousseau *et al.* (2014) summarized interactions with diet-related factors and briefly described those pertaining to anthropometry with selected *CRP* SNPs. As the populations were similar, except for our exclusion of outliers and individuals with missing IL-6 data, similar *CRP* SNP*environment interactions were observed. To increase statistical power, the investigation here used the co-dominant genetic model of heritability and also took IL-6 levels into consideration. In summary, unadjusted CRP medians, stratified to genotypes (Table 5.3), differed in those harboring the minor alleles of the five-SNP group as well as rs2808630, rs3093068, rs3093062 and rs3093058. As median IL-6 concentrations did not differ between genotypes, the role of IL-6 in these differences in CRP concentrations was limited. CRP differences could, therefore, be ascribed to the genetic factors and possible differences in

other variables not included in our study. The continuous variables listed in Tables 5.1 and 5.2 were compared between each genotype of the investigated SNP, with differences found only for rs1417938 (heart rate, BMI and WC) and rs3093068 (HDL-c). The fact that our results indicate that CRP differed across the *CRP* genotypes even when similar IL-6 concentrations were present might indicate that the relationship between IL-6 and CRP is not the same when harboring different genotypes and that genetics plays an important role in the CRP phenotype.

IL-6 was found in higher concentrations across most of the CRP genotypes when stratifying according to JIS cut-off values for those presenting with at-risk WCs, while elevated CRP concentrations were observed for all genotypes in those individuals considered to have elevated WC (Figure 5.2, Table 5.4). Median CRP levels were below the proposed 3 mg/L in individuals in the normal WC group.

Our study was not without its limitations. Left-censored data for IL-6 could mask the true association between anthropometry and IL-6 when harboring certain *CRP* SNPs. In future studies, increased sensitivity could be achieved if an enzyme-linked immunosorbent assay-based methodology were to be used as the LOD of some assays is as low as 0.2 pg/mL (RnD Systems, 2017). A crucial physiological aspect is the fact that CRP has a half-life time of 19 hours, whereas IL-6 has a half-life time of approximately 6 hours (Ablij & Meinders, 2002). This implies that, when measuring CRP and IL-6 at the same time point (in our case early morning), CRP concentrations might have been determined by IL-6 concentrations prior to measurement. IL-6 concentrations follow a circadian rhythm, with morning levels being substantially lower than values measured in the evening (Nilsonne *et al.*, 2016). CRP, in contrast to IL-6, does not present with time-of-day variation, but variation throughout the inflammation cycle, which, therefore, makes it a valuable predictor of CVD risk (Meier-Ewert *et al.*, 2001) when excluding those with acute inflammation. Future studies investigating the regulatory effects of IL-6 on CRP should aim to include the circadian variability of IL-6 to shed more light on this aspect. Here, we included only black South Africans in order to limit genetic heterogeneity and our results should not be extrapolated to other ethnic groups without caution. Despite these limitations, this was the first large-scale integrated epidemiological investigation to determine the interplay between anthropometry, IL-6 and *CRP* polymorphism in relation to CRP, and these results will be of great value for the scientific community at large.

5.6 Conclusions

CRP did not mimic IL-6 across the twelve *CRP* genotypes included in this study, even though both were elevated in individuals with increased waist girths. The newly proposed WC cut-off points for predicting cardiometabolic risk created sub-Saharan black participant subgroups with different inflammatory states. We believe that the elevated CRP concentrations observed

in black individuals are more likely to be associated with anthropometric and related determinants.

This study provides us with important mechanistic insight into the potential cause of increased CRP in the black South African population. Gene-modification techniques are, unfortunately, not currently a viable therapy. In the interim, however, public recommendations should focus on modifiable alternatives. Although genetics may be a major component of elevated CRP, management of WC so that it is within the normal range remains a beneficial therapeutic intervention in the arsenal of health care providers to reduce the global CVD burden.

5.7 Conflict of interest

The authors declare no conflict of interest.

5.8 Acknowledgments

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5.9 Disclosure Statement

Any opinion, findings, conclusions or recommendations expressed in this material are those of the authors and the NRF does not therefore accept any liability in this regard.

Authors have declared no conflict of interest.

5.10 References

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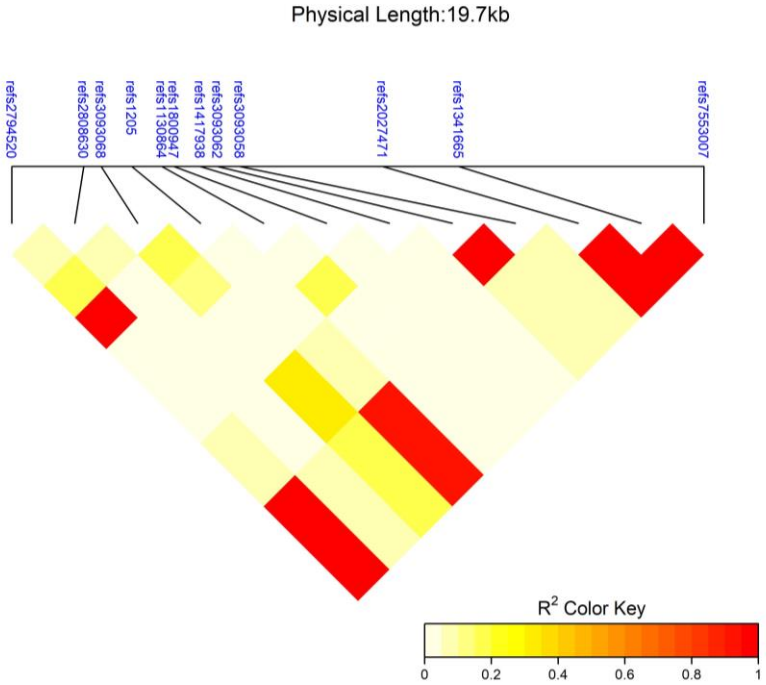
Supplementary table 5.1: Comparison between recommended WC cut-off values for increased cardiovascular risk

Modulating factor		Median IL-6 (pg/mL) [IQR]							Median CRP (mg/L) [IQR]						
			JIS WC recommendations			Ekoru *et al.* (2017)				JIS WC recommendations			Ekoru et al. (Ekoru *et al.*, 2017)		
		Whole group n = 1327	Normal WC n = 879	At-risk WC n = 448	p-value	Normal WC n = 808	At-risk WC n = 519	p-value	Whole group n = 1327	Normal WC n = 879	At-risk WC n = 448	p-value	Normal WC n = 808	At-risk WC n = 519	p-value
Sex	*Men*	2.69 [1.06 – 5.42]	2.56 [1.06 – 5.22]	4.33 [2.25 – 8.71]◊	0.019	2.77 [1.06 – 5.5]	2.52 [1.06 – 4.94]◊	0.8	2.15 [0.69 – 6.74]	1.95 [0.62 – 6.58]	5.32 [2.52 – 9.35]	0.003	1.74 [0.57 – 6.14]	3.79 [1.17 – 7.65]	0.002
	Women	2.74 [1.06 – 4.96]	2.09 [1.06 – 4.64]	3.11 [1.88 – 5.06]	<0.0001	2.14 [1.06 – 4.64]	3.11 [1.92 – 5.08]	<0.001	3.42 [1.10 – 9.12]	1.82 [0.57 – 4.93]	5.94 [2.57 – 13.6]	<0.001	1.83 [0.59 – 4.92]	6.11 [2.67 – 14.0]	<0.001
	p-value	*NS*	*NS*	*NS*	---	*NS*	*NS*	---	<0.0001	*NS*	*NS*	---	0.48	<0.001	---
Location	*Rural*	2.53 [1.06 – 5.18]	2.16 [1.06 – 5.40]	2.68 [1.66 – 4.9]	0.01	2.01 [1.06 – 5.21]	2.91 [1.7 – 5.13]	<0.001	3.06 [0.81 – 8.04]	1.96 [0.57 – 5.61]	5.83 [2.63 – 12.5]	<0.001	1.83 [0.56 – 5.24]	5.59 [2.52 – 12.2]	<0.001
	Urban	3.13 [1.56 – 4.98]	2.5 [1.06 – 4.66]	3.59 [2.37 – 5.30]	<0.0001	2.73 [1.06 – 4.93]	3.09 [1.87 – 4.97]	0.08	2.80 [0.96 –8.69]	1.82 [0.64 – 6.00]	5.94 [2.37 – 14.0]	<0.001	1.82 [0.62 – 5.89]	5.30 [2.02 – 13.5]	<0.001
	p-value	0.02	*NS*	0.01	---	0.02	*NS*	---	*NS*	*NS*	*NS*	---	NS	NS	---
HIV satus	*Positive*	2.69 [1.06 – 5.27]	2.46 [1.06 – 5.56]	2.94 [1.59 – 4.31]	NS	2.69 [1.06 – 5.79]	2.52 [1.06 – 4.26]	0.49	2.64 [0.86 – 7.72]	2.21 [0.69 – 7.01]	3.12 [1.33 – 12.3]	0.2	1.72 [0.56 – 5.22]	5.77 [2.34 – 12.2]	0.07
	Negative	2.71 [1.06 – 5.01]	2.27 [1.06 – 4.8]	3.12 [1.97 – 5.13]	<0.0001	2.26 [1.06 – 4.85]	3.02 [1.86 – 5.09]	<0.001	3.05 [0.89 – 8.34]	1.82 [0.57 – 5.52]	6.11 [2.68 – 13.5]	<0.001	2.19 [0.69 – 6.97]	3.37 [1.38 – 13.7]	<0.001
	Never tested	4.15 [2.40 – 5.72]	3.46 [2.05 – 4.85]	6.01 [6.01 – 6.01]	NS	2.75 [1.8 – 3.81]	9.25 [7.63 – 10.86]	0.13	3.48 [0.99 – 9.53]	2.80 [0.38 – 4.15]	11.32 [11.32 – 11.3]	0.66	3.48 [2.15 – 15.4]	5.85 [3.12 – 8.59]	1
Tobacco	*Formerly*	3.40 [2.09 – 5.90]	3.40 [2.3 – 6.29]*	2.71 [1.76 – 5.59]	NS	4.35 [2.31 – 7.64]	2.52 [1.97 – 5.52]	0.17	2.54 [1.09 – 9.48]	2.21 [0.88 – 8.35]	8.24 [2.97 – 12.59]	0.11	2.13 [0.57 – 18.11]	3.97 [2.16 – 9.14]	0.23
	Currently	2.78 [1.06 – 5.33]	2.61 [1.06 – 5.28]	3.24 [1.79 – 5.28]	0.015	2.62 [1.06 – 5.26]	3.18 [1.7 – 5.31]	0.04	2.75 [0.80 – 7.67]	1.93 [0.64 – 6.69]	4.84 [2.4 – 12.04]	<0.001	1.87 [0.62 – 61]	4.9 [2.26 – 12.0]	<0.001
	Never smoke	2.57 [1.06 – 4.63]	2.02 [1.06 – 4.36]	3.09 [1.98 – 4.96]	<0.0001	2.03 [1.06 – 4.53]	3.00 [1.86 – 4.90]	<0.001	3.29 [0.92 – 8.61]	1.71 [0.56 – 4.92]	6.86 [2.7 – 14.1]	<0.001	1.63 [0.54 – 4.85]	6.08 [2.33 – 13.8]	<0.001

◊ Significant difference in cytokine concentrations between two WC classifications recommendations. *Significantly higher cytokine concentrations observed in this group compared to other groups.

5.11 Supplemental figure 5.1:



Supplemental figure 5.1: Pair-wise LD patterns in the PURE data represented as r^2 values among SNPs at the *CRP* locus. The color gradient indicates level of LD from red indicating complete linkage disequilibrium to white indicating linkage equilibrium. Tagged SNP, named after the SNP with most widely published upon in literature, were chosen from SNPs in full LD, i.e. those in red blocks.

5.12 Supplementary table 5.2:

Median IL-6 and hs-CRP concentrations stratified to Ekoru *et al.* (2017) WC categories and *CRP* SNPs

SNP	Genotype	IL-6 (pg/mL)					CRP (mg/L)				
		Normal WC		Elevated WC		Between group p-value	Normal WC		Elevated WC		Between group p-value
		n	IL-6	n	IL-6		n	CRP	n	CRP	
rs2808630	T/T	607	2.35 [1.06 – 4.85]	369	3.02 [1.85 – 5.15]	<0.001	606	2.01 [0.67 – 5.83]	369	5.88 [2.50 – 12.9]	<0.0001
	C/T–C/C	201	2.36 [1.06 – 5.43]	150	2.90 [1.67 – 4.73]	*NS*	201	1.16 [0.50 – 3.77]	150	4.30 [1.67 – 11.5]	<0.0001
			NS		*NS*			0.002		0.06	
rs3093068	C/C	305	2.45 [1.06 – 5.25]	204	3.04 [1.97 – 5.34]	0.02	305	1.32 [0.47 – 3.77]	204	3.64 [1.59 – 8.46]	<0.0001
	C/G–G/G	502	2.28 [1.06 – 4.89]	314	2.96 [1.73 – 4.90]	0.003	501	2.19 [0.71 – 6.02]	314	6.77 [3.25 – 14.4]	<0.0001
			NS		*NS*			0.0001		<0.0001	
rs1205	C/C	479	2.25 [1.06 – 4.91]	304	3.10 [1.86 – 5.28]	<0.001	478	2.17 [0.69 – 6.35]	304	7.01 [3.26 – 15.1]	215
	C/T–T/T	329	2.49 [1.06 – 5.25]	215	2.93 [1.71 – 4.75]	0.199	329	1.46 [0.47 – 4.41]	215	3.67 [1.50 – 8.17]	<0.0001
			NS		*NS*			<0.001		<0.0001	
rs1130864	C/C	595	2.44 [1.06 – 5.20]	213	2.19 [1.06 – 4.85]	<0.01	595	1.78 [0.58 – 5.65]	402	5.25 [2.05 – 13.1]	<0.0001
	C/T–T/T	402	2.97 [1.79 – 4.98]	117	3.11 [1.81 – 5.10]	<0.01	212	1.94 [0.60 – 5.06]	117	6.11 [2.83 – 11.6]	<0.0001
			NS		*NS*			*NS*		*NS*	
rs1800947	C/C	805	2.35 [1.06 – 5.12]	515	3.01 [1.80 – 5.06]	<0.001	804	1.82 [0.59 – 5.57]	515	5.47 [2.27 – 12.3]	<0.0001
	C/G	3	2.02 [1.54 – 2.94]	4	2.19 [1.55 – 3.77]	1	3	0.37 [0.29 – 1.97]	4	5.64 [3.82 – 7.98]	0.11
			NS		*NS*			*NS*		*NS*	
rs1417938	A/A	754	2.25 [1.06 – 4.99]	503	2.99 [1.79 – 5.03]	<0.0001	754	1.75 [0.57 – 5.51]	503	5.49 [2.27 – 12.3]	<0.001
	A/T	51	3.11 [1.06 – 4.85]	14	4.25 [2.41 – 6.69]	0.34	50	2.71 [0.68 – 5.36]	14	5.05 [3.46 – 23.0]	<0.05
			NS		*NS*			*NS*		*NS*	
rs3093062	G/G	564	2.38 [1.06 – 5.18]	351	3.09 [1.90 – 5.32]	<0.001	564	1.55 [0.53 – 4.65]	351	4.45 [1.79 – 9.54]	<0.0001
	A/G–A/A	242	2.26 [1.06 – 4.72]	167	2.75 [1.73 – 4.61]	0.14	241	2.46 [0.93 – 7.18]	167	7.60 [3.57 – 17.0]	<0.0001
			NS		*NS*			<0.0001		<0.0001	

Concentrations presented as median [25th – 75th percentile]. Wilcoxon Rank Sum Test used to test the significance between genotypes.* Significance level of p <0.05 between genotypes of SNP. rs1205 presents a haplogroup containing rs2794520, rs1341665, rs2027471 and rs7553007 in high LD; rs3093062 also represents rs3093058. P-values were adjusted using the methods of Bonferroni. Abbreviations: A, adenine; BMI, body mass index (kg.m^{-2}); C, cytosine; G, guanine; hs-CRP, high sensitivity C-reactive protein; IL-6, Interleukin-6; NS, not significant (p > 0.05); rs, reference SNP cluster ID; T, thymine.

CHAPTER 6: CONCLUSION

6.1 General discussion

Globally, a surge in the number of deaths attributable to NCDs, and particularly CVDs, has been observed, and this has resulted in the WHO establishing global targets, colloquially referred to as the "25x25" targets, in a bid to halt or, ideally, reduce the mortality rates. In Chapter 2 we indicated that by addressing the targets aimed at lifestyle changes, reductions in chronic inflammation would result (Summarised in Figure 6.1). The inflammatory origins of NCDs, especially CVDs, have come to the fore (Ruiz-Núñez *et al.*, 2013), with the cytokine CRP being of particular interest based upon the associated augmented risk of developing CVD with increasing concentrations of CRP (Ridker, 2016). Black individuals (Nazmi & Victora, 2007), including those from South Africa (Nienaber-Rousseau *et al.*, 2014), are more likely to present with elevated CRP concentrations than other ethnicities and, although polymorphisms in the *CRP*-gene were determined to affect the CRP phenotype (Nienaber-Rousseau *et al.*, 2014), not all variation can be explained by genetics alone. The interplay between modifiable (i.e. behavioural and environmental) and non-modifiable (i.e. genetic[3]) factors is of importance, as identifying modifiable factors could, in turn, present healthcare providers with possible intervention avenues to be explored. We examined whether specific non-genetic factors could potentially explain the CRP phenotype observed in a group of black South Africans more reliably than those previously reported (Nienaber-Rousseau *et al.*, 2014). A review of the current literature identified three possible non-genetic factors specific to the black South African population that may have acted as potential sources of the elevated CRP concentrations in this population and these were investigated here.

[3] Although new technologies such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) exist that present treatment opportunities for genetics-based disorders, there is currently an ethical debate about whether such technologies should be used as a treatment for non-life-threatening conditions such as to modify genetic susceptibility where lifestyle changes could achieve similar effects (Mulvihill *et al.*, 2017).

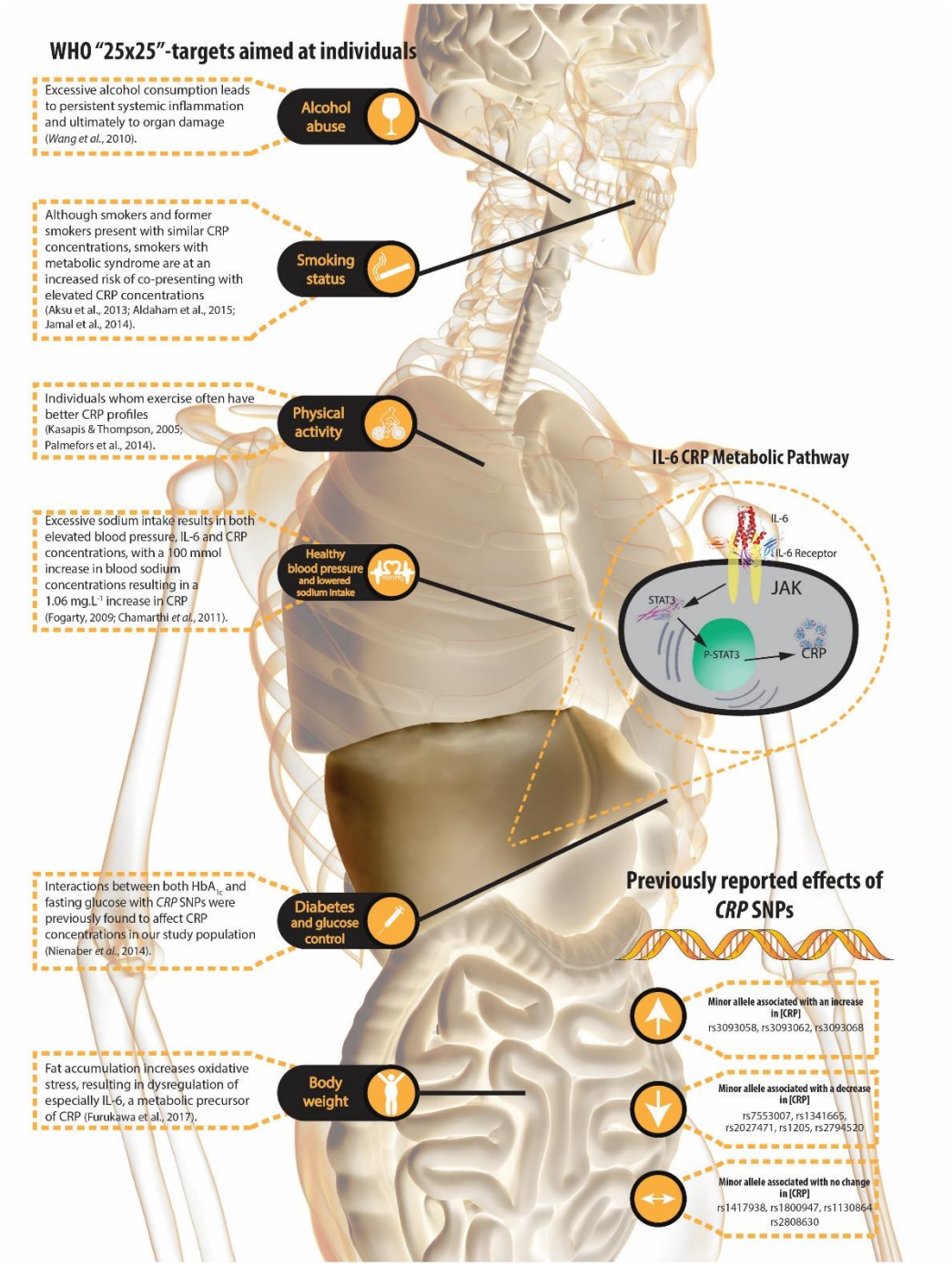


Figure 6.1: Summary of the "25x25" WHO targets aimed at lifestyle changes that individuals can make and how these targets affect systemic inflammation
Abbreviations: [ ], concentration, CRP, C-reactive protein; IL-6, interleukin-6; JAK, Janus kinase; rs, reference SNP; SNP, single nucleotide polymorphism; (P)-STAT3, (phospho)-signal transducer and activator of transcription; WHO, World Health Organisation.

