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NON-COMMUNICABLE DISEASE RISK IN BLACK SOUTH AFRICANS: DISSECTING THE ROLE OF GLUCOCORTICOIDS

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(1445737)



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Co-supervisor: Associate Professor Zané Lombard

A Thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy (Paediatrics)

Johannesburg, South Africa

December 2020

DECLARATION

I **Siphiwe Ndumiso Dlamini (1445737)**, declare that:

- ✓ I am a registered PhD student in the department of Paediatrics, Faculty of Health Science, at the University of Witwatersrand.
- ✓ This thesis was written by myself and that the research described in the thesis is the result of my own work, with the following exceptions:
  - i. Recruitment of the participants and collection of the basic data: questionnaire anthropometry, blood pressure, and blood biomarkers (except glucocorticoids) were conducted by the respective members of the cohorts. However, I conducted all data collations, exploration and cleaning, and all calculations and statistical analyses involved in the thesis. I conducted glucocorticoid measurements, with supervision by Tracy Snyman (Scientist at the NHLS Chemical pathology department).
  - ii. While I was not involved in the genotyping of the AWI-gen samples, I was involved in genotyping the samples of all women described in research paper 2.
- ✓ This thesis has not been submitted for any examination or degree at any other institution.
- ✓ I am aware that plagiarism, the use of someone else's work without acknowledgement or permission, is not acceptable. Hence, all figures and work of others were appropriately acknowledged in this thesis.

Signature: 

Date: 22 December 2020

DEDICATION

This thesis is dedicated to my family: my wife Silindile, my mother Babongile, my daughter Genesis, my two sons Siphesihle and Thabiso, and my younger brother Qhawe.

ORAL AND POSTER PRESENTATIONS

Oral presentations

- Genetic associations of common variants at *CYP17A1* and *SERPINA6/A1* loci with blood pressure and measures of central and whole-body adiposity in black South African women. **Siphiwe N Dlamini**, Zané Lombard, Andrew Crawford, Brian R Walker, Julia H Goedecke. *Society for Endocrinology, Metabolism, and Diabetes of South Africa (SEMDSA)*. 7th May 2017.
- Associations of morning serum glucocorticoids with features of the metabolic syndrome in black South African women. **Siphiwe N Dlamini**, Tracy Snyman, Zané Lombard, Andrew Crawford, Brian R Walker, Julia H Goedecke. *Wits Faculty of Health Sciences Research Day*. 6th September 2018.
- Associations of morning serum glucocorticoids with features of the metabolic syndrome in black South African women. **Siphiwe N Dlamini**, Tracy Snyman, Zané Lombard, Andrew Crawford, Brian R Walker, Julia H Goedecke. *International Congress of Endocrinology (ICE)*. 2nd December 2018.

Poster presentations

- Associations between *CYP17A1* polymorphisms and the metabolic syndrome in black South Africans. **Siphiwe N Dlamini**, Zané Lombard, Ananyo Choudhury, Michèle Ramsay, Lisa K Micklesfield, Julia H Goedecke. *Berzelius Symposium of Obesity and Type 2 Diabetes – Understanding the role of ethnicity.* 11th – 12th September 2019.

RESEARCH PAPERS

Research findings arising from this PhD project were written up and presented as three separate research papers. Research paper 1 has been submitted to a scientific journal, while the other two papers have only been prepared for submission. These research papers are listed below, and my contributions are also indicated.

Research Paper 1

Siphiwe N Dlamini, Zané Lombard, Tracy Snyman, Andrew A Crawford, Nigel J Crowther, Shane A Norris, Lisa K Micklesfield, Brian R Walker, Julia H Goedecke. Associations between fasting serum glucocorticoid concentrations and the metabolic syndrome in ageing black South African adults. *The Journal of Clinical Endocrinology and Metabolism*. Submitted and under review.

Research Paper 2

Siphiwe N Dlamini, Zané Lombard, Andrew A Crawford, Shane A Norris, Lisa K Micklesfield, Brian R Walker, Julia H Goedecke. Associations of common variants at *CYP17A1* and *SERPINA6/A1* with the metabolic syndrome and its components in black South African women. *Prepared for submission*.

Research Paper 3

Siphiwe N Dlamini, Ananyo Choudhury, Michèle Ramsay, Lisa K Micklesfield, Shane A Norris, Tracy Snyman, Nigel J Crowther, Andrew A Crawford, Brian R Walker, Zané Lombard, Julia H Goedecke. *CYP17A1* and *SERPINA6/A1* polymorphisms are associated with components of the metabolic syndrome in black South African men and women of African ancestry. *Prepared for submission*.

My contributions to each research paper

I was responsible for the study design, data collation, data exploration and cleaning, and statistical analyses of each study. I was also responsible for the selection of participants and samples, as well as serum steroid extractions. I performed the measurements of serum glucocorticoid concentrations (used in Research paper 1 and 3) using Ultra-high-pressure liquid chromatography, under the direction of Tracy Snyman. I conducted the genotyping of samples used in research paper 2, with the assistance of a laboratory technician at the Western General Hospital, Edinburgh, United Kingdom. I was also responsible for writing the initial draft of each research paper and revising the papers following suggestions and minor edits from the co-authors.

ABSTRACT

Background: Circulating glucocorticoid concentrations are associated with the metabolic syndrome and its components in non-Africans. Likewise, common variants at *CYP17A1* and *SERPINA6/A1*, two loci that were linked to the inter-individual variations in circulating glucocorticoid concentrations, have also been associated with the components of the metabolic syndrome in non-Africans. However, there is a dearth of studies in populations of African ancestry.