In the PURE study cohort, interactions between dietary factors, *CRP* genotypes and CRP concentrations have previously been investigated. From this, it was determined that obesity, carbohydrate and lipid intake, as well as triglyceride and cholesterol concentrations, were the major influencers of *CRP* gene-mediated changes in CRP concentrations (Nienaber-Rousseau

et al., 2014). Based upon this prior knowledge, we considered other factors that could have impacted on CRP status.

South Africa has a history of a socio-economic divide which still exists even after the dawn of the new political era (Micklesfield *et al.*, 2013). As factors pertaining to SES are known to affect health (O'Neill *et al.*, 2007), and adults experience a burden of elevated CRP based upon their childhood SES (Liu *et al.*, 2017), factors in the individual's immediate living environment may be a potential source of altered CRP. In Chapter 3, therefore, we investigated whether specific socio-economic factors result in variability of CRP concentrations in a group of black sub-Saharan Africans, and determined whether the effects of *CRP*-genotypes on CRP concentrations are heterogeneous. Although we found no specific SES-factors pertaining to living environment that affected systemic inflammation, we determined that completing twelve or more years of formal education predicted 18.9% reduced CRP concentrations compared with individuals with no formal schooling, but with similar physiological and demographical markers (Table 3.4). Lower BMIs were associated with longer periods of formal schooling in the PURE study (Pisa *et al.*, 2012), and as CRP is elevated in individuals with increased body adiposity (discussed with reference to Chapter 5), the predicted lower CRP in higher levels of education could best be explained by the lowered adiposity of the individual. We also presented the first indication that smoking status affects CRP concentrations differently, i.e. smokers harbouring the C-allele at rs3093068 locus presented with elevated CRP concentrations compared with non-smoking individuals and former smokers. We indicate that formal education has life-long effects on general health, and, in particular, that those with a better educational background were less likely to present with elevated CRP concentrations. Our results are also indicative of the fact that elevated CRP concentrations and poorer markers of cardiovascular health coincide and, therefore, establish the risk associated between CRP concentrations and future CVD risk in black South Africans. None of the SES factors, apart from smoking status, interacted with the included *CRP* genotypes, and similar CRP concentrations were observed across the different SES factors. Although we did not indicate an overall effect of smoking and excessive alcohol use on CRP concentrations, refraining from these activities is always to be recommended. This study is indicative of the fact that the CRP phenotype observed in black South Africans is attributable to other, non-SES factors, excluding education, such as anthropometry, genetics and vitamin D, which altered the risk of presenting with elevated CRP.

In Chapter 4, we observed that not only are black women more likely to present with elevated CRP, but they often co-present with vitamin-D deficiency due to a decreased production of 25(OH)D, which is associated with an increased risk of presenting with systemic low-grade inflammation, as reiterated by our findings published in the International Journal of Environmental Research and Public Health (Myburgh *et al.*, 2018). Although this study found no direct

interactions between 25(OH)D and *CRP* polymorphisms, indicating that the association between vitamin D deficiency and increased CRP was not likely to be due to 25(OH)D interacting with the *CRP* gene, it does provide evidence to support the correlation often observed between CRP and 25(OH)D concentrations in other ethnicities and was the first indication of this association for a group of black South Africans. Women presenting with the co-burden of low 25(OH)D status and elevated CRP also presented with high blood pressure and increased adiposity, which are both risk factors for the future development of CVD. Furthermore, this study reiterates the importance of abdominal adiposity as a contributing factor to elevated CRP concentrations, which was subsequently investigated in Chapter 5.

In this study, we tested the effects of IL-6, a cytokinal precursor in the synthesis pathway of CRP, known to be increased in viscerally obese individuals, as a potential mediator of CRP concentrations (Crichton *et al.*, 1996; Fontana *et al.*, 2007; Ganter *et al.*, 1989; Memoli *et al.*, 2007; Pini *et al.*, 2012; Ridker, 2016). We also compared the effectiveness of two WC thresholds indicative of cardiometabolic risk to stratify markers of inflammation in our study population. Both IL-6 and CRP concentrations were increased in individuals with an at-risk WC, while there was also a moderate positive correlation between IL-6 and CRP concentrations. In comparing two different WC cut-off values for metabolic risk, both were able to stratify between normal and elevated CRP concentrations. Importantly, women presented with higher CRP concentrations than men, mainly as a result of increased WC observed in the female participants. An important cultural aspect, that of an increased body weight being preferred by some black South Africans (Micklesfield *et al.*, 2013), may be to blame for the larger WC frequently observed in these women. Differences were observed in CRP concentrations of individuals with varying *CRP* genotypes and similar WC risk thresholds in the presence of similar IL-6 concentrations, this being indicative of a substantial effect on CRP concentrations of *CRP* genetics, and that other factors arising from increased WC may be partly to blame. In normal WC-risk groups, CRP concentrations were, however, below the 3 $mg.L^{-1}$ cut-off indicative of future CVD risk for all the included *CRP* polymorphisms, while elevated CRP concentrations were observed in all genotypes of individuals with an elevated WC. Therefore, aiming to maintain a healthy WC is recommended for all individuals, regardless of genetic composition.

Following the results of this study, as well as the integration of previously reported data from this cohort, the main factors determined to influence CRP concentrations in our cohort of black South Africans are summarised in Figure 6.2. The predictive nature of these factors should be considered in the physiological context within which they may arise. For instance, our adjusted

models predicted a 12.4% increase in CRP concentrations with a single unit[4] increase in HbA_{1C}, which has a limited physiological range of between 4.3–6.4% in non-diabetic individuals (Dayeh *et al.*, 2014). We, however, reported small differences in median HbA_{1C} concentrations between individuals with normal and elevated CRP concentrations (0.20%, Table 3.2), as well as between those stratified into different WC categories (0.30%, Table 5.2), with large differences in CRP concentrations between WC thresholds observed (1.82 vs 5.47 $mg.L^{-1}$) based on the work of Ekoru *et al.* (2017). This is, therefore, indicative of the limited contribution of HbA_{1C} towards elevated CRP concentrations in the studied population. Similarly, substantial reductions in CRP (22.0%) were predicted with a 1 $mmol.L^{-1}$ increase in HDL-c; however, median HDL-c concentrations differed by 0.14 $mmol.L^{-1}$ between those with normal and those with elevated CRP, thus contributing towards only ~3% of CRP variation. A reduction of 1 cm in WC predicted a decrease of between 2.6–3.1% in CRP concentrations, and eliciting this response will co-affect the other modifiable factors, such as lipoprotein concentrations, IL-6 and glucose control, identified as predictors of CRP concentrations.

From Figure 6.2, it can be concluded that CRP concentrations are associated with various factors, and that holistic approaches are needed in any endeavour to capture the complexity of this inflammatory protein. From this model, and the manuscripts contained in this thesis, we can conclude that the most important modifiable factor in managing inflammatory risk is WC. As a 10 cm reduction in WC may result in as much as a 31% reduction in CRP, interventional efforts should focus on educating individuals in achieving/maintaining a healthy visceral circumference. Not only will a lowered WC have direct regulatory effects on CRP, but it will further improve other predictors identified in our research. Improved WC in a population will most likely be brought into effect by focusing efforts on improving diets and increasing physical activity. Proper dietary advice, and access to wholesome food-sources, will result in better glucose control, reduced sodium intake and improved lipid profiles, and may also have a limited effect on vitamin D status, while improving physical activity will improve heart rate, reduce the prevalence of hypertension, and if done outside, could hypothetically increase 25(OH)D concentrations due to increased sun exposure, thereby decreasing CRP. Reductions in WC is also important for the maintenance of normal IL-6 concentrations, which affect other inflammatory and metabolic pathways aside from that of CRP.

[4] HbA_{1C} is reported as %. Changes in HbA_{1C} are referred to as a unit change, so as to not cause confusion between a unit HbA_{1C} increase and a relative % increase.

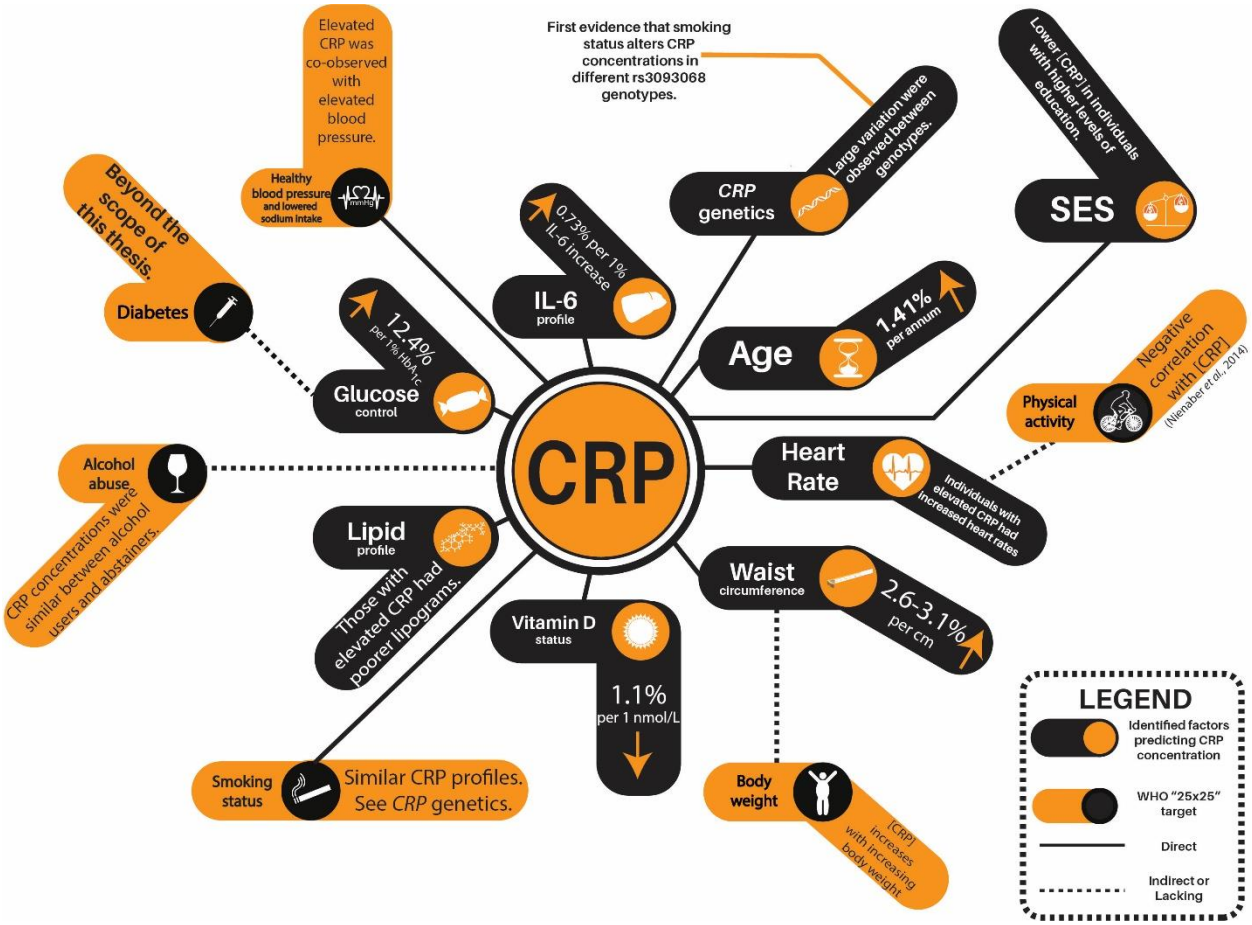


Figure 6.2: Factors influencing CRP concentrations in a group of black South Africans.
Abbreviations: [ ], concentrations; CRP, C-reactive protein; HbA1c, glycated haemoglobin; IL-6, interleukin-6; WHO, World Health Organisation.

As indicated in Chapters 1 and 2 of this thesis, achieving the targets set for individuals in the "25x25" recommendations will greatly reduce the burden of NCDs and CVD, in particular. This may, to some extent, be due to attaining reduced inflammatory profiles in individuals. Although the effects of achieving these targets should be seen holistically, the importance of halting the rise in obesity was an important target that came to the fore in our research. Nienaber-Rousseau *et al.* (2017), in a manuscript co-authored by the Ph.D. candidate (Annexure 3), indicated that within five years, WC increased significantly in this study population, which may have had undesired effects on the inflammatory state of the population. However, since the year 2010, much has changed, and the South African government should be commended for some of the new regulations that have been implemented in a bid to meet the "25x25" targets. South Africa was the first country to implement mandatory sodium targets for certain food products, with industry quickly adopting these targets (Swanepoel *et al.*, 2017). This was also investigated by the Ph.D. candidate, although it lies outside the scope of the current thesis (Annexure 3). More work is needed, however, to ensure that South Africans reduce their inflammatory, and in particular, their CRP, burden.

6.2 Limitations, strengths and proposed focus of future research

In the preceding chapters, the limitations and strengths specific to those sub-studies were described. Here follows a summary. In addressing our main aim, the presented study was limited by the following aspects. First, this study was a cross-sectional study and as such data were limited to a single observation point. Although cross-sectional studies have merit in laying the groundwork for future studies, longitudinal analysis would better elucidate the actual effects of the factors we included in this study. We specifically chose a cross-sectional study design, however, as the study within which this research was conducted had a fairly large attrition rate and, since it was rare to be homozygous for some of the variant alleles, having greater numbers was very important. As our analysis was exploratory in nature, we wanted to have sufficient statistical power when stratifying to ensure that valid conclusions could be drawn. As mentioned in Chapter 3, in measuring the SES factors, no data were collected on the length of time that the individuals had access to these SES factors, such as period of access to electricity and municipal water sources. If longitudinal data were included, this would better illustrate the effects of, for instance, having access to improved water sources or electricity. It would also be beneficial to indicate how and indeed, if, changes in vitamin D status and WC over time would have influenced the inflammatory status of the study volunteers. During the author's tenure as Ph.D. candidate, he co-authored another research output (Annexure 3), in which changes in adiposity in the studied population were longitudinally investigated. The results indicated that the women in this cohort, especially, experienced increases in BMI, as well as increased abdominal adiposity, which may have altered their risk of developing CVD. In future research, we will characterise the effects of this increased WC on the inflammatory status.

Secondly, the study included only genetic polymorphisms located in the *CRP* gene, although previous GWAS studies have established that other regions of the genome also affect CRP status (Dehghan *et al.*, 2011). It would have been ideal to have access to whole-genome data for the study cohort; however, as our study is still a preliminary investigation into the factors affecting CRP status, the focus on arguably the most important gene in the CRP-cascade, i.e. the *CRP* gene itself, remains a valuable addition to the current literature base. Little is known about *CRP* genetics in black populations and, therefore, our research further expands what is reported about this gene in black South Africans. In focusing our attention first and foremost on covering all aspects related to the *CRP*-gene, which encodes the protein, future efforts can further develop and build upon the knowledge generated in this thesis.

As with any study, we could not address all the confounding factors that may have affected CRP concentrations in our study population. Limited data was available regarding chronic medication usage, the duration thereof as well as the adherence to treatment regimens for the 2005 study population, which may have affected some individual participant's results. Different anti-

inflammatory medications also have differing effects on CRP concentrations i.e. some result in increased CRP concentrations, while others decrease circulating CRP (Tarp, 2012). Future pharmacogenetic studies in our study population would be relevant but were beyond the scope of the current research endeavour.

Our study was also limited by our another word of physical activity data, however, from our review of the literature, stronger associations between inflammatory markers and anthropometric measurements were reported. Since our study was exploratory in nature, we decided to first confirm these associations in our study population, paving the way for future longitudinal analyses which would include changes in physical activity and body composition. Other residual confounders, not measured in the PURE study, may also have have resulted in elevated CRP concentrations, the effects of which could potentially be minimised by revisiting the themes of this thesis in longitudinal analyses.

A strength of our study was that we stratified according to the internationally recommended cut-off values for CRP concentrations to indicate future CVD risk (i.e. >3 $mg.L^{-1}$ is associated with an increased CVD risk). Some ambiguity exists in the current literature as to whether the imposed 3 $mg.L^{-1}$ is too low for black individuals who are reported to have increased CRP levels to begin with. Although the aim was not to do a sensitivity analysis to find accurate CRP concentrations indicating increased CVD risk, we have noted that using the 3 $mg.L^{-1}$ value in our stratification process selected individuals who generally had increased heart rate, higher blood pressure and at-risk lipogram results (Table 3.3) and therefore are of the opinion that it is a valid cut-off value for black South Africans.

6.3 Final remarks and conclusion

In our search for factors associating with CRP concentrations in black South Africans, we demonstrated that specific socio-economic factors had little to no association on CRP concentrations, but that education is of the utmost importance in sustaining life-long favourable health choices. Further, we provided the first ever evidence that smoking affects the different genotypes of rs3093068 differently, with smokers with the C-allele having a similar risk of presenting with elevated CRP as those with the already at-risk allele. In women, we found that many individuals presented with the co-phenotype of elevated CRP and deficient/insufficient vitamin D status. These co-burdened individuals had significantly higher blood pressure compared with individuals who were reported as controls, which may be indicative of an increased risk of developing CVD later in life. Fortification efforts and safe sun exposure should be increased to elevate 25(OH)D levels in these women. Lastly, the importance of increased adiposity in the aetiology of increased CRP concentrations was evaluated, comparing two different WC cut-off criteria for cardiometabolic risk. Not only did persons with increased body

weight and WC have higher CRP, but they also had a poorer cardiovascular prognosis. We, therefore, recommend that individuals should aim to achieve/maintain a healthy WC, together with regular exercise and a healthy diet.

In conclusion, our research, which included several novel findings, indicated that CRP remains a valuable marker in identifying individuals at risk of developing CVD, regardless of their ethnic background, and that the current 3 $mg.L^{-1}$ threshold for CRP is sensitive to stratify individuals with significantly poorer markers of cardiovascular health in a black South African population. CRP concentrations are influenced by both the genetics of the individual and other non-genetic factors, of which WC is the most readily modifiable factor. South Africans should achieve/maintain healthy body weight, and especially, aim to achieve/maintain a normal WC, as a means to reduce their future CVD risk.

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ANNEXURE 1: ETHICAL CLEARANCE CERTIFICATES

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12 May 2017

Prof GW Towers
Nutrition-CEN

Dear Prof Towers

APPROVAL OF YOUR APPLICATION BY THE HEALTH RESEARCH ETHICS COMMITTEE (HREC) OF THE FACULTY OF HEALTH SCIENCES

Ethics number: NWU-00004-17-S1

Kindly use the ethics reference number provided above in all correspondence or documents submitted to the Health Research Ethics Committee (HREC) secretariat.

Study title: Interactions of CRP-SNPs with selected contributing factors in determining CRP concentrations in black South Africans

Study leader/supervisor: Prof GW Towers

Student: PH Myburg

Application type: Single study

Risk level: Minimal

You are kindly informed that your application was reviewed at the meeting held on 16/02/2017 of the HREC, Faculty of Health Sciences, and was approved on 12/05/2017.

The commencement date for this study is 12/05/2017 dependent on fulfilling the conditions indicated below. Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation up to a maximum period of three years when extension will be facilitated during the monitoring process.

After ethical review:

Translation of the informed consent document to the languages applicable to the study participants should be submitted to the HREC, Faculty of Health Sciences (if applicable).

ANNEXURE 2: PUBLISHED ARTICLE

International Journal of
Environmental Research and Public Health

MDPI

Article

CRP Genotypes Predict Increased Risk to Co-Present with Low Vitamin D and Elevated CRP in a Group of Healthy Black South African Women

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Abstract: Low 25-hydroxyvitamin D (25(OH)D) and elevated C-reactive protein (CRP) concentrations are independently associated with adverse health outcomes, including cardiovascular disease (CVD). Although an inverse association between these factors has been described, the underlying mechanisms remain unknown. We postulate that environment–gene interactions, through which 25(OH)D interacts with single nucleotide polymorphisms (SNPs) within the *CRP* gene, modulate CRP; that certain *CRP* genotypes predispose individuals to a co-phenotype of low 25(OH)D and elevated CRP concentrations; and that this co-phenotype is associated with higher CVD risk. Twelve *CRP* SNPs were genotyped, and both 25(OH)D and CRP were quantified, in 505 black South African women. Alarmingly, 66% and 60% of the women presented with deficient/insufficient 25(OH)D and elevated CRP concentrations, respectively. CRP concentrations were higher in individuals with lower 25(OH)D concentrations. However, no 25(OH)D–*CRP* genotype interactions were evident. Several genotypes were associated with an altered risk of presenting with the co-phenotype, indicating a genetic predisposition. Women presenting with this co-phenotype had higher blood pressure and increased anthropometric measures, which may predispose them to develop CVD. We recommend increasing vitamin D fortification and supplementation efforts to reduce inflammation among black women with vitamin D deficiency, thereby possibly curbing diseases contingent on the co-phenotype described here.

Keywords: 25-hydroxyvitamin D; 25(OH)D; calcidiol; calciferol; C-reactive protein; nutrigenetics; single nucleotide polymorphisms; SNPs; Tswana

1. Introduction

Cardiovascular disease (CVD) is the leading cause of mortality, with 80% of the global burden carried by developing countries [1]. In South Africa, at the time of this study, one third of all deaths were attributable to CVD [2]. Since then, an increase in CVD-related deaths has been reported [3]. The role of chronic inflammation in the etiology of CVDs has recently come to the fore. Although the inflammatory response is regarded as crucial for survival, dysregulation of this process has detrimental effects, including the development of CVDs [4,5].

Several biochemical markers of inflammation have been identified, including tumor necrosis factor-α, interleukin 1 (IL-1) and 6 (IL-6), and C-reactive protein (CRP). CRP is by far the most widely investigated biomarker of low-grade systemic inflammation, as it has a stable half-life with

well-established associations with disease, and has known cut-off values [6]. People with a basal CRP concentration greater than 3 mg/L are at higher risk of experiencing cardiovascular events [7]. Circulating CRP concentrations can be influenced by demographic factors (age, sex, and ethnicity) as well as environmental and behavioral factors (alcohol intake, diet, socio-economic status, and tobacco use) [8–12]. Among dietary factors, an inverse association between vitamin D and CRP has been established [13,14]. A recent meta-analysis [15] indicated that vitamin D supplementation decreased CRP concentrations by 1.08 mg/L (95% confidence interval (CI), −2.13, −0.03), while those with baseline CRP levels of >5 mg/L registered a more significant reduction of 2.21 mg/L (95% CI, −3.5, −0.92). Various genetic factors have been reported to affect CRP concentrations, with twin and family studies indicating substantial heritability of between 35% and 40% [16]. Furthermore, interactions have been observed between *CRP* single nucleotide polymorphisms (SNPs) and dietary intake influencing circulating CRP concentrations [17]. In contrast, infection with the human immune deficiency virus (HIV) is not associated with CRP [18], an observation re-established by the present study.

Black individuals tend to have higher circulating CRP concentrations than those from other groups [19]. African Americans, included in a meta-analysis, presented with CRP concentrations of 2.6 mg/L compared to 2.51 mg/L in Hispanics, 2.03 mg/L in white Americans, and 1.01 mg/L in East Asians [20]. Elevated CRP concentrations are also typical in black South Africans [17]; comparisons show them to be higher (3.61 mg/L) than those of their white (1.13 mg/L) compatriots [21].

Ethnicity also affects vitamin D status. Darker skin (type V and VI skin on the Fitzpatrick scale [22]), as observed in African individuals, seems to have originated in persons living in areas with high ultra-violet (UV) radiation (UVR) [23]. Even though it is a photoprotective mechanism, darker skin reduces the synthesis of vitamin D [24] as measured by circulating 25-hydroxyvitamin D (5(OH)D), or calcidiol, concentrations. Darker-skinned humans require approximately six times more UVR exposure than their fairer-skinned counterparts to produce similar amounts of vitamin D [25]. South Africa, ranging between the ~22° and 34° southern latitudes, experiences relatively intense UVR, although not as extreme as at the equator [25]. Black South Africans present with lower 25(OH)D concentrations than dark-skinned people living closer to the equator [26]. The rapid urbanization observed in most African countries, resulting in reduced exposure to UVR, has been proposed as a major contributing factor to the low 25(OH)D status observed in black Africans [27]. Other risk factors associated with low 25(OH)D status include age, obesity, HIV infection, and smoking [28–30].

Apart from influencing inflammation, vitamin D itself is related to disease risk. 25(OH)D has preventive effects on a range of chronic maladies, including CVD [31]. Two meta-analyses found an increase in the risk of developing ischemic heart disease, as well as an augmented risk of symptomatic ischemic stroke for the participants in the lowest quartiles of 25(OH)D concentrations [32,33]. Another meta-analysis reported an inverse association between 25(OH)D concentrations and the risks associated with all-cause mortality (relative risk (RR) of 1.35 (1.22–1.49)) and CVD (RR: 1.35 (1.13–1.61)) [34].

As elevated concentrations of CRP are associated with increased CVD risk, whereas CVD risk is reduced with elevated levels of 25(OH)D, a hypothesis has been proposed that 25(OH)D might influence CRP [35]. However, there is no evidence of a direct pathway by which 25(OH)D or its metabolized product, 1,25-dihydroxyvitamin D (or calcitriol), affects the expression of *CRP* (or vice versa) [13]. Vitamin D-mediated mechanisms for a reduction in vascular damage have been proven experimentally, with the inhibition of cholesterol uptake by macrophages and the suppression of the renin gene [14]. In vitro studies have also described the diminished production of IL-6 in monocytes treated with vitamin D_3 (cholecalciferol, or calciol) compared to untreated cells [35]. IL-6, synthesized by macrophages, is transported to the liver, where the transcriptional activation of *CRP* is mediated via Signal Transducer and Activator of Transcription factor 3 (STAT3) [36,37]. These physiological mechanisms might, therefore, act as potential indirect pathways by which vitamin D and its metabolites could influence CRP concentrations. Thus, having elevated CRP as well as low 25(OH)D concentrations, which are both independently associated with increased CVD risk, may exacerbate disease development.

Because black individuals tend to be predisposed to lower 25(OH)D and higher CRP concentrations, investigations that involve this particular population will increase the chance of observing sufficient numbers of the phenotypes of interest. An inverse association between 25(OH)D and inflammation has been described, but the underlying mechanisms remain unknown [13]. Moreover, it has to be established whether vitamin D status influences *CRP* differently in individuals harboring specific *CRP* genotypes in modulating CRP concentrations. We determined whether vitamin D status possibly interacts with these *CRP* genotypes to affect the CRP concentrations. In addition, we tested whether *CRP* SNPs affected 25(OH)D concentrations. Studying environment–gene interactions is important, as identifying these interactions and modifying behaviors accordingly could improve health outcomes. Furthermore, we determined whether specific *CRP* genotypes predispose individuals to a co-phenotype of low 25(OH)D and elevated CRP, as well as whether this co-phenotype is associated with a higher CVD risk in black South African women. This research is important, because unraveling possible mechanisms for the observed relationship between vitamin D and CRP leads to a better understanding of the foundation of this relationship and paves the way for designing targeted approaches to treat the corresponding elevated CRP concentrations and low 25(OH)D concentrations in black individuals. This research might also indicate whether efforts to increase responsible sunlight exposure and include more vitamin-D-rich foods in their diet are, and/or whether supplementation with vitamin D is, desirable for black South African women.

2. Materials and Methods

2.1. Ethical Considerations

For this cross-sectional investigation, we used data collected for the South African arm of the Prospective Urban and Rural Epidemiology (PURE-SA) study, at baseline (2005). Ethical approval, in accordance with the Declaration of Helsinki as revised in 2004 [38], was obtained for the larger study from the Health Research Ethics Committee of the Faculty of Health Sciences, North-West University (NWU–HREC, ethics number: 04M10). Ethical approval was also granted for this affiliated study (ethics number: NWU-00004-17-A1). Goodwill permission was granted to the PURE study by mayors, household heads, community leaders of the communities included, and tribal chiefs before the research started. Participants were well-advised about the research project and were asked to sign an informed consent form, after sufficient time for reflection, to indicate their agreement to take part in the study. Subjects could withdraw at any time, or withhold any information they were not comfortable sharing.

2.2. Research Design and Study Population

The PURE-SA study aims to investigate the development of chronic lifestyle diseases, with a focus on CVDs, by stratifying populations at different levels of urbanization [39]. Four communities were selected in 2005 in South Africa, based on their degree of urbanization, and grouped into either being urban (Location A) or rural (Location B). The initial sampling strategy is explained elsewhere [40].

Eligible participants were all apparently healthy adults who were older than 30 years. On the day of enrollment, individuals with elevated body temperatures (above 38 °C) were excluded to reduce the number of volunteers with acute infections. Further exclusion criteria were that potential volunteers were not allowed to use chronic medication, to have any known lifestyle disease, be pregnant or lactating, or to have a known infection, such as tuberculosis-causing agents and/or the human immune deficiency virus (HIV) (details in [40]). Sampling was conducted between August and November 2005, which is late winter to late spring in the southern hemisphere. Of 6000 individuals screened, 2010 were included at baseline. The 25(OH)D status of a subset of 660 randomly selected women was determined, because of constrained budgets and the fact that women are more likely to develop skeletal disorders associated with low 25(OH)D status.

2.3. Biochemical and Blood Pressure Measurements

Fasting participants, defined as *sans* food and beverages (water permitted) from the evening before enrollment, arrived at the study site, upon which professional nurses obtained blood samples. Blood tubes were centrifuged at 2000× *g* for 15 min at 10 °C. Plasma, serum, and buffy-coat were aliquoted and snap-frozen on dry-ice pellets before storage at −70 °C. Serum high-sensitivity CRP concentrations were measured on a Sequential Multiple Analyzer Computer using a particle-enhanced immunoturbidometric assay (Konelab TM auto analyzer, Thermo Fisher Scientific, Vantaa, Finland). Total 25(OH)D (sum of D_2 and D_3) in serum was quantified using a Roche Elecsys 2010 COBAS system (functional sensitivity: 10.0 nmol/L; Roche Diagnostics, Indianapolis, IN, USA). Lipograms, including high-density lipoprotein cholesterol, triglycerides, and total cholesterol, were performed using a Konelab 20i auto analyzer (Thermo Fisher Scientific, Vantaa, Finland). The Friedewald equation was used to calculate the low-density lipoprotein cholesterol (LDL-c) in those with triglyceride concentrations below 400 mg/dL [41]. Research nurses—trained in voluntary counseling and the testing of HIV, adhering to the UNAIDS/WHO policy statement on HIV testing as well as the protocols set by the National Department of Health of South Africa—gave all participants pre-test counseling. Volunteers could then decide whether they wanted to be tested, with specific signed informed consent obtained for HIV testing after pre-test counseling. HIV determination was conducted using a rapid First Response HIV 1-2.O card test (Transnational Technologies Inc., PMC Medical, Nani Daman, India). Persons testing positive were re-tested using a second card test, developed by Pareeshak (BHAT Bio-Tech, Bangalore, India) to affirm HIV status. All participants, irrespective of their HIV status, were given post-test counseling individually. Blood pressure was measured in duplicate with an Omron automatic digital blood pressure monitor (Omron HEM-757) after 5 min of sitting in a calm environment.

2.4. Anthropometric Measurements

Body weights (kg) were measured twice on calibrated and tared scales, with the mean recorded, while participants were lightly clothed and their arms hanging freely at their sides. Heights (cm), with volunteers' heads in the Frankfort plane, bodies fully extended while inhaling, were measured twice to the nearest 10 mm, using stadiometers, and the mean was reported in meters. Body mass index was computed as kg/m^2.

2.5. Factors Pertaining to Lifestyle

Participants responded to various interviewer-administered questionnaires in a language of their choice. These test instruments included questions on medical history and tobacco use. Nutritional information from the previous 30 days was obtained using validated, interviewer-based quantitative food frequency questionnaires (qFFQs) and employing food portion books standardized for the population under investigation [42]. qFFQs' data were entered into FoodFinder 3 (Medical Research Council, Tygerberg, South Africa) and analyzed by the Medical Research Council of South Africa for nutrient content.

2.6. Genetic Analyses

Determination of the genotypes via a BeadXpress analysis was performed by the National Health Laboratory Service located at the University of the Witwatersrand, Johannesburg. For details on the genetic analyses, please refer to Nienaber-Rousseau et al. [17].

2.7. Environmental Data

Locations A and B were compared using the means of data from 1 August (late winter) to 1 December 2005 (late spring). Environmental factors were investigated using satellite data obtained

from an online repository, Giovanni [43]. Average mean temperature, UV index, erythemal dose rate, and total ozone column were downloaded as Google Earth data files (.kmz files).

2.8. Statistical Analyses

As previously mentioned, 25(OH)D concentrations were available for 660 randomly selected women. Only individuals for whom 25(OH)D concentrations, CRP concentrations, *CRP* genetic data, and all anthropometric markers were available were included in our statistical analyses (n = 534). Furthermore, women with 25(OH)D or natural log-transformed (ln)CRP concentrations greater than 5 standard deviations were excluded as outliers. The final number of participants was 505. Statistical analyses were conducted in R [44].

Numeric variables were visually inspected for normality as well as measures of skewness. Non-parametric variables (CRP) were log-transformed, yet still reported as median and interquartile ranges. Women were grouped as two phenotypes: the case phenotype including individuals with deficient/insufficient 25(OH)D (<75 nmol/L) and elevated CRP (>3 mg/L), and a control phenotype consisting of the remaining volunteers. Pairwise comparisons using the Wilcoxon ranked-sum test were performed to identify significant differences in stratified continuous variables. Comparative tables were created with the compare Groups library in R [45] using non-parametric methods. Spearman correlations were used for testing associations between numeric variables. Multivariate linear models predicting lnCRP concentrations from continuous 25(OH)D values were constructed using backward step-wise linear regressions and evaluated based on the Akaike Information Criterion (AIC). Adherence to Hardy–Weinberg equilibrium (HWE) was tested by a chi-squared (χ^2) test using SNPassoc, and linkage disequilibrium (LD) was calculated using the LDheatmap library of the R package.

Variables identified in regression analyses were evaluated for co-linearity. Possible environment–SNPs interaction was determined using SNPassoc [46] while including covariates obtained from the linear regression model. To determine whether *CRP* SNPs influence vitamin D status, 25(OH)D was used as the dependent variable. To evaluate the risk associated with certain *CRP* SNPs to present with the phenotype of low 25(OH)D combined with elevated CRP concentrations, the case and control phenotypes were entered as dependent variables.

Where applicable, p-values were adjusted using the methods suggested by Bonferroni. Significance was defined as an α level of 0.05.

3. Results

3.1. Association of 25(OH)D Concentrations/Status with Circulating CRP Concentrations

Median concentrations for 25(OH)D and CRP were 68.2 nmol/L and 4.13 mg/L, respectively, indicating that the 25(OH)D status of the women in our cohort was insufficient, while they also presented with elevated inflammation based on CRP. CRP concentrations decreased across increasing 25(OH)D categories, with the median CRP concentration being significantly lower in the sufficient 25(OH)D group compared to both the deficient and insufficient subdivisions (Figure 1). The largest variability in CRP concentrations was observed for those in the 25(OH)D-deficient category, with decreasing variability in the insufficient and sufficient groups. In the population investigated, 42% (n = 216) of individuals presented with both 25(OH)D concentrations lower than 75 nmol/L and elevated CRP concentrations above 3 mg/L (case phenotype).

In Table 1, we have summarized the demographic characteristics of the case and control phenotypes: 25(OH)D concentrations decreased with age. The distribution between rural and urban cases and controls was similar. In addition, the environmental exposure that could have influenced vitamin D status did not differ between the rural or urban areas. Individuals representing the case phenotype were significantly older. Similar distributions were also observed in respect of smoking and HIV status. The median dietary intake of vitamin D sources did not differ for the two groups either.

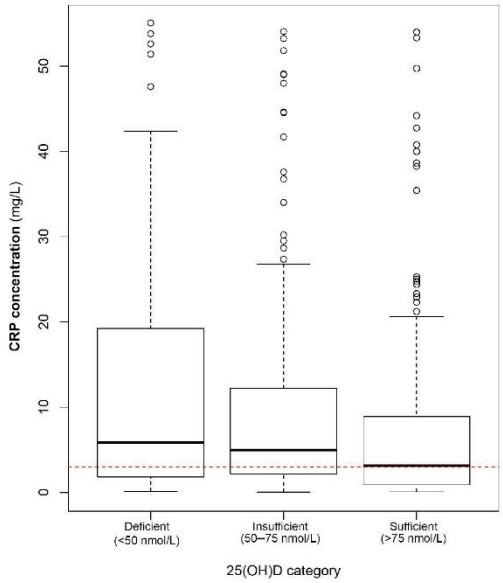

Figure 1. Median C-reactive protein (CRP) concentrations across different categories of 25(OH)D status ($p = 0.001$). Pairwise Wilcoxon ranked-sum test with Bonferroni adjustment revealed that women with sufficient 25(OH)D concentrations had significantly lower CRP concentrations than those with deficient or insufficient 25(OH)D. Outliers depicted as open circles. The red dashed line indicates the cut-off value for CRP concentrations with elevated CRP being greater than 3 mg/L.

Table 1. Comparisons of demographical and biochemical factors in the cohort stratified by control and case phenotypes.

Variable	Controls ($n = 289$, 57.2%)	Cases ($n = 216$, 42.8%)	p-Value
Urban/Rural	144 (49.8%)/145 (50.2%)	126 (58.3%)/90 (41.7%)	*NS*
Age (years)	53.0 (49.0; 59.0)	56.0 (51.0; 63.0)	<0.001
Smoking status: Former/Current/Abstainer	6 (2.10%)/138 (48.3%)/142 (49.7%)	7 (3.24%)/96 (44.4%)/113 (52.3%)	*NS*
HIV positive/negative	26 (9.03%)/262 (91.0%)	15 (6.98%)/200 (93.0%)	*NS*
Vitamin D intake (µg/day)	2.00 (1.02; 3.30)	2.05 (1.02; 3.66)	*NS*
Menorhea/Amenorhea	64 (23.0%)/214 (77.0%)	37 (17.2%)/178 (82.8%)	*NS*

Data presented as median (25th and 75th percentiles) for continuous data and number of observations (percentage) for categorical data. Abbreviations: 25(OH)D, 25-hydroxyvitamin D; HIV, human immune deficiency virus; *NS*, not significant ($p > 0.05$).

Differences in CVD risk markers between the two phenotypes are presented in Table 2. Those representing the control phenotype had lower blood pressure. However, heart rate was similar in the two groups. Anthropometric markers indicated that cases had significantly higher body weight and increased waist circumference. More obese individuals were also observed in the case phenotype. HDL-c was significantly higher in the control group; however, after adjusting for age, waist circumference, and LDL-c, the significance was eliminated. Furthermore, no statistical differences were observed for LDL-c, triglycerides, or energy intake, although energy intake was lower for the controls than for the case phenotypes.

Table 2. Markers of cardiovascular disease (CVD) risk among the control and case phenotypes.