The present study aimed to investigate relationships between circulating glucocorticoid concentrations, and *CYP17A1* and *SERPINA6/A1* single nucleotide polymorphisms (SNPs), and the metabolic syndrome and its components, in Africans, whose phenotype and genetic profiles differ to Europeans.

Methods: Firstly, an epidemiological study was conducted to determine the associations between circulating corticosterone and cortisol concentrations, and the metabolic syndrome and its individual components in 1104 adult men and women of African ancestry. A pilot study was then conducted to investigate the relationships between common variants at previously identified *CYP17A1* and *SERPINA/A1* genetic loci, and the metabolic syndrome and its components in 858 adult women of African ancestry. In the final study, I tested these genetic associations in a larger sample (n = 4431) which included both men and women of African ancestry, but also included SNPs at *CYP17A1* and *SERPINA6/A1* loci that had not been previously identified. In the same study, the hypothesis that these genetic association studies were mediated by circulating glucocorticoids was also investigated in a subsample of 877 men and women.

Results: The epidemiology study demonstrated glucocorticoid- and sex-specific relationships between fasting serum glucocorticoid concentrations and the metabolic syndrome and its components, in men and women of African ancestry. Fasting serum

cortisol concentrations were associated with greater odds of having the metabolic syndrome and higher systolic and diastolic blood pressure, but lower LDL and higher HDL cholesterol concentrations, in both men and women. In contrast, fasting corticosterone concentrations were only associated with higher insulin sensitivity, in men and women, but not independently of BMI. Sex-specific associations were observed, such that both fasting serum cortisol and corticosterone concentrations were associated with higher fasting plasma glucose and HbA1c concentrations in men, but lower HbA1c concentrations in women. The pilot study, which was conducted in women only, demonstrated that rs2749527 and rs7147098, two *SERPINA6/A1* SNPs that were previously associated with circulating cortisol concentrations in Europeans, were also associated with higher fasting plasma glucose and diastolic blood pressure, respectively. In the final genetic association study, *CYP17A1* SNPs (n = 2) were associated with higher waist circumference in both men and women. Other *CYP17A1* SNPs (n = 5) were associated with fasting insulin and the homeostatic model assessment of insulin resistance in men only, whilst other *CYP17A1* SNPs (n = 2) were associated with higher fasting cortisol concentrations in women only. In the same study, *SERPINA6/A1* SNPs (n = 2) were associated with diastolic blood pressure in both men and women, while in women only, different *SERPINA6/A1* SNPs (n = 5) were associated with lower high-density lipoprotein (HDL) cholesterol, and another SNP (n = 1) with higher HDL cholesterol.

Conclusions: The study demonstrated evidence of association between circulating glucocorticoid concentrations and the metabolic syndrome and its components in a population of African ancestry. These findings support the hypothesis that glucocorticoids play a role in the development of the metabolic syndrome. The study demonstrated for the first time in individuals of African ancestry that the associations of corticosterone with the components of the metabolic syndrome were not similar to those observed with

cortisol. Hence, the findings also support the hypothesis that corticosterone has a distinct role in human health and disease. The main and novel finding in the overall thesis was that the associations between circulating glucocorticoid concentrations, *SERPINA6/A1* and *CYP17A1* SNPs, and key components of the metabolic syndrome, exhibited sex-specificity in individuals of African ancestry. Therefore, future studies investigating the metabolic syndrome and its components in populations of African ancestry should include assessment of sex interactions.

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Firstly, I would like to thank God giving me the strength and patience to finish my PhD project. “But by the grace of God I am what I am: and His grace which was bestowed upon me was not in vain; but I laboured more abundantly than they all: yet not I, but the grace of God which was with me” (1 Corinthians 15:10).

I am highly grateful to my supervisor, Prof Julia Goedecke, for the valuable guidance and consistent support she displayed throughout my PhD, for giving me a chance to be in her research team, for her financial support, and enabling me to see other parts of the world. I also appreciate my co-supervisor, Prof Zané Lombard, who gave valuable guidance in the field of genetics and bioinformatics, provided a wonderful working environment, and introduced me to several useful people in her field.

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I am very thankful to Prof Michele Ramsay, Prof Shane Norris, Prof Lisa Micklesfield, Prof Nigel Crowther for granting me permission to use retrospective data and serum samples from their study cohorts. I also thank Tracy Snyman for the valuable training in glucocorticoid determination using the LCMS, and for using her experience in processing the results. The assistance provided by SAMRC/Wits DPHRU students, Asanda Mtintsilana and Chifhiwa Rachikombo, during sample selection is also appreciated.

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Department of Health, the UKMRC, the Newton Fund, GSK and NRF. Opinions expressed and conclusions arrived at, are those of myself and are not necessarily to be attributed to the research sponsors.

CONTENTS

DECLARATION i

DEDICATION ii

ORAL AND POSTER PRESENTATIONS iii

Oral presentations iii
Poster presentations iii
RESEARCH PAPERS iv

Research Paper 1 iv
Research Paper 2 iv
Research Paper 3 iv
My contributions to each research paper v
ABSTRACT vi

ACKNOWLEDGEMENTS ix

CONTENTS xi

LIST OF ABBREVIATIONS AND SYMBOLS xv

LIST OF FIGURES xix

LIST OF TABLES xxi

PREFACE xxiii

Chapter 1 Literature Review 1

1.1. Non-communicable diseases 2
1.2. NCD risk factors in South Africa 3
1.2.1. Obesity 3
1.2.2. Modifiable risk factors 4
1.2.3. Non-modifiable risk factors 5
1.3. The metabolic syndrome 7
1.3.1. Previous clinical definitions of the metabolic syndrome 8
1.3.2. The harmonized clinical definition 9
1.3.3. Prevalence of the metabolic syndrome in South Africa 10