Variable		Controls (n = 289, 57.2%)	Cases (n = 216, 42.8%)	p-Value	Adjusted p-Value
Systolic blood pressure (mmHg)		133 (118; 148)	138 (124; 159)	<0.001	<0.01
Diastolic blood pressure (mmHg)		87.0 (78.0; 96.0)	91.0 (83.8; 101)	<0.001	<0.01
Heart rate (BPM)		73.0 (65.0; 83.0)	72.0 (63.0; 85.0)	*NS*	*NS*
Waist circumference (cm)		79.3 (70.8; 87.7)	86.4 (74.8; 95.5)	<0.001	<0.001
Hip circumference (cm)		98.0 (89.5; 106)	106 (94.1; 119)	<0.001	0.01
WHR		0.81 (0.76–0.87)	0.81 (0.76–0.86)	*NS*	*NS*
Weight (kg)		60.2 (52.1; 72.8)	72.0 (56.5; 85.5)	<0.001	<0.01
BMI (kg/m^2)		24.7 (21.3; 29.0)	29.9 (23.3; 35.2)	<0.001	<0.001
BMI category	*Underweight*	29 (10.0%)	22 (10.2%)	<0.001	<0.001
	Healthy	122 (42.2%)	45 (20.8%)		
	Overweight	80 (27.7%)	42 (19.4%)		
	Obese	58 (20.1%)	107 (49.5%)		
Total cholesterol (mmol/L)		5.21 (4.38; 6.33)	5.30 (4.52; 6.20)	*NS*	*NS*
High-density lipoprotein cholesterol (mmol/L)		1.48 (1.14; 1.92)	1.36 (1.07; 1.76)	0.03	*NS*
Low-density lipoprotein cholesterol (mmol/L)		3.34 (2.64; 4.23)	3.58 (2.69; 4.41)	*NS*	*NS*
Triglycerides (mmol/L)		1.22 (0.90; 1.79)	1.32 (0.92; 1.78)	*NS*	*NS*
Dietary intake (kJ)		6620 (5056; 9265)	7432 (5294; 9283)	*NS*	*NS*

Adjusted for age, waist circumference, and LDL-c. Abbreviations: BMI, body mass index; BPM, beats per minute; CRP, C-reactive protein; mmHg, millimeters of mercury; WHR, waist hip ratio. Data presented as median (25th and 75th percentiles) for continuous data and number of observations (percentage) for categorical data. BMI categories' cut-off values: Underweight <18.5 kg/m^2; healthy 18.5–24.9 kg/m^2; overweight 24.9–29.9 kg/m^2; obese >29.9 kg/m^2. Values presented in accordance with the International System of Units: to convert kJ to Cal multiply by 0.24.

3.2. Quantification of the Associations of 25(OH)D with CRP Concentrations

Spearman correlation analyses (results not shown) revealed that 25(OH)D was inversely, albeit weakly ($\varrho > -0.20$; $p \leq 0.05$), associated with age. No other factors were associated with 25(OH)D with a correlation greater than 0.20, so that these are not reported here except for the correlation with CRP presented later. CRP was moderately associated with anthropometric markers (all $\varrho > 0.30$; $p < 0.05$) and lipid profile markers of which LDL-c ($\varrho = 0.13$; $p < 0.05$) presented with the strongest correlation. Similar to vitamin D status, other variables did not correlate strongly with CRP even though these correlations were statistically significant.

A weak, yet significant, negative correlation was observed between 25(OH)D and CRP ($\varrho = -0.15$; $p < 0.05$). Converting 25(OH)D from nmol/L to mg/L, using a conversion factor of 0.0004 (Equation (1)), indicated that a one-unit increase in 25(OH)D was associated with a 0.15 mg/L decrease in CRP concentration. Vitamin D intake did not correlate with 25(OH)D or CRP concentrations.

$$\begin{gathered} 1\ \text{nmol/L}\ 25(\text{OH})\text{D} = 0.4\ \text{ng/mL}\ 25(\text{OH})\text{D} = 0.0004\ \text{mg/L}\ 25(\text{OH})\text{D} \\ 0.0004\ \text{mg/L}\ 25(\text{OH})\text{D} \equiv -0.15\ (\text{CRP})\text{mgL} \end{gathered} \quad (1)$$

The linear relationship between 25(OH)D as a factor influencing lnCRP concentrations was modelled in two ways: for the first model, we adjusted for age; and in the second, we adjusted for age, anthropometrical marker (see discussion below), and LDL-c. These covariates were chosen owing to the likelihood of these variables influencing the model based on their previous association with CRP concentrations, as well as having the lowest AIC score. As there is a large degree of co-linearity between anthropometric markers, each marker (i.e., BMI, waist and hip circumference, and weight) was entered into the model separately, and models were evaluated based on their resulting AIC value. The lowest AIC value was observed for waist circumference and LDL-c; therefore, these markers were used as a proxy for all other anthropometric and lipid profile markers, respectively. Dietary sources of vitamin D did not affect the model ($p > 0.05$). The unadjusted model (Model 1) accounted for 2.1% of the variance of lnCRP (calculated from adjusted R^2 values; $p = 0.001$). For Model 1, a 1.1% reduction in CRP concentration (converting lnCRP to CRP by using $(e^{\beta} - 1) \times 100)$) for each 1 nmol/L increase in 25(OH)D was observed. In Model 2, when adjusting for age, waist circumference, and LDL-c, 1.8% of the lnCRP variation could be explained ($p < 0.00001$). The inverse relationship between vitamin D status and CRP was slightly intensified when controlling for the covariates. Here, for each 1 nmol/L increase in 25(OH)D, CRP decreased by 1.1%. Excluding individuals with CRP concentrations above

10 mg/L—the clinical cut-off point for acute inflammation—resulted in similar trends being observed (0.71% and 0.82% reduction per 1 nmol/L increase of 25(OH)D for unadjusted and adjusted models, respectively; results not shown).

3.3. SNP Interaction

All genotyped SNP frequencies reflected the assumptions of what would be expected under Hardy–Weinberg equilibrium. Previously, in our population, LD was reported between rs2027471 with rs1341665 and rs3093058 with rs3093062 [17]. In the subset of women studied here, the same LD pattern (Figure 2) was observed. Here, linkage was also detected for a haplogroup linking rs7553007, rs1341665, rs2027471, rs1205, and rs2794520 (Figure 2).

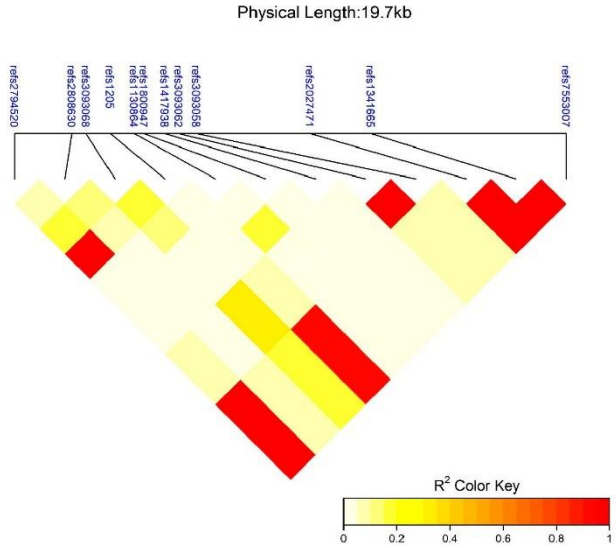

Figure 2. Linkage disequilibrium heatmap indicating linkage between 12 single nucleotide polymorphisms (SNPs) on the *CRP* gene.

Using the association analyses provided by the SNPassoc package in R, trends were investigated based on models of interaction between 25(OH)D concentrations with *CRP* SNPs on lnCRP concentrations. Associations were investigated under co-dominant, dominant, additive, and recessive genetic models, and the genetic model with the lowest AIC values was included (Supplementary Table S1). The minor alleles of the five SNPs (i.e., rs7553007, rs1341665, rs2027471, rs1205, and rs2794520) in high linkage were previously reported to be associated with decreases in circulating CRP [17], which was echoed by our results. Three SNPs were also significantly associated with increased CRP concentrations: rs3093058, rs3093062, and rs3093068.

To quantify whether 25(OH)D concentrations interacted with any of the 12 *CRP* SNPs to affect lnCRP concentrations, factorial analyses of co-variance with age, LDL-c, and waist circumference as covariates were performed. In the 12 SNPs investigated, 96.0% of the genetic variation could be grouped into six haplotypes. No interactions were observed between 25(OH)D concentrations and the identified haplotypes to affect lnCRP concentrations (p for trend = 0.68). No significant associations between *CRP* SNPs and 25(OH)D were found under either the co-dominant or dominant model.

To investigate whether a genotype was associated with an increased risk of presenting with either deficient or insufficient 25(OH)D and elevated CRP concentrations, odds ratios (OR) were calculated

using the SNPassoc library in R for each of the 12 SNPs while adjusting for age, LDL-c, and waist circumference (Table 3). The minor alleles of SNPs previously associated with significant increases in CRP concentrations were found to be at higher odds of co-presenting with insufficient/deficient 25(OH)D concentrations and vice versa. The minor alleles of the five SNPs in LD were associated with a reduced risk of presenting with the phenotype of inadequate 25(OH)D combined with elevated CRP concentrations (cases) compared to the phenotype presenting with either sufficient 25(OH)D or normal (<3 mg/L) CRP concentrations. Of these, rs3093068, rs3093062, and rs3093058 presented with increased odds (1.54, 1.64, and 1.67, respectively), while reduced odds were observed in rs2794520 and rs7553007 (0.65 and 0.67, respectively) for individuals harboring the minor alleles. A trend towards significance for ORs for carriage of the minor alleles at two *CRP* SNPs (rs2027471 and rs1341665) to present with the co-phenotype was observed (OR: 0.05; $p > 0.05$).

Table 3. Genetic predisposition to develop insufficient/deficient 25(OH)D combined with elevated CRP concentrations adjusting for age, low-density lipoprotein cholesterol (LDL-c), and waist circumference.

SNP ID	Allele	Control Phenotypes	%	Case Phenotypes	%	Odds Ratio (95% CI)	*p*-Value
rs2794520	C/C C/T–T/T	166 123	57.4 42.6	141 70	66.8 33.2	0.65 (0.44–0.95)	0.03
rs2808630	T/T C/T–C/C	198 91	68.5 31.5	154 61	71.6 28.4	0.79 (0.53–1.18)	*NS*
rs3093068	C/C C/G–G/G	123 164	42.9 57.1	72 143	33.5 66.5	1.54 (1.05–2.26)	0.03
rs1205	C/C C/T–T/T	171 118	59.2 40.8	142 73	66 34	0.72 (0.49–1.05)	*NS*
rs1130864	C/C C/T–T/T	216 73	74.7 25.3	170 45	79.1 20.9	0.86 (0.56–1.34)	*NS*
rs1800947	C/C C/G	289 0	100 0	213 2	99.1 0.9	0	*NS*
rs1417943	A/A A/T	277 12	95.8 4.2	203 10	95.3 4.7	1.7 (0.70–4.13)	*NS*
rs3093062	G/G A/G–A/A	214 74	74.3 25.7	137 78	63.7 36.3	1.64 (1.10–2.45)	0.02
rs3093058	A/A A/T–T/T	215 74	74.4 25.6	135 78	63.4 36.6	1.67 (1.12–2.50)	0.01
rs2027471	T/T A/T–A/A	167 122	57.8 42.2	142 73	66 34	0.68 (0.46–1.00)	0.05
rs1341665	G/G A/G–A/A	167 122	57.8 42.2	142 73	66 34	0.68 (0.46–1.00)	0.05
rs7553007	G/G A/G–A/A	164 125	56.7 43.3	141 74	65.6 34.4	0.67 (0.46–0.98)	0.04

Cases are those presenting with the phenotype of deficient or insufficient 25(OH)D together with elevated (>3 mg/L) CRP concentrations. Controls were individuals with normal CRP and/or sufficient 25(OH)D concentrations. The reference group comprised those homozygotes for the wild-type allele. Abbreviations: 25(OH)D, 25-hydroxyvitamin D; A, adenine; C, cytosine; CI, confidence interval; G, guanine; rs, reference SNP cluster ID; T, thymine.

4. Discussion

In this research, we confirmed the existence of an inverse relationship between CRP and vitamin D and attempted to unravel the mechanisms involved. From this study, we know that vitamin D status does not directly modulate CRP via *CRP* SNPs nor do *CRP* SNPs influence vitamin D, even though these SNPs influence CRP concentrations (data presented elsewhere). Since we found that the double phenotype of high CRP and low vitamin D was associated with particular *CRP* SNPs, we hypothesized that negative feedback mechanisms were at play (will be described later). In cases where these genotypes were co-observed with low 25(OH)D concentrations, poorer CVD markers were also observed, such as elevated blood pressure.

The majority (65.5%) of the South African women in our cohort had 25(OH)D concentrations lower than the recommended 75 nmol/L. Moreover, 42.8% of them presented with the co-burden of low vitamin D status combined with elevated CRP concentrations. Having deficient or insufficient 25(OH)D and elevated CRP concentrations was previously shown to increase the risk of developing

various chronic non-communicable conditions, such as CVD [5,31,34]. In our study, volunteers classified as cases (those presenting with both low vitamin D status and high CRP concentrations) had significantly higher blood pressure and anthropometrical markers (even when adjusting for waist circumference), precursors in the etiology of CVD, than controls. We established that vitamin D correlated inversely with CRP, which is in accordance with a previous report [13]. Moreover, we found that CRP decreased within increasing strata of 25(OH)D categories as recommended by the Endocrine Society [47] and confirmed this inverse association with our regression models. Furthermore, we proposed that 25(OH)D might interact with SNPs located on the *CRP* gene, thereby influencing CRP concentrations. Contrary to our hypothesis, none of the *CRP* SNPs investigated showed any interactions with circulating 25(OH)D; thus, vitamin D does not modulate *CRP* genotypes to influence CRP concentrations. The inverse association between vitamin D and CRP is therefore not due to nutrigenetic effects. A recent Mendelian association study also found a lack of association between 25(OH)D and genetic markers influencing CRP concentrations [13] even though they detected a negative correlation between 25(OH)D and CRP concentrations.

We also investigated whether *CRP* SNPs influenced 25(OH)D concentrations directly to learn whether a possible unknown backward feedback mechanism might exist or whether certain *CRP* SNPs predispose individuals to having heightened inflammation, with concurring maladies, leading to reduced UV exposure resulting in altered vitamin D status, but found no associations. We then determined whether harboring CRP genotypes could increase the odds of co-presenting with both high CRP and low vitamin D status. Carriage of the minor allele at three SNPs (rs3093068, rs3093058, and rs3093062) was associated with increased odds, while harboring the variant allele at two SNPs (rs2794520 and rs7553007) resulted in lower odds to present with the phenotype of insufficient/deficient 25(OH)D (<75 nmol/L) and elevated CRP concentrations (>3 mg/L). This is, to our knowledge, a novel addition to the existing literature, as these genetic effectors were not previously reported in this context.

How blood pressure is influenced by vitamin D status remains inconclusive [48], although there are suggestions of causative pathways [49]. Li [50] hypothesized that vitamin D could have an influence on the renin–angiotensin system (RAS), which was substantiated by a study that reported how low concentrations of 25(OH)D upregulated the RAS [51]. In our population, 25(OH)D was reported to be associated with carotid wall thickening and arterial stiffness [52], both being attributes observed in individuals with increased CRP concentrations. This is a possible mechanism whereby low 25(OH)D could result in increased arterial stiffness, and in turn result in increased blood pressure [53], leading to elevated CRP concentration by negative feedback mechanisms. Individuals with the case phenotype were also investigated in another population, where cases had worse pro-inflammatory marker panels than controls [54]. However, with increasing CRP concentrations, it was reported that the anti-inflammatory effects of vitamin D decreased substantially and most of the other pro-inflammatory markers were upregulated [54]. These risk factors are further exacerbated by the presence of abdominal adiposity observed in our population [55], thereby further increasing the risk of developing CVD by means of increased pro-inflammatory factors (such as IL-6) released by adipose tissue. Another possibility is that low vitamin D status has harmful side effects that are pro-inflammatory themselves; alternatively, synergistic effects between these two factors may exist, explaining the co-existence of low vitamin D with high CRP, as was the case in Kuwaiti women [54]. Low concentrations of 25(OH)D have been linked to an increased risk of developing CVD [56], although definite conclusions remain ambiguous [57]. Similarly, CRP has been strongly associated with increased CVD risk [6]. Future studies should explore possible mechanisms for the inverse association between 25(OH)D and CRP further, as well as whether the presence of both low vitamin D status and inflammation might heighten disease risk.

Differences in 25(OH)D were observed among individuals residing in the two different locations (A and B) that were investigated here (Supplementary Table S2). Although 25(OH)D and CRP values were closer to the recommended concentrations in rural participants, neither median 25(OH)D nor CRP concentrations met the recommended guidelines in both population subdivisions. Rural-urban differences in 25(OH)D disappeared when adjustments for age were made. Although age was

reported as a non-significant contributor to our linear models, it was included in analyses as a possible covariate based on four previously reported reasons. First, aging results in decreased concentrations of 7-dehydrocholesterol in the epidermis, which in turn reduces the response to UV light and subsequently results in decreased formation of pre-vitamin D_3 [58,59]. Second, a decline in absorption, transport, or liver hydroxylation of orally ingested vitamin D sources was reported in older individuals [60]. Increased frailty with advancing age may also result in individuals spending less time outdoors, affecting their exposure to UV sources. Lastly, age, sex, and ethnicity are recommended factors to adjust for when conducting predictive analyses for CRP [12], with sex and ethnicity controlled for in our black, female population. It could, however, be argued that environmental and climate differences, such as reduced UVR, could have affected 25(OH)D concentrations between individuals located in the two different locations of our study. These places, A and B, whence we drew our samples, were on similar latitudes; when measured using the equator as a reference the difference between them was 14 km. In terms of elevation above sea level, they differed by less than 80 m. The likely differences in UV radiation were therefore small. Data pertaining to environmental factors influencing vitamin D synthesis in individuals were not available from ground observations in these localities and, therefore, satellite observations were used. Similar mean average temperatures near the earth's surface, UV indices, erythemal dose rates, and total ozone columns were observed between the two areas from which we recruited volunteers. Another factor that could have affected 25(OH)D concentrations in rural participants is differences in lifestyle, as observed in Asian [61], other African [62], and European [63] populations. As no data were available pertaining to sun exposure in our study, no inference about differences in lifestyle was possible. Low 25(OH)D concentrations have also been linked to an increased risk of obesity [31]; however, for the population investigated similar anthropometric markers were observed across differing 25(OH)D status. The lack of association between BMI and 25(OH)D concentrations was reported in another South African study, where recruitment was done in a province that neighbors the one from which we selected our volunteers [64]. This may indicate that the correlation between anthropometric markers and 25(OH)D status does not apply to black individuals living in South Africa, which necessitates further investigation.

Nutritional intake of vitamin D in our population did not differ between the three 25(OH)D categories and was well below the recommended 15 μg/day [27]. Dietary vitamin D also failed to influence the linear regression modeling of lnCRP concentrations, which might be due to the extremely low dietary intake observed. A low intake of dietary vitamin D is common in African populations, with only margarine being fortified with vitamin D in South Africa [27]. Dietary sources of vitamin D did not significantly contribute to vitamin D status [65], as ingested vitamin D is more readily excreted [66]. This result aligns with the fact that the primary factor contributing to 25(OH)D concentration is exposure to UV light [24], resulting in vitamin D_3 (cholecalciferol or calcidiol) synthesis from 7-dehydrocholesterol (a precursor of cholesterol); after hydroxylation by the liver and kidneys, vitamin D_3 becomes 25(OH)D and then 1,25-dihydroxyvitamin D. That said, a study controlling caloric intake of obese women showed that replenishment of 25(OH)D by supplementation with vitamin D at 2000 IU per day led to participants in that group losing more weight, having smaller waist circumferences, and a 46% higher reduction in CRP concentrations compared to those in a placebo group [67]. Because a placebo group was included in this other study, the reduction in CRP was attributed to 25(OH)D values stabilizing at sufficient levels [67], not simply a reduction of body composition markers. We attributed the elevated CRP concentrations observed in our urban population to increased abdominal adiposity, as urban individuals had increased waist circumferences. In their review, Brooks et al. [68] reported that the association between inflammation, as measured by CRP, and abdominal adiposity is highly correlated, even when correcting for BMI. Women with increased waist circumferences were previously reported to be at greater risk of co-presenting with elevated CRP concentrations [69], with our results indicating that waist circumference was also the largest effector contributing to CRP concentrations. Reductions in abdominal adiposity in response to dietary interventions were previously reported to reduce CRP concentrations [70]. These dietary interventions included supplementation with fish oil tablets, and although not stated in the original work,

fish oil naturally contains bioavailable vitamin D, which could have contributed to the reduction in CRP concentrations [71]. Here, we report a predicted 0.15 mg/L reduction in CRP concentration with a 1 nmol/L increase in 25(OH)D concentrations as determined from Spearman correlation. Recommendations intended to achieve sufficient 25(OH)D status should, therefore, aim to include responsible guidelines for exposure to sunlight, as well as an increase in the intake of good dietary sources of vitamin D to improve health in a country such as South Africa, which has a history of health policies failing its citizens.

At the time of our study, South Africa was rife with HIV denialism by government and stigmatization of seropositive individuals, which resulted in a large segment of the population not knowing their HIV status. Overall, 8.1% of our study population was first diagnosed as being HIV-positive during our investigation. Median values, when excluding HIV-positive individuals, were similar, in terms of both 25(OH)D and CRP concentrations, to those of the whole group. Both 25(OH)D [72] and CRP [73] concentrations can be affected by the use of anti-retroviral (ARV) treatments; however, as these individuals were first diagnosed during this study, they were not receiving ARVs and were therefore not excluded.

Our study was not without limitations. Because gender is a factor that contributes to both 25(OH)D and CRP concentrations, including men in our sample would have made the study more informative. Future studies should aim to explore the relationship between vitamin D and inflammation in men as well as women. Furthermore, no data were available on sun exposure time, which could have contributed to explaining the variance in 25(OH)D concentrations. However, measuring 25(OH)D concentrations is a strength of our study as it avoids the necessity for UVR, sun exposure time, and even dietary vitamin D intake data. The cohort was also randomly selected, without prior genetic screening. Future studies, including more extensive numbers of minor allele carriers, could establish associations between SNPs where our population had too few genotypes, resulting in lowered statistical power. Because 1,25(OH)2D is epigenetically active [74], future investigations to explore a possible mechanism to explain the anti-inflammatory effects of vitamin D could also incorporate epigenetics.

Our study cohort was very well-described, with data available on daily vitamin D intake, and was particularly well-characterized in terms of demographic, genetic, and biochemical factors that could address the variance in circulating CRP concentrations. The unique population investigated here was ideal, as individuals presented with both low 25(OH)D and elevated CRP concentrations. Including both CRP concentrations, known to fluctuate quite extensively, and genetic constants with known effects on CRP phenotype further strengthens the data presented here.

5. Conclusions

Disturbingly, 43% of our female cohort presented with both elevated CRP concentrations and deficient/insufficient 25(OH)D levels; because the combination is linked to CVD, this may compound their disease risk and predispose them to future disease. A negative association was identified between 25(OH)D and circulating CRP concentrations, but no vitamin D–gene interactions were observed between common SNPs on the *CRP* gene and 25(OH)D in this study. Moreover, *CRP* SNPs did not influence vitamin D status. Several genotypes were, however, associated with an altered risk of presenting with the co-phenotype of insufficient/deficient 25(OH)D and elevated CRP, indicating that a genetic predisposition exists. The present research extends past work by demonstrating that the link between 25(OH)D and CRP is not associated with vitamin D–*CRP* gene interactions and that other pathways need to be investigated. This finding is important, as it offers a starting point for unraveling the possible mechanisms for the previously reported inverse relationship between 25(OH)D and CRP, which in turn may ultimately result in therapeutic and policy recommendations to combat CVD.

This paper highlights the necessity of public health efforts in South Africa to assist women to achieve sufficient vitamin D status through responsible exposure to sunlight, increased intake of natural and fortified dietary sources of vitamin D, and for those who require it, vitamin D supplementation. Improved vitamin D status would reduce inflammation and thus possibly curb diseases contingent on both low vitamin D and elevated CRP: the co-phenotype described here.

Supplementary Materials: The following are available online at www.mdpi.com/1660-4601/15/1/111/s1. Table S1. Environment-SNP associations with CRP concentrations based on different genetic models of inheritance. Table S2. Demographic markers associated with differing 25(OH)D status.

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CRP Genotypes Predict Increased Risk to Co-Present with Low Vitamin D and Elevated CRP in a Group of Healthy Black South African Women

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Supplementary Material

Supplementary Table 1. Environment-SNP associations with CRP concentrations based on different genetic models of inheritance.

SNP ID	Allele	Genetic model	n	Mean adjusted CRP (mg/L)	Standard error	Association with CRP	*p*-value	AIC
rs2794520	C/C	Co-dominant	307	4.48	1.08	Lowered CRP	0.03	1665
	C/T		162	3.25	1.12			
	T/T		31	2.94	1.25			
	C/C	Dominant	307	4.48	1.08		0.01	1663
	C/T–T/T		193	3.2	1.11			
rs2808630	T/T	Dominant	352	4.02	1.07		0.62	
	C/T–C/C		152	3.82	1.13			
rs3093068	C/C	Co-dominant	195	3.34	1.11	Increased CRP	0.04	1670
	C/G		243	4.31	1.09			
	G/G		64	5.04	1.19			
	C/C	Dominant	195	3.34	1.11		0.02	1668
	C/G–G/G		307	4.45	1.08			
rs1205	C/C	Dominant	313	4.43	1.08	Lowered CRP	0.02	1676
	C/T–T/T		191	3.22	1.11			
	C/C–T/T	Over-dominant	343	4.26	1.08		0.04	1677
	C/T		161	3.38	1.12			
rs1130864	C/C	Dominant	386	4.22	1.07		0.25	
	C/T–T/T		118	3.21	1.13			
rs1800947	C/C	Co-dominant	502	3.95	1.06		0.68	
	C/G		2	8.03	1.62			
rs1417938	A/A	Co-dominant	480	4.01	1.07		0.94	
	A/T		22	2.97	1.4			
rs3093062	G/G	Dominant	351	3.43	1.08	Increased CRP	0	
	A/G–A/A		152	5.64	1.11			
rs3093058	A/A	Dominant	350	3.38	1.08	Increased CRP	0	
	A/T–T/T		152	5.64	1.11			
rs2027471	T/T	Dominant	309	4.49	1.08	Lowered CRP	0.01	
	A/T–A/A		195	3.24	1.11			
rs1341665	G/G	Dominant	309	4.49	1.08	Lowered CRP	0.01	
	A/G–A/A		195	3.24	1.11			
rs7553007	G/G	Dominant	305	4.53	1.08	Lowered CRP	0.01	
	A/G–A/A		199	3.22	1.11			

Abbreviation: A, adenine; AIC, Akaike Information Criterion; CRP, C-reactive protein; C, cytosine; G, guanine; SNP, single nucleotide polymorphism; rs, reference SNP cluster ID; T, thymine.

Although rural (Location B) and urban (Location A) participants had similar distributions stratified to 25(OH)D status, rural dwellers had higher median concentrations of 25(OH)D (69.4 nmol/L compared to 66.6 nmol/L, $p < 0.05$) and lower median CRP concentrations (3.58 mg/L vs 4.78 mg/L, $p < 0.05$) compared to their urban counterparts. Rural women were, however, significantly younger (median age 53 compared to 56 years), with significantly lower waist circumferences (81 cm compared to 83.3 cm). Correcting for the effects of age on 25(OH)D concentration, and waist circumference on lnCRP concentration, resulted in the rural–urban differences falling away. All HIV-positive individuals (n = 41) were first diagnosed during this study, and both 25(OH)D and CRP concentrations were similar for the HIV-positive and -negative women (whole group medians excluding HIV-positive individuals 25(OH)D: 68.2 nmol/L; CRP: 4.27 mg/L; $p > 0.05$ observed for both 25(OH)D and CRP concentrations when comparing the whole cohort and the cohort excluding HIV-positive individuals). HIV-positive subjects were, therefore, not excluded from further analyses. Smokers had significantly lower 25(OH)D concentrations. However, smoking was found not to be a significant predictor of 25(OH)D concentrations when modeled via linear regression analysis. Vitamin D intake from nutritional sources was low across all three categories of 25(OH)D status, with none of the participants ingesting the recommended 15 μg/day.

Supplementary Table 2. Demographic markers associated with differing 25(OH)D status.

Variable	Deficient <50 nmol/L (n = 81; 16.0%)	Insufficient 50—75 nmol/L (n = 250; 49.5%)	Sufficient >75 nmol/L (n = 174; 34.5%)	*p*-Value
Urban/Rural	51 (63.0%)/30 (37.0%)	132 (52.8%)/118 (47.2%)	87 (50.0%)/87 (50.0%)	*NS*
Age	57.0 [50.0;61.0][a]	55.0 [50.0;62.0][a]	52.5 [49.0;58.0][b]	0.001
Smoking status: Former/Current/Abstainer	2 (2.47%)/53 (65.4%)/26 (32.1%)	8 (3.21%)/103 (41.4%)/138 (55.4%)	3 (1.74%)/78 (45.3%)/91 (52.9%)	0.003
HIV-positive/negative	5 (6.25%)/75 (93.8%)	19 (7.63%)/230 (92.4%)	17 (9.77%)/157 (90.2%)	*NS*
Vitamin D intake (μg/day)	2.12 [1.17;3.49]	1.98 [0.98;3.57]	2.03 [0.99;3.35]	*NS*

Data presented as median [25th and 75th percentiles] for continuous data and number of observations (percentage) for categorical data. Abbreviations: 25(OH)D, 25-hydroxyvitamin D; HIV, human immune deficiency virus; NS, not significant ($p > 0.05$).

ANNEXURE 3: CO-AUTHORED PUBLICATIONS

During the author's tenure as a Ph.D. student, he was involved in two other publications outside the scope of this thesis. These works involved the transitional effects of the population included in the previously presented works, as well as an investigation regarding the implementation of sodium regulation in foodstuffs of South Africa.

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Article

Socio-Demographic and Lifestyle Factors Predict 5-Year Changes in Adiposity among a Group of Black South African Adults

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Abstract: The rising prevalence of obesity and excessive adiposity are global public health concerns. Understanding determinants of changes in adiposity over time is critical for informing effective evidence-based prevention or treatment. However, limited information is available to achieve this objective. Cultural, demographic, environmental, and behavioral factors including socio-economic status (SES) likely account for obesity development. To this end, we related these variables to anthropometric measures in 1058 black adult Tswana-speaking South Africans who were HIV negative in a prospective study over five years. Body mass index (BMI) and waist circumference increased in both sexes, whereas triceps skinfold thickness remained the same. Over the five years, women moved to higher BMI categories and more were diagnosed with central obesity. Age correlated negatively, whereas SES, physical activity, energy, and fat intake correlated positively with adiposity markers in women. In men, SES, marital status, physical activity, and being urban predicted increases in adiposity. For women, SES and urbanicity increased, whereas menopause and smoking decreased adiposity. Among men, smokers had less change in BMI than those that never smoked over five years. Our findings suggest that interventions, focusing on the urban living, the married and those with the highest SES—the high-risk groups identified herein—are of primary importance to contain morbidity and premature mortality due to obesity in black South Africans.

Keywords: central obesity; marital status; marital transition; obesity; socio-demographic; socio-economic status; sub-Saharan Africa; urbanization

1. Introduction

Obesity and excessive adiposity are global public health issues, and their prevalence seems to be ever increasing. This pandemic is associated with a myriad of co-morbidities—such as cardiovascular disease, type 2 diabetes mellitus as well as various cancers—and mortality [1]. The prevalence of obesity in South Africa is the highest in sub-Saharan Africa [2–4]. The 2012 South African Demographic and Health Survey (SADHS) reported an obesity (body mass index (BMI) > 30 kg/m^2) prevalence of 11% and 41%, respectively, for both men and women over the age of 15, with the greatest contribution in the urban areas [4].

The etiology of obesity, and of excessive adiposity, is multifaceted and complex. Unraveling and understanding factors affecting this scourge are critical to halt and, ideally, reverse the problem. An appreciation of factors other than the biomedical—such as socio-cultural, demographic, environmental, and behavioral, including socio-economic status (SES), i.e., an individual's position on a socio-economic scale measured through indicators such as education, income, occupation, and place of residence—are needed [2,5–7].

In developed countries, SES is inversely related to obesity [8,9], whereas in South Africa and other poor regions, the rich (those with the highest SES) are more likely to be overweight or obese than their less well-off counterparts elsewhere [4,6,7]. Having a low SES in low- and middle-income countries (LMIC) is associated with long-term weight gain, predicts BMI, and may contribute to an unfavorable body fat distribution [6,7]. Obesity in poor countries may be explained by overconsumption of energy-dense processed foods, which are relatively cheap and readily accessible [10–12]. Cultural aspects related to body image may also play a role. In many black African communities, being overweight or obese is regarded as a sign of good health, of beauty and affluence, whereas thinness is stigmatized due to its association with HIV/AIDS [2,13]. Moreover, lifestyle factors play a role in the degree of adiposity. For instance, sufficient physical activity is associated with a reduction in body fat among obese individuals, whereas increased levels of sedentarism are associated with obesity [14]. In addition, smoking has been associated with markers of non-communicable diseases (NCDs) including central fat accumulation [15], and in other studies with diminished measures of adiposity [7,16]. Quitting smoking seems to add to adiposity [17]. Previous studies reported conflicting results regarding the association of urbanicity versus rural residency on adiposity [18,19]. In high-income countries, the so-called rich nations, rural dwellers are reported to have higher adiposity, whereas the opposite is the case in LMICs, where urbanized individuals tend to be obese [18,19]. In South Africa, rapid urbanization is leading to the consumption of more westernized diets (comprising mainly energy-dense processed foods containing high amounts of fats [20]), eating meals away from home and insufficient physical activity levels [12,21] and, in turn, increases in obesity [4] that are mirrored by the greater prevalence of obesity-related NCDs [22].

Moreover, marital status circumscribes the social environment. In particular, being married seems to influence physical activity levels, food intake, and smoking habits [23–26]. Several studies point to its influence and highlighted the impact of transitions in and out of marriage on body weight [23–26]. However, information on marital status and transitions on markers of adiposity is lacking for black South Africans.

Numerous studies have been conducted on the associations of socio-demographic factors including measures of wealth on adiposity in Africa [5,6,16,27]. To our knowledge, none has focused on the predictors of adiposity, other than BMI and/or waist circumference (WC) over time, or investigated marital status or transitions among black South African adults, thus making the study reported here unique and original. The aim of this study is to fill a gap in the literature and extend current knowledge by exploring socio-demographic and SES characteristics at baseline in relation to adiposity over a period of five years. Furthermore, smoking and marital status were considered, and transitions in status over time were related to changes in adiposity measures over five years.

Adiposity can be assessed by anthropometry in several ways including by height, weight, BMI, waist and/or hip circumference and skinfold thickness measurements [28]. BMI, expressed as weight

in relation to height, is often used as a proxy to evaluate levels of adiposity [29]. WC is a simple, yet sensitive, measure of central fat distribution and a good predictor of abdominal obesity [30]. Skinfold measurement is an inexpensive and accessible method of subcutaneous body fat assessment; in particular, the triceps site seems to be a valuable determinant of general obesity [31]. For our study, we used all the above measures to indicate adiposity. By not only determining BMI, we avoided the inherent limitation of BMI, which is that muscle mass can vary substantially between individuals of the same height and, therefore, has an imperfect association with body fat and is not always a true indicator of adiposity [32,33]. Additionally, the triceps skinfold is a marker of peripheral subcutaneous fat, whereas WC is abdominal, with both giving a more accurate reflection of total body adiposity, and both complement the BMI.

2. Materials and Methods

2.1. Study Design

This work is nested within the Prospective Urban and Rural Epidemiology (PURE) study, which is aimed at tracking the effects of lifestyle and changing environmental exposures on the development of NCDs [34]. The North West province arm of the PURE project in South Africa (PURE-SA) began with baseline data collected in 2005 and continued with follow-ups at five-year intervals. The current study reports on data collected during baseline and at the first follow-up. Owing to the large attrition rate, data from the third follow-up (in 2015) were not included. Recruitment procedures, study design, and methodology for PURE South Africa have been described in detail elsewhere [35]. Briefly, our sample included 2010 South African black adults older than 30 years and recruited from 6000 randomly selected households in two urban and two rural areas of the North West province. The urban communities were chosen from the established part of the township next to Potchefstroom, a major city in the North West province, and from the informal settlements that surround the township. The rural communities were identified in a remote area 450 km north-west of the city, in areas still under tribal law. Of the 2010 individuals participating at baseline, 722 were lost to follow-up, of whom 211 were deceased, as is shown in Figure 1.

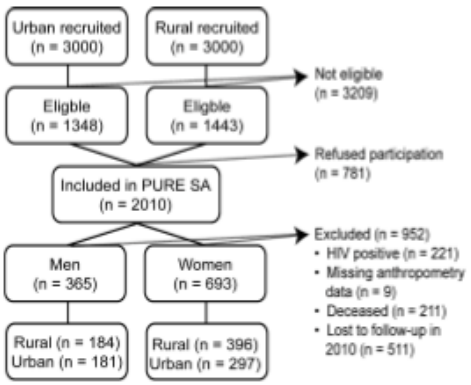


Figure 1. Consort diagram of the Prospective Urban and Rural Epidemiology (PURE) study reported herein.

We further excluded 221 HIV-positive participants and nine others with missing anthropometric data. Hence, a total sample of 1058 (365 men and 693 women) with complete data at baseline and at five-year follow-up were eligible for our study's data analyses. Those who remained in their respective areas of residence throughout the study period were included, whereas change in address to outside the study areas (rural to urban or vice versa) was regarded as loss to follow-up. The participants lost to follow-up were younger and had significantly lower BMI, but a similar mean WC to those who

were followed up [35]. We speculate that the participants lost to follow-up due to relocation could be a result of employment opportunities elsewhere for younger individuals with higher educational status. The study was approved by the Health Research Ethics Committee of North-West University (NWU), Potchefstroom campus (04M10 and NWU-00016-10-A1), South Africa. All participants provided written informed consent. The PURE-SA study conformed to the Declaration of Helsinki as revised in 2004.

2.2. Data Collection

2.2.1. Questionnaires

Structured questionnaires were used by all countries participating in the PURE study to collect socio-demographic (including marital status) and lifestyle information at baseline [34]. The questionnaires also aided in the determination of menopausal status by asking women questions pertaining to the regularity of menses. Questionnaires were administered by trained fieldworkers during home visits and outings to the Metabolic Unit at NWU in their language of choice. Total fat and energy intakes were estimated from validated culturally sensitive quantitative food frequency questionnaires [36]. The nutrient intakes were coded and analyzed using the South African Medical Research Council's food composition database [37]. A modified Baecke's physical activity questionnaire, reported to be reliable and valid when compared with 24-h activity recalls among South Africa adults [38], was used. The physical activity questionnaire consisted of 21 questions organized into three sections: physical activity at work, organised sport, and activity during leisure time [36]. Three levels of occupational physical activity, namely low, middle, and high, were defined in the questionnaire. Questions in each of the indices were scored on a five-point Likert scale, ranging from "1 = never" to "5 = very often". The sum of the four indices is the total physical activity score. A continuous physical activity score was calculated from the responses to the physical activity questionnaire. A score below 2.25 reflects the bottom tertile of physical activity and represents an occupation with no manual labor, commuting by public transport, no sports participation, and some light leisure-time activity, a score between 2.25 and 2.8 reflects a middle-level occupation, no organized sport, and some walking during leisure time, while the top tertile reflects a more active lifestyle with more leisure time activity and some sport participation [39].

2.2.2. Socio-Economic Status Index

A uni-dimensional measure of SES was constructed by adapting a previously described SES index [40]. This index provides a better picture of the complex issue of SES and allowed us to observe poor-rich differences in adiposity measures. Our SES was calculated as the sum of the graded categories—for the educational level attained by the participants, type of occupation, source of household water, access to electricity and type of roofing material—at baseline. The SES index criteria were scored as follows: no formal education, 0; 1–7 years of formal education, 1; 8–12 years, 2; and more than 12 years, 3. For employment, being unemployed scored 0; domestic/informal work, 1; skilled work, 2; and professional work, 3. Source of water scored 1; if water was fetched from a river or dam, a community tap per street block, 2; and piped water inside the house, 3. No access to electricity scored 0, and access to electricity, 1. Use of informal roofing materials scored 1; asbestos, 2; galvanized iron, 3; and tiles, slates, or reinforced concrete, 4. The highest possible score was 14, and arbitrary scores between 2–4, 5–9, and 10–14 were allocated to indicate a low, moderate, and high SES, correlating to the tertiles of scores.