1.3.4. Ethnic disparities in components of the metabolic syndrome: why an interest in populations of African ancestry? 12
1.4. Glucocorticoids 17
1.4.1 The role of glucocorticoids in the metabolic syndrome 17
1.4.2 What are glucocorticoids? 18
1.4.3 Glucocorticoid production and regulation 18
1.4.4 Physiological functions of glucocorticoids 24
1.5. A separate role of corticosterone in human health and disease 26
1.5.1 Lower circulating corticosterone concentrations when compared to cortisol 26
1.5.2 Corticosterone and cortisol may be differently regulated by the HPA axis 30
1.5.3 Corticosterone and cortisol interact differently with ABC transporters 30
1.6. Cross-sectional studies investigating associations between cortisol and the metabolic syndrome and its components 32
1.6.1 Potential confounders in cross-sectional studies of cortisol 32
1.6.2 Cross-sectional studies of cortisol in populations of non-African ancestry 36
1.6.3 Cortisol in populations of African ancestry 42
1.7 Genetics of glucocorticoid concentrations 47
1.7.1 The *SERPINA6/A1* locus 47
1.7.2 The *CYP17A1* gene 49
1.8 Genetic architecture in populations of African ancestry 52
1.9 Literature review summary 53
1.10 Study rationale and research questions 55
Chapter 2 Research paper 1 59
2.1 Abstract 59
2.2 Introduction 60
2.3 Methods 62
2.3.1 Study population and sampling procedure 62
2.3.2 Participant testing procedures 65
2.3.3 Blood sampling and biochemical analyses 65
2.3.4 Definition of the metabolic syndrome 67
2.3.5 Statistical analyses 67
2.4 Results 69
2.4.1 Participant characteristics 69
2.4.2 Associations with the metabolic syndrome and its components 72
2.5 Discussion 77
Chapter 3 Research paper 2 84

3.1 Abstract 84
3.2 Introduction 85
3.3 Methods 87
3.3.1 Study participants 87
3.3.2 Participant testing procedures 87
3.3.3 Blood sampling and biochemical analyses 88
3.3.4 Definition of the metabolic syndrome 89
3.3.5 Selection of tag SNPs 89
3.3.6 Genotyping 92
3.3.7 Data quality control and missingness 92
3.3.8 Statistical analyses 93
3.4 Results 93
3.4.1 Characteristics of the study sample 93
3.4.2 Genetic associations with the metabolic syndrome and its individual components 95
3.4.3 Comparing the minor allele frequencies of the identified SNPs in other populations 97
3.5 Discussion 98
Chapter 4 Research Paper 3 103

4.1 Abstract 103
4.2 Introduction 104
4.3 Methods 106
4.3.1 Study population 106
4.3.2 Participant testing procedures 106
4.3.3 Definition of the Metabolic Syndrome 108
4.3.4 Genotyping, imputation, and data quality control 109
4.3.5 Statistical analyses 110
4.4 Results 112
4.4.1 Study sample characteristics 112
4.4.2 *CYP17A1* SNPs and the metabolic syndrome components 116
4.4.3 *SERPINA6/A1* SNPs and metabolic syndrome components 121
4.4.4 Minor allele frequencies of the identified SNPs 125
4.5 Discussion 127
Chapter 5 General Discussion 135

5.1 General discussion 136
5.2 Study strengths and limitations 143

5.3 Future studies and recommendations 145
5.4 Conclusions 146
References 148

Appendices 199

Appendix A: Ethics clearance certificates 200
Appendix B: Supplementary figures and tables 205
Appendix C: Scatter plots (men and women combined). 209
Appendix D: Scatter plots (BMI: men and women separated). 218
Appendix E: Sex comparison of circulating glucocorticoids. 219
Appendix F: Proportions of the study population above each cut off point. 220
Appendix G: Research paper 1 power calculation results. 221
Appendix H: Research paper 2 power calculation results. 222
Appendix I: LCMS method used to measure glucocorticoids. 223

LIST OF ABBREVIATIONS AND SYMBOLS

11β-HSD	11β-Hydroxysteroid dehydrogenase
3α-HSD	3α-Hydroxysteroid dehydrogenase
AAT	Alpha-1-antitrypsin
ABC	Adenosine triphosphate-binding cassette
ACTH	Adrenocorticotropin hormone
AGVP	African Genome Variation Project
AHA/NHLBI	American Heart Association: National Heart, Lung, and Blood Institute
AIDS	Acquired Immune Deficiency Syndrome
ATP	Adenosine triphosphate
ATP III	National Education Program Adult Treatment Panel III
AWI-Gen	Africa Wits-INDEPTH partnership for Genomic Research
BMI	Body mass index
BP	Blood pressure
Bt20+	Birth to twenty plus
CBG	Corticosteroid binding globulin
CETP	Cholesteryl ester transfer protein
CI	Confidence interval
CNS	Central nervous system
CRH	Corticotropin-releasing hormone
CV	Coefficients of variation
CVD	Cardiovascular disease

DNA	Deoxyribonucleic acid
DPHRU	Developmental pathways for health research unit
ECLIA	Electro-chemiluminescence immunoassay
EIA	Enzyme Immunoassay
ELISA	Enzyme-linked immunosorbent assay
GR	Glucocorticoid receptor
GWAS	Genome-wide association studies
H3Africa	Human heredity and health in Africa
HbA1c	Glycated haemoglobin
HDL	High-density lipoprotein
HOMA-IR	Homeostatic model assessment of insulin resistance
HPA	hypothalamic pituitary adrenal
HSL	hormone-sensitive lipase
HWE	Hardy-Weinberg equilibrium
IDF	International Diabetes Federation
IHD	Ischaemic heart disease
LCMS	Liquid chromatography-mass spectrometry
LD	Linkage disequilibrium
LDL	Low-density lipoprotein
LMICs	Low- and middle-income countries
LPL	Lipoprotein lipase
MAF	Minor allele frequency
MDR1	Multidrug resistance protein 1