2.2.3. Anthropometric Measurements

Anthropometric measurements were performed at baseline and follow-up according to standard methods of the International Society for the Advancement of Kinanthropometry. Height was measured to the nearest 0.1 cm with a stadiometer (Leicester height measure, Seca, Birmingham, UK) and weight

was recorded on a portable electronic scale (Precision Health Scale, A & D Company, Kitamoto-shi, Saitama, Japan) to the nearest 0.01 kg with participants in light underwear and shoes removed. WC was measured at the narrowest point between the lower rib border and the iliac crest, and recorded to the nearest 0.1 cm with a steel tape (Lufkin, Cooper Tools, Apex, NC, USA). Abdominal obesity was defined by WC >94 cm for men and >80 cm for women [41]. Triceps skinfolds measurements were performed on the right arm of the participants with a Harpenden skinfold caliper (Baty International, West Sussex, UK), and the average of two recordings was used for data analysis. BMI was calculated by dividing weight in kilograms by height in meters squared and classified using the WHO categories of BMI, namely, <18.5 kg/m^2 as underweight, 18.5–24.99 kg/m^2 as normal weight, 25–29.99 kg/m^2 as overweight and $\geq$30 kg/m^2 as obese [1]. Relevant changes (Δ) in adiposity variables were determined by subtracting adiposity values determined in 2005 from those recorded in 2010 for each individual, respectively.

2.3. Statistical Analysis

Normally distributed data are presented as means with standard deviation, and non-normally distributed data are presented as medians and interquartile range. Categorical data were analyzed using frequencies and prevalence of specific conditions and expressed as percentages.

Paired *t*-tests were used to assess the differences between baseline and follow-up values for energy and fat consumption as well as adiposity measures (BMI, WC, and triceps skinfold) for men and women according to residence (urban and rural). Because the literature indicates considerable differences in body composition between men and women [32], analyses were carried out separately for the sexes. We calculated the magnitude of changes in adiposity markers and presented these as effect sizes (Cohen's *d*-values), i.e., the mean change over five years divided by the standard deviation of the baseline value [42]. Effect size calculation is a standard way of presenting magnitude of change over time. The BMI and abdominal obesity distributions of men and women in 2005 were compared with the corresponding values in 2010 using the Bhapkar test of equal category thresholds. This test was also used to compare the extent of changes in marital and smoking status between the two time points.

Spearman correlations were used to explore the relationship between baseline socio-economic variables, dietary intake and physical activity score, and changes in adiposity variables. Variables with significant correlations were entered in the full regression models (Model 1 for BMI change, WC change and triceps skinfold change for men and women, respectively—representing six separate regressions). Backwards multiple linear regressions were used to assess the association between baseline SES index, dietary intake and physical activity as predictors; and changes in three different adiposity variables over five years as the dependent variables, for men and women, separately. Potential confounders identified from the literature—namely, age, marital status, urbanization level, and menopausal status (for women only, cessation of menstrual periods via self-report)—were included in the models. In each case, Model 1 was the full model with all identified independent variables. Backwards regression was used in order to identify the relevant predictors that accounted for the most variance in the outcome variables, presented as the maximum R^2 for the model. These identified determinants form Model 2, the best predicting model for each of the changes in adiposity markers.

Marital and smoking status transitions were also investigated, but were limited to those for whom we had marital and smoking status at the two time points and excluded those whose responses were missing (n = 65 for marital and n = 14 for tobacco use) or inconsistent from baseline to follow-up (n = 71) (as defined by Sobal et al. [24] for marital status and n = 87 for smoking status). Continuous marital status, transitions out of marriage, and transitions into marriage while never smoking, quitting smoking, starting smoking, and continuing smoking, respectively, were used as categories and other transitions were not included due to concerns about sample size. The associations of marital and smoking transition with change in adiposity markers were assessed with ANCOVA, stratified for sex while adjusting for age. Statistical significance was set at $p < 0.05$. Data were analyzed with

IBM SPSS version 22 (IBM Company, Armonk, NY, USA), except for the Bhapkar test, which was performed using R software (https://cran.r-project.org/web/packages/irr/irr.pdf) (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Descriptive Baseline Data for the Sample Stratified According to Sex

Results of their 2005 baseline characteristics (Table 1) revealed that most adults (80.8%) reported primary school or no formal education, and 88.4% were domestic or informal workers. Women had significantly higher BMI, body weight, WC, and triceps skinfold values than men ($p < 0.001$). Men revealed a higher energy intake ($p < 0.001$), and more men than women were tobacco users ($p < 0.001$).

Table 1. Baseline descriptive data stratified according to sex.

Variables			Men (n = 365 *)	Women (n = 693 *)	p [a]
Age at baseline (year), mean ±SD			51.9 ± 10.1	51.8 ± 10.2	0.95
Marital status % (n)		Living single Married/cohabiting	42.2 (152) 57.8 (208)	47.5 (317) 52.5 (351)	0.11
Socio-economic variables	Stratum of urbanization % (n)	Urban Rural	49.6 (181) 50.4 (184)	42.9 (297) 57.1 (396)	0.04
	Education % (n)	No formal education Low (1–7 years) Intermediate (8–12 years) High (>12 years)	41.5 (149) 42.1 (151) 15.3 (55) 1.1 (4)	38.4 (257) 44.5 (298) 16.6 (111) 0.6 (4)	0.59
	Employed full-time		59.7 (218)	57.4 (398)	0.77
	Occupation % (n)	Domestic/informal worker Formally trained/skilled Professionals No answer	89.0 (325) 4.1 (15) 0.8 (3) 6.0 (22)	88.0 (610) 2.6 (18) 0.6 (4) 8.8 (61)	0.23
	Type of roofing	Tiles, slates or reinforced concrete Galvanized iron Asbestos Scrap material	3.6 (13) 79.7 (291) 14.2 (52) 2.5 (9)	3.2 (22) 82.0 (568) 12.4 (86) 2.5 (17)	0.82
	Electricity % (n)		88.5 (323)	91.3 (633)	0.23
	Piped water in house % (n)		45.5 (166)	36.4 (252)	0.004
	SES index score		7.83 ± 1.27	7.77 ± 1.13	0.43
Life style	Tobacco use % (n)		63.2 (230)	47.2 (325)	<0.001
	Physical activity score, median (interquartile range)		2.83 (2.52–3.23)	2.90 (2.57–3.25)	0.40
	Energy intake (kg), mean ±SD		8563 ± 3625	7413 ± 3512	<0.001
	Fat intake (g), mean ±SD		50.1 ± 29.5	48.0 ± 32.3	0.33
Adiposity parameters	BMI (kg/m^2)		21.0 ± 4.32	27.6 ± 7.41	<0.001
	Height (cm)		167 ± 6.75	157 ± 6.25	<0.001
	Weight (kg)		58.7 ± 12.7	67.9 ± 18.8	<0.001
	WC (cm)		77.1 ± 10.6	82.9 ± 13.8	<0.001
	Triceps SFT (mm)		9.32 ± 6.09	22.3 ± 9.30	<0.001
	Obese: BMI >30 kg/m^2, % (n)		4.1 (15)	34.9 (242)	<0.001
	Abdominal obesity [Y], % (n)		7.4 (27)	56.1 (389)	<0.001

Normally distributed data are reported as mean ±SD, non-normally distributed data as median and interquartile range, frequencies and percentages of the group; * Sample size varies due to missing values; [a] Level of significance for differences between men and women; [Y] WC >80 cm for women and >94 cm for men; BMI, body mass index; SD, standard deviation; SES, socio-economic status; SFT, skinfold thickness; WC, waist circumference.

3.2. Five-Year Dietary and Adiposity Changes of Men and Women Stratified by Residence

3.2.1. Changes in Dietary Intake

Dietary intakes differed between urban and rural residents for both sexes at baseline, with lower mean energy and fat intakes for rural dwellers than their urban counterparts (all $p < 0.001$). Over five years, energy and fat intakes increased in both rural and urban men and women. Changes in energy and fat intakes were higher for urban than for rural men ($p = 0.003$ and $p = 0.04$, respectively). Change in fat intake was less in urban than in rural women ($p < 0.05$), but the difference in energy intake change between urban and rural women was not significant ($p > 0.05$). Detailed analyses of dietary

factors pertaining to adiposity were not within the scope of this investigation and were limited to factors known to change with urbanization.

3.2.2. Changes in Adiposity

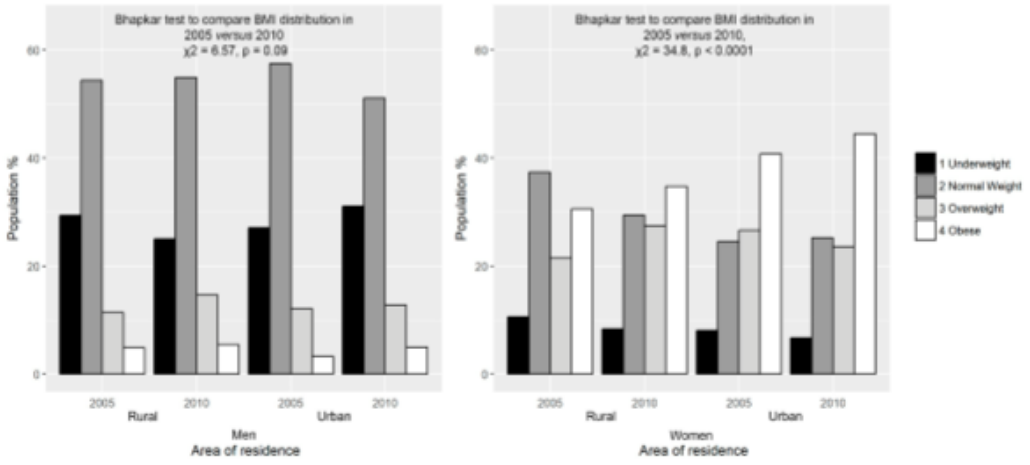
Over five years, men displayed a mean WC increase of 1 cm ($p < 0.001$) and a mean BMI increase of 0.42 kg/m^2 ($p < 0.001$) with no change in mean triceps skinfold value (0.1 mm, $p = 0.58$). Similarly, mean WC of women enlarged by 2.3 cm ($p < 0.001$) and BMI by 0.90 kg/m^2 ($p < 0.001$); however, mean triceps skinfold thickness remained unchanged ($p = 0.72$). The effect sizes—calculated as the mean change divided by standard deviation of the baseline values of the overall changes in BMI, WC, and triceps skinfold of both the men ($d = 0.19$, 0.09 and 0.16, respectively) and women ($d = 0.2$, 0.20 and 0.03, respectively)—were small (Table 2).

Table 2. Changes in adiposity over five years stratified by sex and residence.

Anthropometric Variables	Residence	Men			Women		
		Baseline	Follow-Up	Δ	Baseline	Follow-Up	Δ
Weight (kg)	Urban	58.5 ± 12.6	58.9 ± 14.1	0.46 ± 5.00	70.6 ± 19.5	71.7 ± 20.6	1.09 ± 6.36
	Rural	59.0 ± 13.0	60.1 ± 13.3	1.04 ± 4.98	65.7 ± 18.1	68.4 ± 18.5	2.52 ± 6.37
BMI (kg/m^2)	Urban	20.9 ± 4.18	21.3 ± 4.90	0.34 ± 2.15	28.7 ± 7.58	29.4 ± 8.06	0.62 ± 2.80
	Rural	21.1 ± 4.47	21.6 ± 4.67	0.50 ± 1.77	26.7 ± 7.17	27.9 ± 7.34	1.11 ± 2.82
WC (cm)	Urban	76.6 ± 10.3	78.4 ± 11.5	1.83 ± 5.95	84.7 ± 3.45	88.2 ± 1.56	3.45 ± 7.65
	Rural	77.5 ± 10.9	77.8 ± 10.5	0.27 ± 4.13	81.4 ± 14.0	82.7 ± 13.2	1.13 ± 6.18
Triceps SFT (cm)	Urban	8.6 ± 5.50	9.2 ± 5.87	−0.6 ± 3.69	21.1 ± 8.50	23.8 ± 11.4	0.81 ± 8.17
	Rural	10.1 ± 6.81	9.4 ± 6.41	0.7 ± 4.79	23.1 ± 9.77	24.4 ± 9.98	−0.37 ± 7.98

BMI, body mass index; SFT, skinfold thickness; WC, waist circumference.

The results of adiposity status based on BMI categories stratified by residence for men and women at the two time points are illustrated in Figure 2a,b. Greater agreement between baseline and follow-up categories was found in rural than in urban men (Figure 2a). Most rural (83.2%) and urban (78.9%) male subjects remained in the same BMI category over five years.



(**a**) (**b**)

Figure 2. Adiposity status based on BMI of men (**a**) and women (**b**) stratified by residence at baseline and five-year follow-up.

The Bhapkar tests for agreement between BMI subdivisions in 2005 and 2010 revealed that, although there was a trend to shift from underweight and normal weight to overweight and obese categories, the distribution among the categories at baseline and follow-up showed marginal homogeneity in the total group of men ($\chi^2 = 6.57$, $p = 0.09$). Less agreement was found in rural than among urban women. Almost three-quarters (74.6%) of rural and 79.5% of urban women were in the same BMI segment at both time points. Overall, there was a significant difference in distribution between the BMI categories of women in 2005 and 2010 ($\chi^2 = 34.8$, $p < 0.0001$), with a general shift from underweight and normal weight to overweight and obese categories (Figure 2b). Whereas most increases in BMI categories can be regarded as detrimental, moving from being underweight to normal weight is beneficial: a small group (5.48% men and 2.45% women) progressed from the underweight to normal weight BMI category.

There was no difference in the prevalence of abdominal obesity in rural men at baseline and at follow-up (8.7% versus 7.6%). However, abdominal obesity increased in urban men from 6.1% to 11.7% over 5 years ($p < 0.001$). At baseline, 63.6% of urban women were identified with abdominal obesity compared to 50.5% of rural women ($p = 0.001$). At follow-up, abdominal obesity levels increased to 69.7% in urban and 55.1% in rural women ($p < 0.001$) (Figure 3). According to the Bhapkar test, the prevalence of abdominal obesity increased significantly over five years in women ($\chi^2 = 12.8$, $p < 0.0001$), but not in men ($\chi^2 = 2.69$, $p = 0.10$).

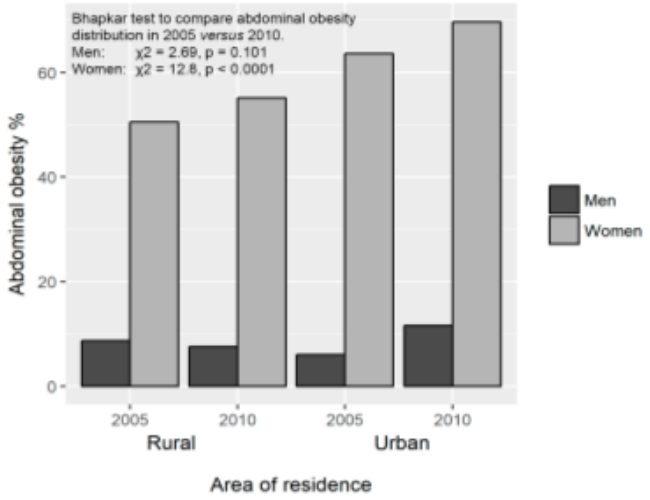


Figure 3. Abdominal obesity prevalence stratified by sex and residence at the two time points.

3.3. Association between Changes in Adiposity with Demographic, Socio-Economic and Lifestyle Variables

The Spearman correlation analysis revealed that none of the variables correlated with markers of adiposity in men (Table 3). Age was negatively correlated with changes in BMI ($r = -0.16$, $p = 0.001$) and with differences in triceps skinfold values ($r = -0.12$, $p = 0.001$), whereas the physical activity score at baseline was positively correlated with change in BMI ($r = 0.13$, $p = 0.001$) for women (Table 3). A positive correlation between change in WC and SES index ($r = 0.14$, $p < 0.001$) as well as level of education at baseline were established ($r = 0.11$, $p = 0.001$) for women (Table 3). Similarly, fat and energy intakes at baseline were also marginally positively correlated, albeit weakly, with changes in WC of women ($r = 0.09$, $p < 0.05$ for both).

Table 3. Spearman correlations between changes in adiposity and baseline dietary intake, socio-economic, and lifestyle variables.

Variable		Men			Women		
		Δ BMI (kg/m²)	Δ WC (cm)	Δ Triceps SFT (mm)	Δ BMI (kg/m²)	Δ WC (cm)	Δ Triceps SFT (mm)
		(*n* = 364)	(*n* = 363)	(*n* = 358)	(*n* = 691)	(*n* = 685)	(*n* = 569)
Age (year)		0.04	0.07	0.002	−0.16 **	−0.07	−0.12 **
Socio-economic	Education level	−0.02	0.03	0.01	0.0	0.11 *	0.08
	Occupation (graded)	0.04	0.04	−0.02	0.03	0.03	−0.03
	SES index	−0.04	0.05	0.04	0.04	0.14 **	0.07
Lifestyle	Physical activity score	0.10	0.05	−0.02	0.13 *	−0.02	0.08
	Energy intake (kJ)	−0.08	0.02	0.05	−0.06	0.09 *	−0.05
	Fat intake (g)	−0.03	0.08	0.10	−0.05	0.09 *	0.02

* Significant at $p < 0.05$; ** significant at $p < 0.01$; Δ, change; BMI, body mass index; WC, waist circumference; SES, socio-economic status; SFT, skinfold thickness.

3.4. Multivariate Analysis of Changes in Adiposity (Dependent) and Predictor (Baseline Socio-Economic and Lifestyle) Variables

Multiple linear regression analysis—for the association between changes in adiposity (dependent) and predictor (baseline demographic, socio-economic, and lifestyle factors, including dietary intake, physical activity, and tobacco use) variables—is presented in Table 4. In the final model (Model 2), using change in BMI as dependent variable, marriage (22%; $p < 0.001$) appeared to be the most attributable determinant followed by baseline physical activity score (12%; $p < 0.05$) among men. No baseline factors predicted changes in BMI among women.

The factors that determined changes in WC at baseline among men and women were baseline WC ($p < 0.0001$), SES ($p = 0.03$, both) and urban versus rural residence ($p = 0.006$ and $p < 0.0001$, respectively). Marital status predicted change in WC in men (accounting for 27% of the variance; $p < 0.001$), but not women. WC at baseline, SES index, marital status and area of residence explained 13.8% variation in WC change among men (Table 4; Model 2). A smaller percentage (10.7%) of variation in WC change of women was explained by baseline WC, age, SES and residential area (Table 4; Model 2).

Table 4. Multiple regression analysis for the association between changes in adiposity variables (Δ BMI, Δ WC, and Δ triceps SFT in men and women, respectively) as dependent variables and predictor variables.

Predictor Variables	Δ BMI		Δ WC		Δ Triceps SFT	
	Men	Women	Men	Women	Men	Women
Model 1: Full model with all variables						
Baseline BMI	−0.07	−0.08	N/A	N/A	N/A	N/A
Baseline WC	N/A	N/A	−0.28 **	−0.26 **	N/A	N/A
Baseline triceps SFT	N/A	N/A	N/A	N/A	−0.48 **	−0.36 **
Baseline age	−0.08	−0.09	0.02	−0.05	−0.02	−0.06
Baseline SES index	0.003	0.05	0.13 *	0.09 *	0.10	0.10 *
Baseline physical activity score	0.11	0.05	0.02	0.02	−0.04	0.05
Baseline fat intake (g)	0.09	−0.01	−0.005	−0.04	0.06	0.06
Baseline tobacco use *0 = never used, 1 = ever used*	−0.07	−0.02	−0.09	0.01	−0.10	−0.10 *
Baseline marital status *0 = single, 1 = married/cohabiting*	0.22 **	0.03	0.27 **	0.07	0.14 *	−0.06
Stratum of urbanization *0 = rural, 1 = urban*	−0.07	0.00	0.15 *	0.23 **	0.09	0.02
Baseline menopausal status *0 = premenopausal, 1 = postmenopausal*	N/A	−0.09	N/A	−0.03	N/A	−0.10 *
Adjusted R^2	0.054	0.024	0.131	0.100	0.228	0.129

Table 4. *Cont.*

Predictor Variables	Δ BMI		Δ WC		Δ Triceps SFT	
	Men	Women	Men	Women	Men	Women
Model 2: Model with best fit						
Baseline BMI	–	−0.08	N/A	N/A	N/A	N/A
Baseline WC	N/A	N/A	−0.28 **	−0.26 **	N/A	N/A
Baseline triceps SFT	N/A	N/A	N/A	N/A	−0.48 **	−0.36 **
Baseline age	−0.09	−0.09	–	−0.07	–	−0.06
Baseline SES index	–	0.05	0.12 *	0.09 *	0.12 *	0.09 *
Baseline marital status *0 = single, 1 = married/cohabiting*	0.22 **	–	0.27 **	–	0.13 *	–
Stratum of urbanization *0 = rural, 1 = urban*	–	–	0.15 *	0.22 **	0.12 *	
Baseline physical activity score	0.12 *	0.05	–	–	–	–
Baseline menopausal status *0 = premenopausal, 1 = postmenopausal*	N/A	−0.08	N/A	–	N/A	−0.11 *
Baseline tobacco use *0 = never used, 1 = ever used*	–	–	−0.09	–	–	−0.10 *
Adjusted R^2	0.057	0.030	0.138	0.107	0.231	0.132

Numbers are beta values and data are adjusted for baseline variables: specific relevant anthropometric measures of adiposity, age, SES index, physical activity score, fat intake, energy intake, tobacco use, marital status, menopausal status (for women only) and stratum of urbanization; * $p < 0.05$; ** $p < 0.001$; Δ, change; BMI, body mass index; NA, not applicable; WC, waist circumference; SES, socio-economic status; SFT, skinfold thickness.

Using triceps skinfold measurement as the dependent variable in the final model (Table 4; Model 2), SES, marital status and place of residence were positive predictors of change, whereas, baseline triceps skinfold thickness was a negative contributor for men. These determinants explained 23.1% of variation in triceps change. In women, baseline triceps values, menopausal status, and tobacco use were negative contributors, whereas SES was the only positive predictor of this adiposity marker, explaining 13.2% of triceps change (Table 4).

3.5. Marital Transitions and Changes in Adiposity

Even though the Bhapkar test indicated significant discrepancies between marital status in 2005 and 2010 ($\chi^2 = 50.7$; $p < 0.0001$), changes in markers of adiposity did not differ between constant marital status and alterations (transitioning in or out of marriage).

3.6. Smoking Status Changes and Changes in Adiposity

The Bhapkar tests revealed that smoking status differed from 2005 to 2010 for men ($\chi^2 = 1008$; $p < 0.0001$) and women ($\chi^2 = 811$; $p < 0.0001$), respectively. The relationship between the categories [i.e., never smoked (unchanged smoking status), stopped smoking (transitioning out of), started to smoke (transitioning into), continued to smoke (unchanged smoking status)] and the adiposity markers (change in BMI, WC, and triceps skinfold) were assessed while adjusting for age. For men, smoking status from 2005 to 2010 influenced change in BMI ($p = 0.04$). The *post hoc* Bonferroni test revealed that change in BMI for those that have never used tobacco differed significantly from continued users ($p = 0.03$). Continued smokers had less change in BMI [0.19 kg/m^2; 95% CI (−0.097; 0.48)] over five years than those that never smoked [0.93 kg/m^2; 95% CI (0.51; 1.35)].

4. Discussion

The novelty of our work lies in the identification of factors associated with increases in adiposity (not limited to BMI alone) over time and not just a mere snapshot of the determinants. In addition, we investigated for the first time marital status and the influences of transitions on markers of adiposity. Our study highlights the remarkable prevalence of excessive adiposity, especially among women, and showed an increase in BMI and WC with subcutaneous fat at the triceps skinfold site remaining

the same in black South African adults over a five-year period. Thus, markers of adiposity increased over time, and the accumulated adipose tissue was probably distributed abdominally. Urbanicity was a predictor of change in WC for both men and women, whereas marital status was a determining factor of change in adiposity for men. We also found that increasing SES leads to elevated adiposity markers in both genders. No differences between unchanged marital status or transitioning in or out of marriage in relation to changes in adiposity markers over time were observed. However, among men, being a continuous smoker resulted in a smaller change in BMI over five years when compared to those that never smoked.

Our results, in terms of the prevalence of obesity, are comparable to rates reported in the general black South African population. For instance, the National Nutrition and Health Examination Survey described an equally high national prevalence of 39.9% obesity (BMI > 30 kg/m^2) among black South African women [3]. The overall prevalence of obesity in this study, further confirms the rising concern that South Africa is in the nutrition-related NCD phase of the nutrition transition [3,10]. The higher prevalence of overweight subjects, obesity and abdominal obesity among women compared to the men in our study is in agreement with other investigations in sub-Saharan Africa [3,4,16,18,43]. A cultural perception among black Africans, for whom overweight or obese women are regarded as being more beautiful, symbols of happiness, well looked-after by their husbands, and being affluent, could be a reason for this continent-wide phenomenon [2,13].

The significantly greater intake of energy and fat by urban than by rural dwellers observed in our study could also be associated with the higher measures of obesity that we observed, particularly among urban women. High dietary intakes of energy and fat have been positively associated with measures of obesity [10,11,44]. Although there was no significant difference between the fat intake of men and women in our study, energy intake was greater for men. Dietary fat and energy intake were not predictors of increases in adiposity in our regression models, which could indicate that our dietary assessment method may not be sensitive enough to detect individual differences in fat and energy intake.

The level of urbanization, that is, being urban dwellers, significantly determined increases in WC of both men and women in this study. According to Cohen [45], food abundance, novelty, and variety are some of the factors that contribute to the effects of urban environments on increasing adiposity. Studies in sub-Saharan Africa have also demonstrated higher BMI in urban compared to rural people [18,43]. Abdominal and overall obesity were reported to be higher among urban Kenyans compared with their rural counterparts [6]. The picture is different in western countries, as inhabitants of rural areas have been reported to display higher measures of obesity than urban dwellers [19]. Even though measures of obesity were higher in our urban women compared to their rural counterparts, we observed a trend of increased obesity in both urban and rural areas over the five-year period (Figure 2b). This reflects findings that increase in adiposity parameters could be an indication of nutrition transition even in the rural areas with its resultant adverse effects as previously observed [10,11].

It has been reported that the association between indicators of SES and BMI varied depending on the socio-economic development of a country [5,8]. McLaren [8] used the human development index to compare low-, middle-, and high-income countries and found that the association between socio-economic indicators and obesity was mostly positive in LMICs and largely negative in rich nations. Earlier studies reported that SES was a significant predictor of BMI and that SES was inversely associated with BMI among European populations [7,46]. Our findings of a positive association between SES index and WC in both men and women are in line with studies in other LMICs [8,16]. The SES index also positively predicted increased WC in both men and women. Future studies can elaborate on our findings by gathering additional information on the ownership of household assets such as cars, televisions, and microwave ovens to create an asset index, which could complement or perhaps even increase the sensitivity of the SES index used here.

An inverse association between educational status and BMI was found among women in Sweden [7], whereas a positive association between BMI and educational status was observed in our study, as well as among Ghanaians [43]. The educational level of the women in our study was also positively associated with gain in WC. However, in our study it is important to keep in mind that the majority of the women (83%) were educated only up to primary school level. The trend was similar for the men as only 16% of them had school education beyond primary school. Our results are in keeping with another South African report; Sartorius et al. [16] observed an association between primary or secondary schooling with increased risk of obesity, whereas those with tertiary education were not at higher risk than those with no schooling.

It is well known that physical activity is inversely associated with measures of obesity [14]. Earlier studies showed that habitual physical activity levels are low in black South African women of the North West province [38,39], similar to what we found in both men and women in our study. However, urban men in the present investigation reported a higher level of physical activity than urban women. Contrary to expectations, we found that baseline physical activity levels correlated positively with BMI change in women and positively predicted BMI alterations in men. This could partly be explained by BMI being a measure of lean mass together with fat mass, and therefore, changes in BMI do not reflect increased adiposity only. Physical activity has been shown to predict lean mass accrual [47]. Moreover, physical activity did not positively correlate with nor predict any of the other adiposity markers thus corroborating our hypothesis.

Smoking is associated with central fat accumulation [15] and has been shown to have a strong negative association with the BMI of women in Europe [7] and South Africa [16]. Tobacco use had no relationship with changes in BMI or WC, but was a negative predictor of changes in triceps skinfold thickness for women in our study. For men in our study, continued smoking were associated with lower rates of increase in BMI than those that never smoked over a five-year period. In our study, changes in adiposity between those that quitted smoking, did not differ from other smoking status categories as was reported by Cois and Day [17].

In the current investigation regression models explained only between 3% and 23.1% of the variations in change in adiposity over five years. This level of explanation seems low, but is comparable to the variation of adiposity change accounted for in the WHO MONICA study [48], where only 4% of variation in WC was explained in men and 5% in women, when other anthropometric parameters were excluded. The innate restrictions of the SES index, dietary intakes and epidemiological data on physical activity may clarify the low variation explained by the regression models in our study, but also in other studies.

Being married or cohabiting (married under common law) was a significant determinant of increased adiposity (WC, triceps skinfold thickness, and BMI) of men, but not in the women. The higher percentage of obese and abdominally obese married men compared to single obese men in our study agrees with a recent report on South Africans [16]. A study in an Iranian population showed that marriage was associated with an increased risk of obesity in both genders [23]. Reports on Americans indicated that men's and women's weights were differently associated with marital changes [24,26]. In a cross-sectional study, married men were significantly more likely to be obese than single men, whereas marital status was not associated with obesity among women [26]. In a later report on Americans, unmarried women who married gained weight whereas single men lost more weight than married men over 10 years. The reason(s) for this phenomenon is not clear. Possible causes could be marriage-associated social environments and lifestyle changes, such as reduced physical activity, increased food consumption, and different smoking habits [23,24]. We also hypothesize that the cultural view of a larger body being the ideal among women may have influenced the men as well, and that larger married men might also be viewed as being well-cared-for by their wives. Marital transitions were also investigated in our study, because it was postulated by others that they trigger change in one's lifestyle that might alter health behavior [24,25]. In other publications, transitions out of marriage through widowhood were more important than through divorce or transitions into

marriage in triggering weight change, because the latter two are transient [25]. However, we did not observe any changes in markers of adiposity between continuous or marital transition categories in our population.

Our work has strengths and limitations. It was performed on black men and women of Tswana ancestry in one province. However, local data are important for informing weight-loss intervention that might be culture sensitive. Moreover, the results may not be generalizable to the greater South African population that also includes minority groups, which were not studied in our investigation. A strength of our study was that the sample included men and women over a wide range of BMI and that we deployed two additional indicators of adiposity (WC and triceps skinfold thickness), because BMI does not distinguish fat from muscle mass. The smaller number of male than female subjects in this study might be problematic, but low participation rates among men is common in epidemiological studies in South Africa [49]. Despite the limitations often encountered in epidemiological data, the scientific rigour of our study renders our results sound. For this reason, we believe that our findings are solid grounds upon which to make recommendations that are crucial for informing intervention and treatment efforts of excessive adiposity in the South African context.

5. Conclusions

Our longitudinal findings over five years showed that black African adults experienced increases in BMI and WC. Residing in an urban environment and having a higher SES played a significant role in increasing the adiposity of our participants. Marital status, among men, also seems to be a contributor to excessive adiposity. Based on the current study and other statistics on obesity in South Africa, if we do not intervene in addressing the issue, the long-term expectation is that obesity will increase further in future. We postulate that for effective evidence-informed prevention or treatment in our population, focusing on the urban living, married people, and those of higher SES—the high-risk groups identified herein—will be essential to reduce the prevalence and extent of adiposity with time. Targeted interventions might be more effective in containing the adverse consequences for health, and even death, with the associated economic burden due to excessive adiposity than general, unfocused intervention strategies.

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Original research article

Sodium content of foodstuffs included in the sodium reduction regulation of South Africa

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ABSTRACT

As a first step in combating the high hypertension rate in South Africa, the Government has recently implemented a mandatory regulation (R.214) pertaining to the sodium content of foodstuffs with targets for 2016 and 2019. We aimed to measure the sodium content and establish whether industry is complying with the targets set in Regulation R.214. The sodium content of ten food products, randomly selected from each of the 13 food categories as described in the regulation (R.214) were measured by means of atomic absorption spectrometry subsequent to microwave digestion. The majority of the food products tested comply with the targets for 2016 (72%) and almost half of the products with the 2019 targets (42%). The highest variation was observed in the "*all fat and butter spread*" (20%) category, as well as the "*raw-processed meat sausages*" (32%). All of the food categories, except for "*flavoured potato crisp, excluding salt-and-vinegar*" and "*flavoured ready-to-eat savoury snack and potato crisp, salt-and-vinegar only*", complied with the 2016 target. South Africa is at the forefront of countries implementing mandatory legislation for the reduction of sodium levels in food. These data provide valuable information with regards to baseline measurements and regulation compliance, therefore enabling future endeavours pertaining to sodium regulation in South African foodstuffs.

1. Introduction

High salt (sodium) intake has been linked to increased prevalence of hypertension (Kotchen et al., 2013), stomach cancer (D'Elia et al., 2012) and kidney disease (Deckers et al., 2014). Worldwide, the mean sodium intake exceeds 2000 mg/day (5 g salt per day), which is the daily allowance recommended by the World Health Organization (WHO) (WHO, 2012). Recently, Swanepoel et al. (2016) indicated that mean sodium intake in three different populations (n = 692) was 7.8 g per day, with 92.8% of the included participants not meeting the WHO's recommendation.

One of the leading causes of death in South Africa is hypertensive heart disease, with the number increasing each year (Nojilana et al., 2016). In response to this public health problem and the overwhelming evidence that supports population-wide sodium reduction strategies, South Africa was one of the 75 countries to develop sodium reduction strategies in order to reach the targeted 2000 mg/day recommended by the WHO before 2025 (WHO, 2012). One of the steps taken by the South African National Department of Health was to reduce non-discretionary sodium intake by passing a regulation in 2013, limiting the sodium content of certain processed foods (R.214:March 2013, amended in 2016) (South African Government, 2013). South Africa was the first country to implement mandatory sodium reduction targets for certain foods.

The first targeted reduction in sodium came into effect in June 2016. Monitoring of sodium in the 13 food categories is not only important to evaluate regulation compliance but is also essential in determining the success of the national sodium reduction strategy of South Africa. Continuous monitoring will provide valuable information that can improve the strategy in South Africa and globally. Regulation and monitoring of sodium in food also requires standardisation of the methodology in terms of the protocol followed as well as in the

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preparation of the different food samples. Within the Regulation R.214, the following methodology is recommended: "*For all foodstuff categories, suitable sodium potentiometric method or elemental analysis, with either flame atomic absorption spectroscopy or inductively coupled plasma, for determining typical total sodium content which shall be applied for monitoring and law-enforcement purposes; provided that these methods may also be used for routine testing or for the purpose of nutritional information labelling of the typical total sodium content by manufacturers.*"

In a recent review (Webster et al., 2014) investigating which type of programmes are more likely to have an impact on sodium reduction, it was reported that 35 countries decided to implement voluntary targets for industry. Nine countries had established mandatory sodium targets, all of which had a target for bread and only two countries (South Africa and Argentina) had mandatory targets for a range of food products. The majority of these countries did report a reduction of sodium in the targeted food products, even though compliance sought was mostly voluntary. The implementation of mandatory targets in some countries is relatively new and therefore the impact will be determined only at a later stage.

The United Kingdom, which is often used as the success story in terms of sodium reduction, implemented voluntary targets in 2006 and in 2011. They reported an overall reduction of 7% in the sodium content of their identified food categories (Eyles et al., 2013). It is therefore crucial for countries that implemented mandatory sodium reduction, like South Africa, to monitor the sodium in the food supply chain. This will enable us to compare the efficiency of mandatory *versus* voluntary sodium targets in countries such as South Africa and the United Kingdom.

The aims of this research were to describe sodium measurement methodology in detail and establish whether industry is complying with the targets set in Regulation R.214. In addition, we compared the sodium as analysed with the sodium values given on the food labels at the time of testing.

2. Materials and methods

2.1. Samples and preparation

The laboratories that were used for sample preparation as well as sodium analysis were kept clean in terms of the bench space and equipment used. All the samples were also handled with caution and precision to prevent contamination. Where possible, ten random food samples were identified from each of the 13 food categories included in Regulation R.214. Different food brands were collected for each food category and included all the major food companies in South Africa. The collection period was from March to May 2016. Three identical food samples (primary samples) with the same batch numbers and expiry dates were used to form the composite sample for each food product.

Sampling of the food products to form the composite sample was conducted according to the guidelines of Greenfield and Southgate (2003). Each food category had a different method of preparation before analysis (because of the different matrices), as is also indicated in Greenfield and Southgate (2003). To ensure homogeneity within the composite sample, all the primary samples were blended after sampling. The composite samples of each food product were divided into three analytical samples, weighing approximately 0.5 g each. Each of these analytical samples was weighed in duplicate. All of the food products were purchased at a major chain supermarket in Potchefstroom, South Africa and included all the major food companies. The homogeneous analytical samples were kept in air-tight containers until analysis.

With each food category, certified reference material from the National Institute of Standards and Technology (NIST, Gaithersburg, MD) was analysed to ensure accuracy (a total of 13 measured NIST samples). The NIST standard used was Peanut Butter (SRM2387), with a certified sodium content of 4890 ± 140 mg/kg.

2.2. Sodium analysis

In line with the methods prescribed by Regulation R.214 we performed the sodium analysis as follows. After appropriate sampling of each food category, the analytical samples were accurately weighed to the nearest 0.5 g with a Boeco scale, with a precision of 0.001 g (Boeco, Hamburg, Germany). The analytical samples were directly weighed in the Teflon vessels of the microwave digester (ETHOS Easy; Milestone, Shelton, CT). A combination of 8 mL 65% HNO_3 (UltraSpec SupraPURE; De Bruyn Spectroscopic Solutions, Bryanston, South Africa) and 2 mL 30% H_2O_2 (SupraPURE; Merck, Darmstadt, Germany) was added (to make up a total of 10 mL) to each Teflon vessel containing the analytical sample. Each digestion series contained one reagent blank which consisted of the same combination of H_2O_2 and HNO_3 (10 mL) without sample material.

Each food category was digested according to the programme that suited the matrix of the food category, by using the manufacturer's digestion protocol. The digested samples were transferred into 500-mL volumetric flasks and filled up to exactly 500 mL with double-distilled (dd) H_2O, after which dilutions were made so that the final concentration of sodium was within the linear range of the atomic absorption spectrometer (AAS) (240 FS; Agilent, Santa Clara, CA).

2.3. Setting of the AAS optical parameters

The AAS technique was used for all sodium analysis. The AAS signals were measured as absorbance using a standard curve (of absorbance against concentration). The standard curve on the AAS was set up between 1 and 5 mg L^{-1} and standard solutions of sodium concentrations of 1.0–5.0 mg L^{-1} were prepared each day before measurements. Measurements were performed in triplicate. The linear equation of the sodium-calibration curve was unique for each line and was part of the system suitability. The line was based on the following formula: Abs = 0.00396c − 0.00134. The wavelength and slit width were set at 3303.3 nm and 0.2 nm, respectively. An oxidising flame was used, whereby the flame type was set on air/acetylene with an air flow of 13.5 L/min and an acetylene flow of 2.0 L/min. The current of the sodium lamp was set at 5.0 mA. Background corrections with the deuterium lamp (D2) were not done. Method detection limits were determined by analysing seven replicates of a low sodium standard (0.5 mg L^{-1}) and multiplying the standard deviation of the seven replicates by three. The detection limit was then determined to be 0.008 mg L^{-1}. As mentioned, the salt contents of all the food samples were estimated to be between 1 and 5 mg L^{-1}, which is well above the detection limit. Limit of quantification was determined to be 1 mg L^{-1}. The average *r* value of the sodium calibration curve of the AAS analyses across all measurements was 0.999.

2.4. Data analysis

Microsoft Excel (2016) was used for all statistical analysis. The mean and percentage relative standard deviation (%RSD) of nine samples (three analytical samples derived from the composite sample measured in triplicate on the AAS) were calculated for each food product in a food category (see Supplementary Table S1 in online appendix). Subsequently the mean and%RSD of each food category was also calculated. The measured sodium content was compared against the sodium content allowed according to Regulation R.214 (both the 2016 and 2019 targets) and expressed as a percentage of the total number of food products analysed. The percentage difference between the measured sodium and the sodium content stated on the label was also calculated. It is important to note that according to the R.214 regulation a 20% excess of the targeted sodium value is allowed on products without claims.

Table 1
Reproducibility and variance of AAS results based on NIST (peanut butter) samples.

	expected result (mg L^{-1})	AAS results(mg L^{-1})	bias (%)	RSD (%)
NIST1	2.45	2.46	100.45	22.4
NIST2	2.45	2.32	94.94	4.9
NIST3	2.45	2.34	95.80	1.4
NIST4	2.45	2.16	88.44	3.8
NIST5	2.45	2.25	91.83	0.4
NIST6	2.45	2.59	105.87	1.2
NIST7	2.45	2.44	99.70	6.1
NIST8	2.45	2.46	100.56	1.3
NIST9	2.45	2.43	99.50	7.8
NIST10	2.45	2.65	108.28	10.9
NIST11	2.45	2.37	96.85	3.4
NIST12	2.45	2.32	94.75	4.1
NIST13	2.45	2.59	105.72	8.4
Average			**98.67**	**5.85**

AAS, atomic absorption spectrometer; NIST, National Institute of Standards and Technology; RSD, relative standard deviation.