Med	Medication
MR	Mineralocorticoid receptor
N	Total number of observations
n	Number of observations from the total
N/A	Not applicable
NCD	Non-communicable disease
NR	Not reported
NS	Not specified
NT	Not tested
OGTT	Oral glucose tolerance test
OR	Odds ratio
P2	P value after adjusting for multiple testing
PCR	Polymerase chain reaction
QC	Quality control
RAAS	Renin-aldosterone-angiotensin system
RCL	Reactive centre loop
Ref	Reference
RIA	Radioimmunoassay
SA	South Africa
SAMRC	South African Medical Research Council
SAT	Subcutaneous adipose tissue
Sex Int	Sex interaction
SNP	Single nucleotide polymorphism

STD	Standardized
UK	United Kingdom
UPLC-MS	Ultra-pressure liquid chromatography mass spectrometry
USA	United States of America
UTR	Untranslated region
VAT	Visceral adipose tissue
VIF	Variance inflation factor

LIST OF FIGURES

Figure 1.1: Age-adjusted death rates for cardiovascular diseases, diabetes, and renal disease by South African population groups, in 2010. **3**

Figure 1.2: Percentage distribution of body mass index categories in South African men and women (aged 15+). **4**

Figure 1.3: Life expectancy of South African men and women, from 2002 to 2019. **6**

Figure 1.4: Clinical features of Cushing's syndrome. **17**

Figure 1.5: Chemical structures of the human glucocorticoids: cortisol (A) and corticosterone (B). **18**

Figure 1.6: The production of glucocorticoids via the HPA axis. **19**

Figure 1.7: The biosynthesis of glucocorticoid hormones in humans. **20**

Figure 1.8: The binding and release of glucocorticoids by CBG. **22**

Figure 1.9: Metabolic clearance of glucocorticoids by 5α-reductase and 3α-HSD, to generate tetrahydro-glucocorticoids. **23**

Figure 1.10: Serum cortisol concentrations minutes before and after awakening (A) and salivary cortisol concentrations minutes after awakening from different age groups (B). **32**

Figure 1.11: Association results from the meta-analysis of GWAS for morning plasma cortisol in populations of European ancestry. **47**

Figure 2.1: Selection of the study sample used in the present study. **64**

Figure 2.2: Associations between fasting serum glucocorticoid concentrations and odds of developing the metabolic syndrome. **73**

Figure 2.3: Sex-stratified associations of fasting serum corticosterone and cortisol concentrations with the metabolic syndrome components. **76**

Figure 3.1: Selection of the dataset used in the analyses. **91**

Figure 3.2: Genetic associations with the odds of having the metabolic syndrome. **96**

Figure 4.1: A comparison of the prevalence of the metabolic syndrome and its components between men and women of African ancestry. **114**

Figure 4.2: LocusZoom (a) and box and whisker (b) plots for the associations of rs17115144 at *CYP17A1* with waist circumference in men and women of African ancestry. **117**

Figure 4.3: Sex-specific associations of *CYP17A1* SNPs with fasting insulin concentrations and HOMA-IR (a-f), and fasting cortisol concentrations in men and women of African ancestry (g-i). **118**

Figure 4.4: LocusZoom (a) and box and whisker (b) plots for the associations of rs17090691 at *SERPINA6/A1* with diastolic blood pressure in men and women of African ancestry. **121**

Figure 4.5: Sex-specific associations of *SERPINA6/A1* SNPs with HDL cholesterol concentrations and in women of African ancestry. **123**

Figure 4.6: *CYP17A1* enzyme functions in the synthesis of steroid hormones and relationships with components of the metabolic syndrome. **127**

LIST OF TABLES

Table 1.1: Criteria for definition of the metabolic syndrome in populations of African ancestry. **9**

Table 1.2: The prevalence of the metabolic syndrome in South Africa. **11**

Table 1.3: Studies describing circulating corticosterone (± cortisol) in populations of European ancestry. **27**

Table 1.4: Cross-sectional studies that investigated the associations between glucocorticoids and NCD-risk in populations of African ancestry. **44**

Table 1.5: Genome-wide association studies that reported associations between *CYP17A1* SNPs and components/outcome related to the metabolic syndrome. **51**

Table 2.1: Participant characteristics. **71**

Table 2.2: Associations of serum glucocorticoid concentrations with components of the metabolic syndrome. **74**

Table 3.1: Single Nucleotide Polymorphisms included in this study. **90**

Table 3.2: Characteristics of the study sample. **93**

Table 3.3: Genetic associations with the metabolic syndrome and its components. **95**

Table 3.4: Minor allele frequencies of the identified SNPs in the present study sample and other populations. **97**

Table 4.1: Characteristics of the study sample. **113**

Table 4.2 Associations of *CYP17A1* SNPs in men and women of African ancestry. **115**

Table 4.3: Associations of *SERPINA6/A1* SNPs in men and women of African ancestry. **120**

Table 4.4: Minor allele frequencies of the identified SNPs in the present study sample and other populations. **125**

Table 4.5: A summary of previous genetic associations with the significant SNPs identified in this study. **128**

Table 5.1: Summary of the findings from the present study. **137**

PREFACE

Glucocorticoids are well acknowledged for their wide range of physiological functions in humans. Hence, their role in human health and disease has been a subject of interest since their discoveries in the 1940s. Indeed, numerous human studies have been conducted to date, in an attempt to elucidate the role of glucocorticoids in complex diseases, such as the metabolic syndrome. However, most of these studies were conducted in populations of predominantly European and Asian ancestries, and only a handful of studies measured glucocorticoids in individuals of African ancestry.