3. Results

We analysed all 13 food categories stated in regulation R.214, which amounted to 110 different food products (of different brands). The NIST sample was analysed 13 times (before measuring each new food category and freshly prepared every time) on different days and gave an (average) accuracy and coefficient of analytical variation of 98.7% (% bias) and 5.9%, respectively (Table 1). The targeted concentration was 2.45 mg L^{-1} and the average measured concentration was 2.40 ± 0.14 mg L^{-1}. Reagent blanks, which were measured with each of the food categories, came back zero. Among the different food categories, the variation of measured sodium ranged from 12.4% to 31.8% (Table 2). The highest variation was observed in the "*raw-processed meat sausages*" (31.8%) and "*all fat and butter spread*" (20.4%).

All of the food categories, except for "*flavoured potato crisp, excluding salt-and-vinegar*" and "*flavoured ready-to-eat savoury snack and potato crisp, salt-and-vinegar only*", complied with the 2016 target as stipulated in the R.214 regulation. The majority of the food products tested complied with the targets for 2016 (72%) and almost half of the products complied with the 2019 targets (42%) (data not shown).

Focusing on each of the food categories, Fig. 1 summarises the percentage of the food products within each category that complied with the R.214 regulation targets for 2016 and 2019. As seen in Fig. 1, 100% of the food products tested in the "*dry savoury powders with dry instant noodles*", "*all breakfast cereals*", and "*raw-processed meat sausages*" categories complied with the 2016 targets. Ninety percent of the breads tested complied with the 2016 target and 80% with the 2019 target. Seventy percent of the "*flavoured potato crisp, excluding salt-and-vinegar*" category did not comply with any of the targets.

As bread, dry soup powders and fat and butter spreads contribute the most to South Africans' sodium intake, we showed all the samples of these categories in Figs. 2–4. Here they are compared to the label concentrations and the target concentrations of 2016. (Wentzel-Viljoen et al., 2013). Within the "*bread*" category, sodium on all the labels overestimated (and in some cases severely overestimated) the actual sodium content when compared with the AAS analysis (Fig. 2). The 2016 target for sodium in bread was sufficiently met when considering the category average (306 mg/100 g *vs* the target of 400 mg/100 g). The mean sodium content of both the "*dry soup powder (not instant type)*" (Fig. 3) (5078 mg/100 g *vs* the target of 5500 mg/100 g) and "*All fat and butter spreads*" (Fig. 4) (440 mg/100 g *vs* the target of 550 mg/100 g) were under the 2016 target set out in the regulation. Although the means of these food categories were under the 2016 target, it is important to note that certain food companies did not comply, for example "bread 2", "soup 1", "soup 4", "soup 5" as well as "fat spread 10".

In most of the food products analysed, the label overreported the sodium content (Fig. 5). Within the "*Flavoured potato crisp, excluding salt-and-vinegar*" category, a difference of up to 240% was observed in some of the food products and was excluded from the graph in Fig. 5. Most of the labels of the food products in the "*Dry gravy powders and dry instant savoury sauces*" category underreported the sodium content of their products. According to the R.214 regulation, the permitted tolerance for nutrient declaration in the labelling of sodium cannot be more than 20% in excess of the declared or reported sodium value. Although this stipulated in the regulation, we noticed that some of the food products in the "*Dry gravy powders and dry instant savoury sauces*" and "*All butter and fat spreads*" categories had a higher than 20% difference between the reported sodium content and the measured sodium content.

4. Discussion

South Africa is at the forefront of countries implementing mandatory legislation for the reduction of sodium levels in food as part of a sodium reduction strategy to manage hypertension. Future monitoring of the sodium content of more food products should be done routinely to assess whether the sodium content complies with the regulation. Engagement with industry should also form part of future research to investigate and monitor the substantial discrepancies observed between the sodium declared on the label and what was measured. Lastly, a standard method that includes not only the instrument that should be used for sodium measurement, but also the protocol for sample preparation in different food matrices, should be developed for sodium analyses in South Africa. The RSD% (5.9%) of the NIST sample shows a small variation in the AAS analysis itself and that the higher variation seen in some of the food categories could be ascribed to the heterogeneity that is inherent in some food samples. Even though measures

Table 2
Summary of the mean sodium content (mg/100 g) of 10 food products in each of the 13 food categories included in the sodium reduction regulation (R.214).

food category	measured sodium (mean ± SD)	RSD (%)	2016 target	2019 target
bread	306 ± 78.2	15.09	400	380
raw-processed meat sausages	514 ± 477	31.75	800	600
processed meat – uncured	586 ± 422	14.30	1300	1150
processed meat – cured	811 ± 232	15.01	850	650
all fat and butter spreads	440 ± 122	20.40	550	450
all breakfast cereals	329 ± 84.9	14.86	500	400
savoury snacks, excluding salt-and-vinegar flavoured	686 ± 235	15.93	800	700
flavoured ready-to-eat savoury snack and potato crisp, salt-and-vinegar only	1104 ± 79.8	12.36	1000	850
dry savoury powders with dry instant noodles to be mixed with a liquid	1197 ± 295	19.71	1500	800
flavoured potato crisp, excluding salt-and-vinegar	1648 ± 1157	15.05	650	550
stock cubes/powder/granules/emulsions/pastes/jellies	1,3906 ± 8614	13.78	18,000	13,000
dry gravy powders and dry instant savoury sauces	3433 ± 1265	16.43	3500	1500
dry soup powder (not instant type)	5079 ± 1260	14.49	5500	3500

RSD, relative standard deviation. n = 110 food products in the different categories.

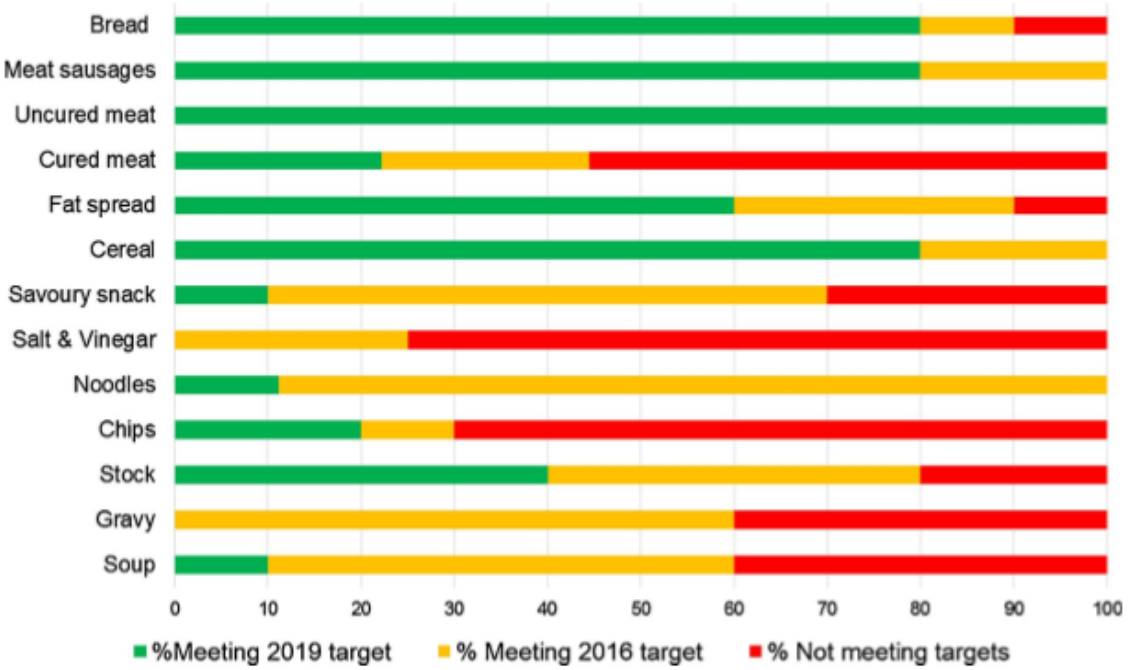

Fig. 1. Food categories' compliance with the sodium target for 2016 and 2019 as stipulated in the R.214 regulation.

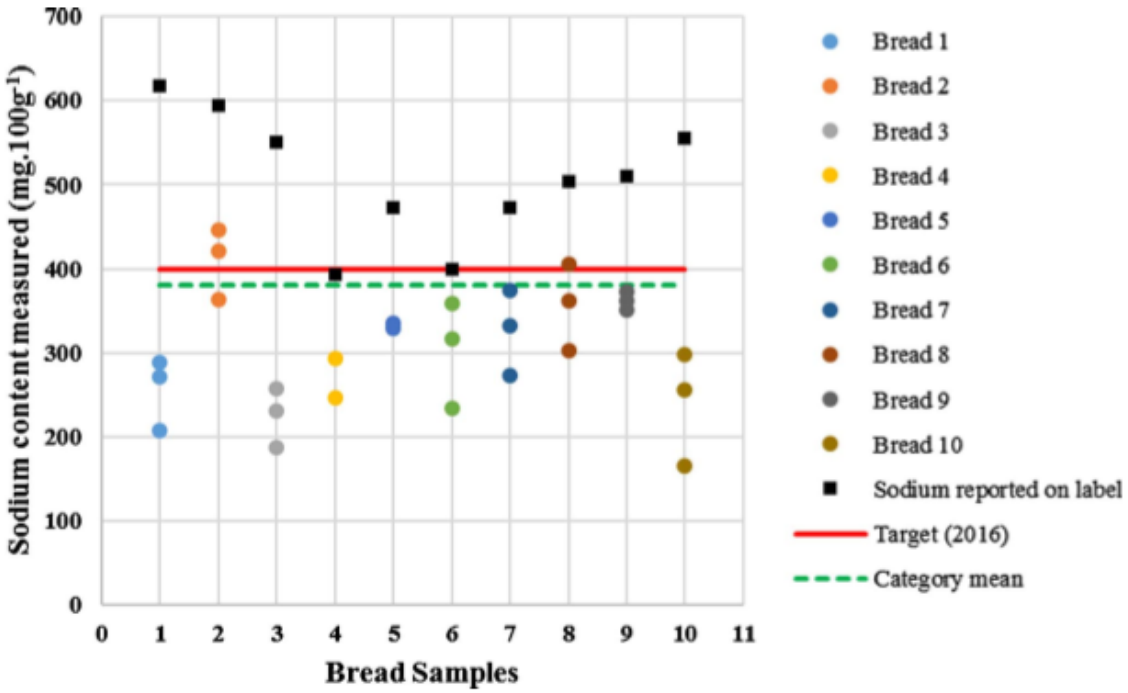

Fig. 2. Variation between food samples as well as comparison between the label as well as the 2016 target within the "*bread*" category.

were in place to ensure that samples were homogenous, this seemed to be one of the greatest challenges in analysing sodium in food products and accounts for a substantial variation in samples.

The data on the sodium content of 13 categories of processed foods provide valuable information with regards to the monitoring and evaluation of the R.214 regulation. The data can serve as a baseline for monitoring product compliance over the next few years. According to the results of this study, 72% of food products tested complied with the 2016 sodium target and almost half with the 2019 target. It should be kept in mind that food sampling was performed between March and May 2016, thus before the target date of 2016.

Food categories that had 100% compliance included "*Raw-processed meat sausages*", "*all breakfast cereals*" and "*dry savoury powders with dry instant noodles to be mixed with a liquid*". Bread, fat spreads and soup powders are the food products that contribute the most to South African sodium intake (Wentzel-Viljoen et al., 2013) and these food categories

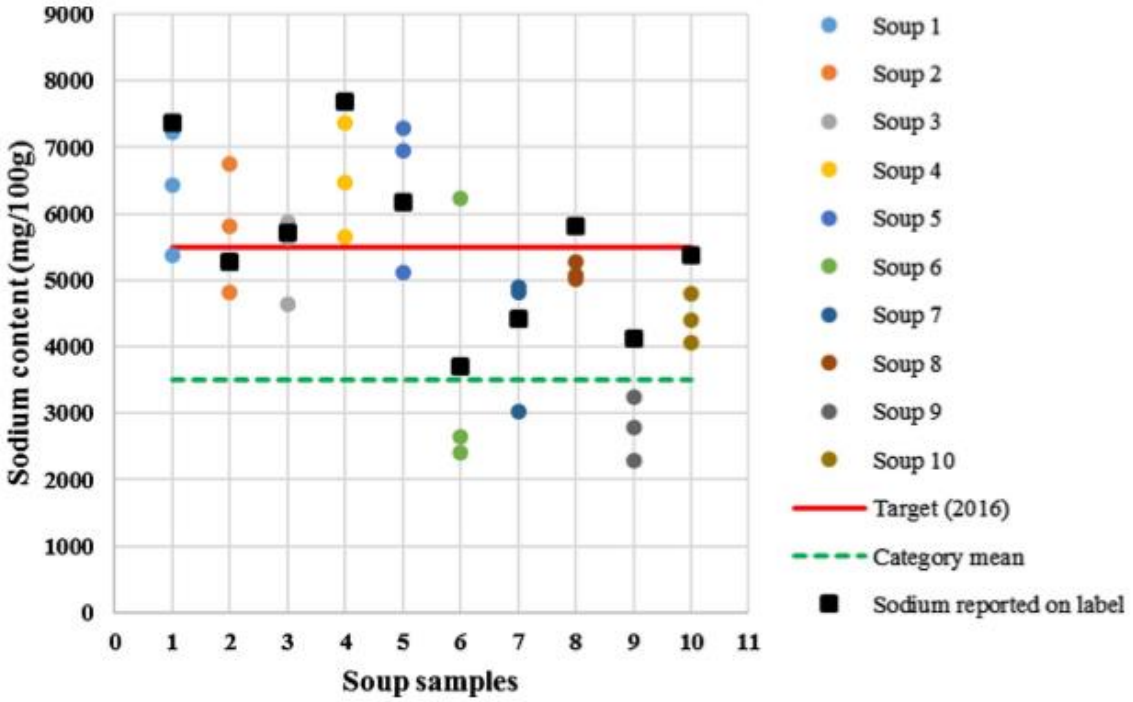

Fig. 3. Variation between food samples as well as comparison between the label as well as the 2016 target within the "*Dry soup powder (not instant type)*" category.

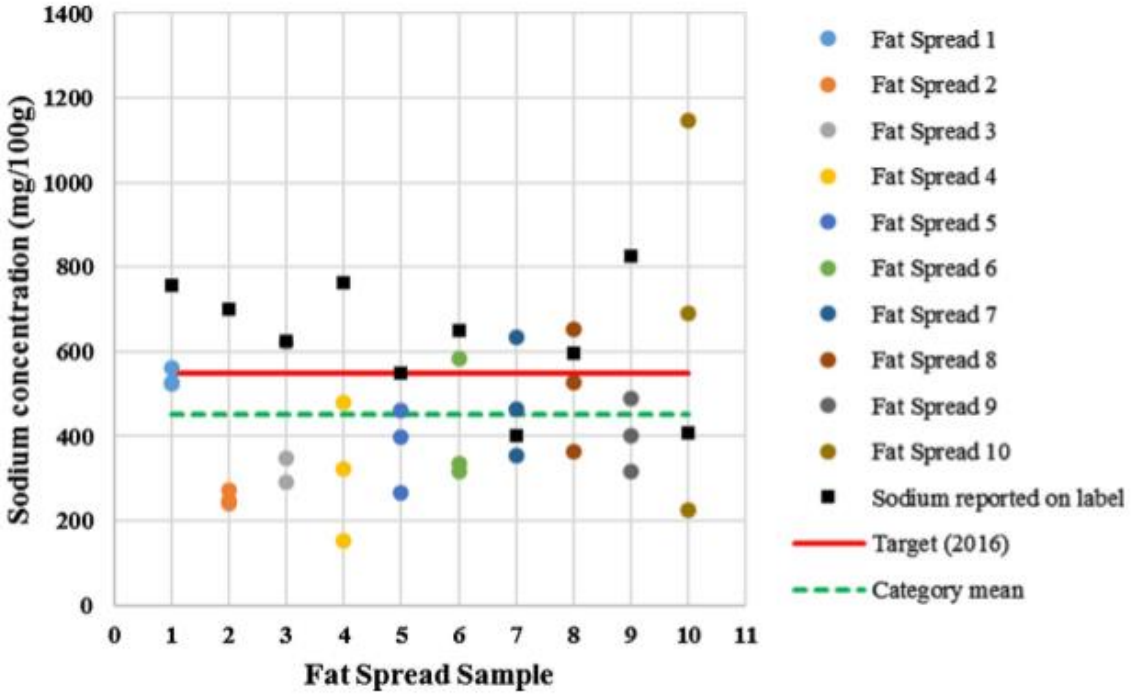

Fig. 4. Variation between food samples as well as comparison between the label as well as the 2016 target within the "*All fat and butter spreads*" category.

had fairly good compliance (bread = 90%, fat spreads = 90% and soup powders = 60%) with the 2016 targets set out in the regulation.

"*Flavoured potato crisp, excluding salt-and-vinegar*" as well as "*Flavoured ready-to-eat savoury snack and potato crisp, salt-and-vinegar only*" had the weakest compliance of 30% and 25%, respectively. These two categories should be carefully monitored after the regulation's target date for 2016.

In most of the food products, the sodium reported on the label and the measured sodium in the food product did not correspond. Differences of more than 20% can be seen across the categories. A possible reason for this could be that re-printing of labels can take time, or that product development and adaptation were not finalised at the

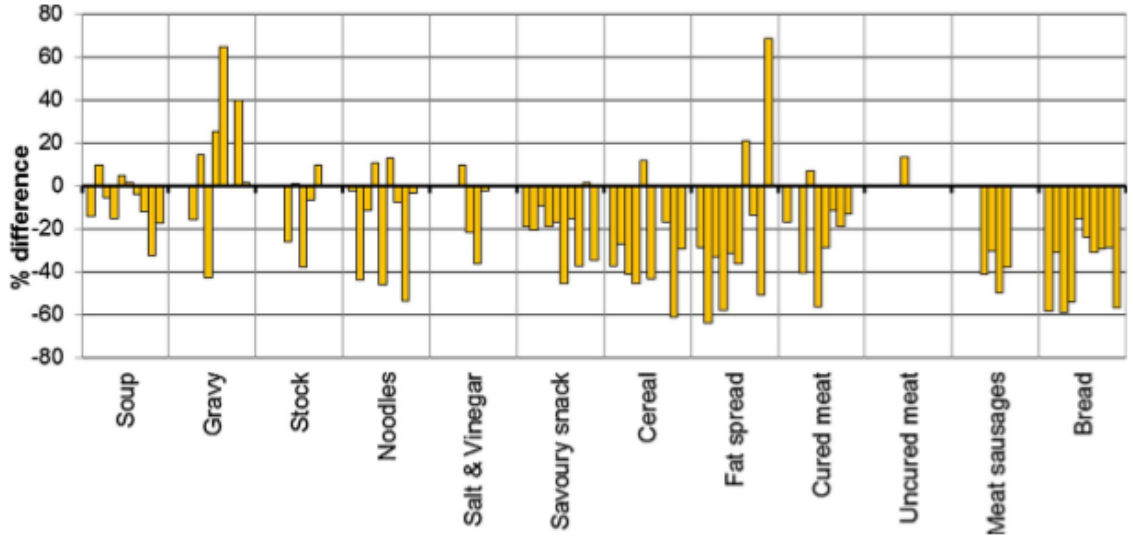

Fig. 5. Difference between the measured sodium content and what is reported on the label for all food categories included in the R.214 regulation.

time of the sampling.

While this research is vital for monitoring aspects, it has some limitations. The first is the sampling of the food products, in that these were not sampled from different areas in the country. However, we did ensure that the batch number and expiry date were the same. The second limitation is that funding permitted the measurement of only 10 food products in each category. Furthermore we could not report on the sodium content of "regular" bread (baked in-house in the bakery) since this bread is not labelled. The majority of the population consumes this kind of bread, since it is cheaper than packaged and labelled bread. The third limitation could be that the research did not measure particle size of the composite or the analytical sample. This could have given a better indication of the homogeneity of sodium in the samples before digestion, and should be conducted in future studies. The fourth limitation is the fact that only one type of food matrix in the form of a NIST sample was used. It should however be noted that peanut butter is a complex and difficult food matrix and was chosen for this reason.

In summary, South Africa was one of the first countries in the world to legislate the sodium content of certain food products. This initiative forms part of a strategy to reduce the sodium intake of the population, in order to manage the high incidence of hypertension. This study is the first to look comprehensively at the compliance of food products with the R.214 regulation as well as a detailed methodology. This study can serve as an encouragement for the Department of Health and for the industry in their efforts to curb the high hypertension rates in the country. These data are vital to the monitoring aspect of the regulation of sodium as a measure of success and will eventually contribute to a reduction in the burden of disease due to hypertension and related cardiovascular diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jfca.2017.07.040.

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