In this thesis, the term “ancestry” is used to define ethnic descent or origin. Hence, populations are mainly divided into five major ethnic groups, based on self-identified ancestry, to include i) African, ii) European, iii) Asian, iv) American, and v) mixed ancestry populations. For simplicity, populations of African ancestry include ethnic groups of African origin which are also referred as “Africans”, “blacks”, “black Africans”, and other nation-based ethnic terms, such as “African Caribbeans” and “African Americans”, in published literature. Likewise, populations of European ancestry include ethnic groups of European origin that are also referred as “Europeans”, “whites”, “white Europeans”, “Caucasians”, and other ethnic based ethnic terms, such as “white Americans” and “white South Africans”, used in published literature. In contrast, populations of Asian ancestry can be further divided into populations of East and Asian ancestry. East Asian ancestry generally refer to all ancestry of nations in East Asia including China, Japan, North and South Korea, etc. South Asian ancestry generally refers to individual with origins such as India, Afhganistan, Pakistan, Bangladesh, Nepal etc.

My interest in investigating the metabolic syndrome in populations of African ancestry, originates from the fact that populations of African ancestry have been shown to present with a unique phenotype when it comes to the relationship among key components of the

metabolic syndrome. There is also evidence to suggest differences in circulating glucocorticoid concentrations and glucocorticoid signalling when comparing women of African ancestry to their European ancestry counterparts. Moreover, genomes of populations of African ancestry are known to be more diverse when compared to those of populations of European ancestry. Hence, such population-based findings as those presented in this thesis are likely to improve the efficacy of future medical methods, particularly, genomic and precision medicine.

The thesis is presented in an 'integrated' format with the results presented as three research papers (Chapter 2-4). The content of the thesis is separated into five chapters as follows:

Chapter 1: Literature Review

Chapter 2: Associations between fasting serum glucocorticoid concentrations and the metabolic syndrome in ageing black South African adults.

Chapter 3: Associations of common variants at *CYP17A1* and *SERPINA6/A1* with the metabolic syndrome and its components black South African in women

Chapter 4: *CYP17A1* and *SERPINA6/A1* polymorphisms are associated with components of the metabolic syndrome in black South African men and women

Chapter 5: General discussion

Chapter 1 Literature Review

This chapter gives an overview of the literature to provide background information on key concepts that are relevant to this thesis. The concepts discussed include the prevalence of non-communicable diseases (NCDs) and the metabolic syndrome in South African and ethnic-based differences in the metabolic syndrome and its components when comparing populations of African ancestry to their European ancestry counterparts. The biology of glucocorticoids including physiological functions and structure are then discussed in relation to their role in the metabolic syndrome. The chapter then summarizes human studies that investigated the role of glucocorticoids in the metabolic syndrome and its components. Finally, the study rationale, research questions, and objectives are described.

1.1. Non-communicable diseases

Regardless of the awareness and extensive global interventions, non-communicable diseases (NCDs) remain a global health challenge. More than 41 million deaths were caused by NCDs alone in 2017, which was over 70% of the total annual human deaths (1). This number is expected to increase substantially over the next decade if adequate measures are not taken to combat the increase in NCDs (2). Notably, more than 76% (31 million) of NCD deaths occurred in low- and middle-income countries (LMICs), as they represent over 82% of the global population (3). The rapid rise in NCD prevalence in LMICs results from multifaceted interactions, including rapid urbanization and an unhealthy lifestyle (4), which accompany several risk factors for NCDs including obesity, increased alcohol intake, smoking, unhealthy eating, and physical inactivity (5). Although in most African countries the current leading causes of death are communicable diseases, it is projected that NCDs will have taken over by 2030 (6).

Within South Africa, NCDs are already among the leading causes of death (7), with more than 57.8% of total deaths being attributed to NCDs in 2017, and more than a third occurring in individuals below the age of 60 years (8). Notably, the NCD mortality profiles showed distinct ethnic differences when comparing the four major South African population groups (populations of African, European, Asian, and mixed ancestries) as shown in Figure 1.1. Age-standardised death rates from cerebrovascular disease, hypertensive heart disease, diabetes, ischaemic heart disease, and cardiomyopathy were all highest among the population of African ancestry (7). Indeed, when compared to their European ancestry counterparts, populations of African ancestry are known to be at a higher risk of developing obesity-related NCDs in urban areas of South Africa (9).

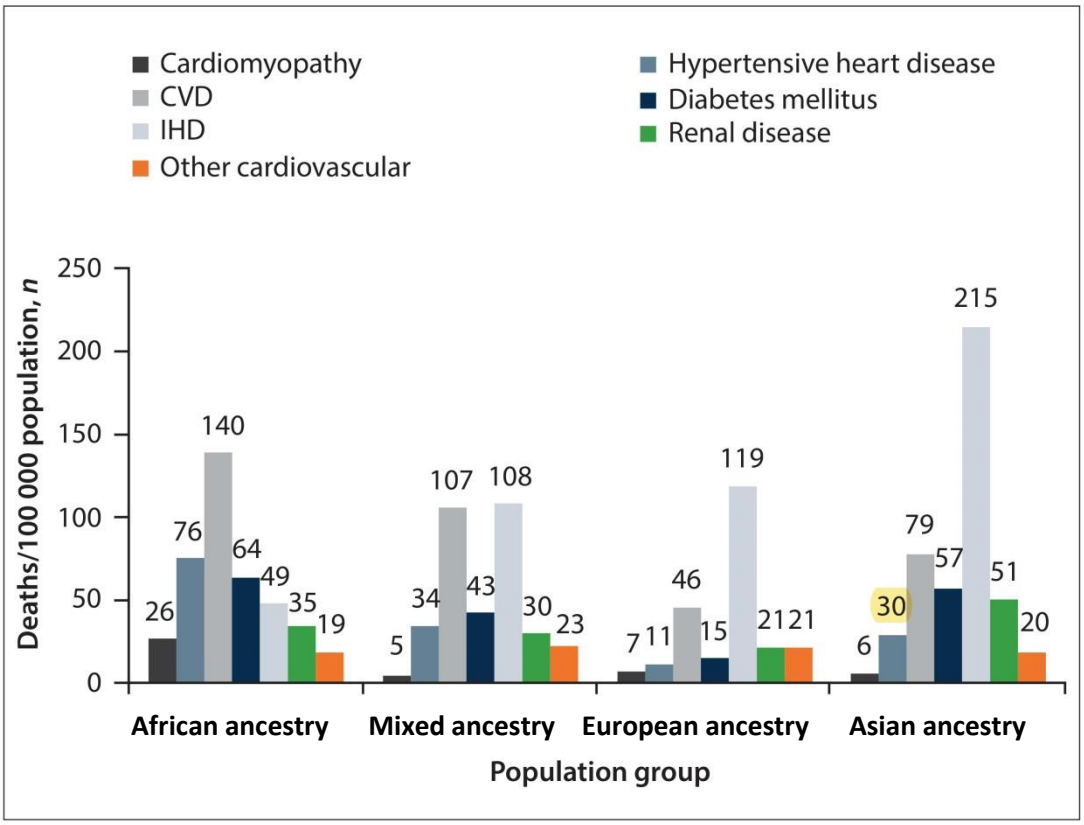


Figure 1.1: Age-adjusted death rates for cardiovascular diseases, diabetes, and renal disease by South African population groups, in 2010.

CVD: Cerebrovascular disease; IHD: Ischaemic heart disease. Age-standardised death rates from cerebrovascular disease, hypertensive heart disease, diabetes, ischaemic heart disease and cardiomyopathy were all highest among the African ancestry group. *Figure used with permission* (7).

1.2. NCD risk factors in South Africa

1.2.1. Obesity

Obesity is one of the critical drivers of the observed high prevalence of NCDs. It is acknowledged as a major risk factor for cardiovascular diseases (10), several cancers (11), and type 2 diabetes (12), and there is considerable evidence that it also associates with chronic respiratory diseases (13).

According to the 2016 South African Demographic and Health Survey (14), the prevalence of obesity was relatively high in South Africa, with more than half of the adult

population being classified as overweight or obese (BMI ≥ 25 kg/m^2). Moreover, the women of South Africa have higher rates of obesity compared to men (41% versus 11% in 2016) as shown in Figure 1.2 (14), with the prevalence being highest among urban-dwelling women (42%) (15).

Obesity generally results from a combination of modifiable (behavioural) risk factors, such as excess energy intake and physical inactivity, and non-modifiable risk factors, such as genetic predisposition.

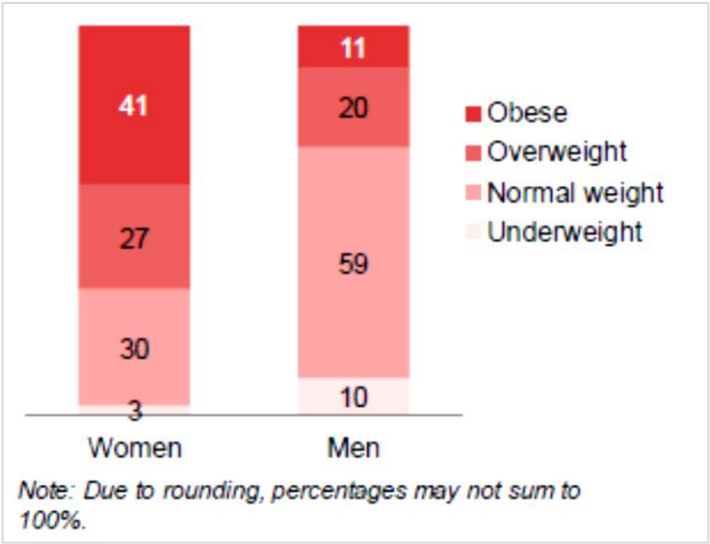


Figure 1.2: Percentage distribution of body mass index categories in South African men and women (aged 15+). Figure used with permission (14).

1.2.2. Modifiable risk factors

Urbanization has been globally associated with rapid increase in obesity and related NCDs (16). Urbanization is often accompanied by increases in several behavioural risk factors including unhealthy diet, physical inactivity, tobacco smoking, and excessive alcohol intake (17). South Africa is currently undergoing rapid urbanization, with at least 63% of the population already residing in urban areas (18), and more than 80% projected to be dwelling in urban settings by 2050 (19).

The rapid urbanization in South Africa has been paralleled with unhealthy eating, particularly excess energy intake (18). Energy intake in this country appears to be partly driven by poverty, as a higher obesity prevalence was generally observed in the low-income groups (20). Furthermore, poor South Africans were shown to primarily consume foods that are cheap and energy-dense (generally high in fats and carbohydrates), but lacking in nutrients (21). The effects of excess energy intake may be exacerbated by the relatively high prevalence of physical inactivity in South Africa. More than 40% of South Africans, aged 15 years and above, were classified as physically inactive in 2000 (22). In parallel with the obesity trends, the prevalence of physical inactivity was relatively high in urban-dwelling adult women (53.6%) (23). Contrary to physical inactivity, which is more common in women than men, the prevalence of smoking and excessive alcohol intake is more common in South African men than women (24). These sex disparities may partly explain the higher prevalence of cardiovascular diseases in South African men in comparison to their female counterparts (25).

1.2.3. Non-modifiable risk factors

Age and sex

As NCD risk factors positively associate with age, the rapid rise of NCD prevalence may also be partly attributed to the improved life expectancy of South Africans. According to the Statistics South Africa 2019 report (26), the life expectancy of South African men and women had increased over the past 20 years (2007-2019) by 11.1 and 9.2 years, respectively (see Figure 1.3). The higher life expectancy in women may partly explain the higher prevalence of age-related NCDs in South African women. The overall increase in life expectancy may be due to reduced prevalence of infectious diseases and improved quality of health care in South Africa (27). For example, death from AIDS (acquired immune deficiency syndrome) decreased by 16.1% from 2007 to 2018 (26).

Nevertheless, the majority of South Africans affected by NCDs are less than 60 years, with individuals between the ages of 40 and 60 years being the most affected (28).

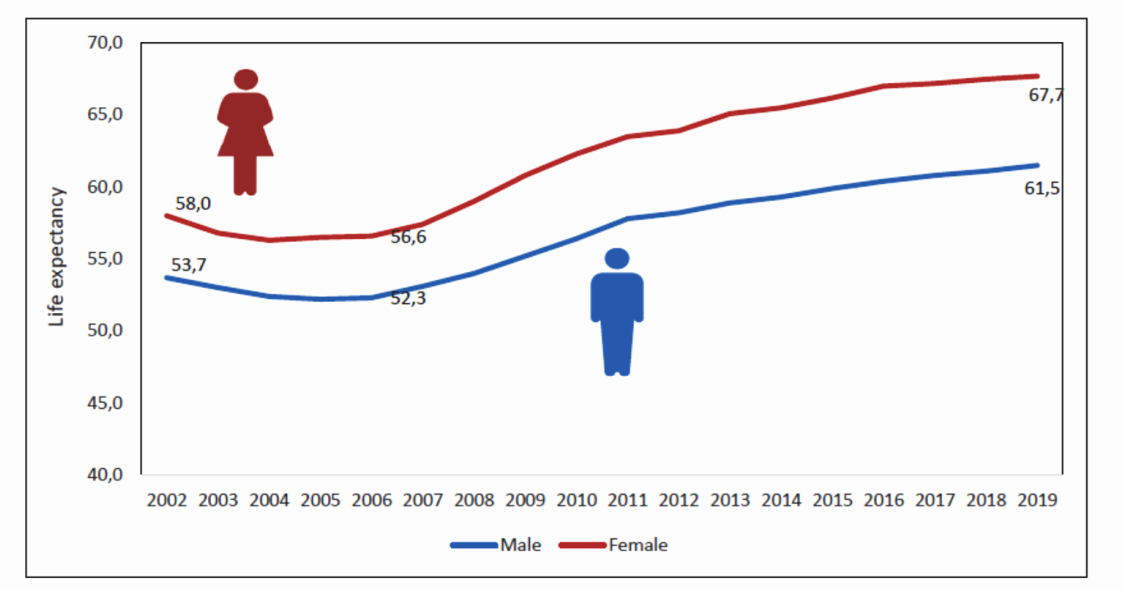


Figure 1.3: Life expectancy of South African men and women, from 2002 to 2019. ***Figure used with permission*** (26).

Genetics

Although behavioural risk factors and age strongly associate with NCD risk factors, not all individuals develop NCDs, suggesting a genetic component. Notably, the heritability range for obesity was estimated at 40-50% in humans (29,30), while the estimates for hypertension, type 2 diabetes, and coronary artery disease were 30-60% (31), 25-80% (32), and 40-60% (33), respectively.

Most of the genes implicated in NCD risk factors were discovered through genetic association studies, which assess whether genetic variations (e.g., allele, haplotype, gene, or chromosome region) significantly associate with a phenotype. Such studies included genome-wide association studies (GWAS) which use an unbiased approach to screen the human genome for single nucleotide polymorphisms (SNPs) that significantly associate with the phenotype of interest (34), and candidate gene studies, which only focus on pre-specified genetic regions (commonly referred as loci) (35).

Although hundreds of SNPs have been implicated in NCD risk factors through GWAS, the majority of these were discovered in individuals of European ancestry (36). Recent studies have suggested a need for population diversity and inclusion of populations of African ancestry in human genomic research (37). Notably, the South African population is highly diverse, composed of approximately 80.7% of African, 8.8% of mixed, 7.9% of European, and 2.6% of Asian ancestries (26). Several of the established genetic loci have been replicated in populations of African ancestry using the candidate gene approach (38–40), and a few using the GWAS approach (41,42). Novel genes implicated in NCD risk factors from GWAS in populations of African ancestry included *AGMO*, which was associated with type 2 diabetes (42), and *ZRANB3*, which was associated with beta-cell mass and insulin response (41).

1.3. The metabolic syndrome

The combination of the modifiable and non-modifiable risk factors can lead to the development of intermediary risk factors, which ultimately lead to NCDs (43). The common intermediary risk factors include elevated waist circumference, insulin resistance, elevated blood pressure, and dyslipidaemia. Although these can occur individually, they are usually clustered together in an interrelated fashion. When this occurs in any individual, the condition is referred as the metabolic syndrome (44).

The constellation of the intermediary risk factors was first reported in the 1920s by Joslin (45) and Kylin (46), who observed that type 2 diabetes was associated with elevated blood pressure and circulating uric acid. Although elevated uric acid is primarily caused by gout, it is also common in the metabolic syndrome (47). Hence, this condition was first termed “The hypertension-hyperglycaemia-hyperuricemia syndrome” (46). In the 1940s, it was observed that elevated waist circumference significantly predisposed individuals to type 2 diabetes, cardiovascular diseases, and gout (48). Finally, the term “metabolic

syndrome” was first used in the late 1950s, after it was observed from the Framingham study that the intermediary risk factors such as obesity, type 2 diabetes, elevated blood pressure, and dyslipidaemia were occurring at a greater degree than expected by chance (49). After the first use of the term “metabolic syndrome”, several studies describing the metabolic syndrome were reported in the 1960s and 1970s (50–53). As the interrelationships between the components of the metabolic syndrome were largely unknown, there was no clear clinical definition for the diagnosis until it was proposed by Reaven that insulin resistance was the primary link (54).

1.3.1. Previous clinical definitions of the metabolic syndrome

Several attempts were made to devise a clear clinical definition of the metabolic syndrome. The first formal definition was formulated by a World Health Organization (WHO) consultation group in 1998 (55). The group proposed that the diagnosis should be based on markers of insulin resistance in addition to two of the following risk factors: obesity, elevated blood pressure, reduced high-density lipoprotein (HDL) cholesterol, elevated triglycerides, or microalbuminuria. Another clinical definition was proposed in 2002 by the National Education Program Adult Treatment Panel III (ATP III) (56). Their definition did not require a demonstration of any single factor. Instead, the ATP III definition stated that diagnosis should be based on the presence of any three of the following five components: elevated waist circumference, elevated fasting glucose levels (or type 2 diabetes), elevated triglycerides, reduced HDL cholesterol, and elevated blood pressure. Other definitions were proposed in 2005 by the American Heart Association: National Heart, Lung, and Blood Institute (AHA/NHLBI) (57) and the International Diabetes Federation (IDF) (58). The AHA/NHLBI definition was similar to that proposed by the ATP III and did not rely on a single component. In contrast, the IDF definition mandated elevated waist circumference as a required risk factor for diagnosis.

1.3.2. The harmonized clinical definition

The existence of different clinical definitions led to inconsistencies in epidemiological studies relating to the metabolic syndrome. Thus, the Joint Interim Statement was released in 2009 by various organizations (IDF, AHA/NHLBI, International Atherosclerosis Society, and International Association for the Study of Obesity), which proposed a harmonized clinical definition of the metabolic syndrome (44). According to the Joint Interim Statement, a clinical diagnosis of the metabolic syndrome requires the presence of three or more of the following components: elevated waist circumference, elevated triglycerides, reduced HDL cholesterol, elevated blood pressure, and elevated fasting glucose (specific cut-points described in Table 1.1). These are commonly referred to as "components of the metabolic syndrome". Due to lack of data from populations of African ancestry, the recommended cut-points indicated in Table 1.1 were largely based on studies conducted in populations of European ancestry.

Table 1.1: Criteria for definition of the metabolic syndrome in populations of African ancestry (44)

Component	Categorical cut point
Elevated waist circumference	≥ 94 cm in men, ≥ 80 cm in women
Elevated triglycerides	≥ 1.7 mmol/L (and/or using cholesterol-lowering medication)
Reduced HDL-cholesterol	< 1.0 mmol/L in men (and/or using triglyceride medication), < 1.3 mmol/L in women (and/or using HDL cholesterol medication)
Elevated blood pressure	≥ 130 mmHg for systolic and/or ≥ 85 mmHg for diastolic and/or using blood pressure medication
Elevated fasting glucose	≥ 5.6 mmol/L and/or using diabetes medication

The table is adapted from (44). The presence of three or more of any of the listed components defines the metabolic syndrome.

1.3.3. Prevalence of the metabolic syndrome in South Africa

Several cross-sectional studies were conducted in South Africa to estimate the prevalence of the metabolic syndrome. These studies are summarized in Table 1.2, which was modified from a previous review (59), and only included studies with at least 100 participants. The majority of these studies reported sex comparisons, and generally indicated a higher prevalence of the metabolic syndrome in women (range: 8% to 86.6%) compared to men (range: 7% to 74.1%) (60–72). This was no surprise as the metabolic syndrome is highly linked to obesity, and the prevalence of obesity is much higher in South African women compared to men (14).

Due to the greater risk of developing the metabolic syndrome in South African populations of African ancestry in comparison to their European ancestry counterparts (9), most of these observational studies of the metabolic syndrome were conducted in South African populations of African ancestry (61–64,71–76), and only a few compared the prevalence to other ethnic groups (60,66). The prevalence of the metabolic syndrome was generally higher in South African women of African ancestry (range: 8% to 85.2%) compared to South African men of African ancestry (range: 7% to 46.5%) (60–64,66,69–76). The differences in the prevalence in each study may be due to differences in the age range of the participants, the criteria used for diagnosis, and exposure to different environmental factors (e.g. rural versus urban areas) (59).

Table 1.2: The prevalence of the metabolic syndrome in South Africa

Ref	Location & ancestry	Study design & setting	N	Age (years)	Criteria used	Prevalence (%)	
						Men	Women
(73)	Potchefstroom (African)	Cross-sectional (Urban)	102	31.3 ± 8.6	IDF	N/A	24.8
(60)	Johannesburg (African & European)	Cross-sectional (Urban; with type 2 diabetes)	500	>30	IDF	European: 74.1 African: 46.5	European: 86.6 Africans: 85.2
(74)	Cape Town (African)	Cross-sectional (Urban)	225	27.0 ± 7.0	IDF	N/A	9.9
(61)	KwaZulu-Natal (African)	Cross-sectional (Rural)	947	>15	Harmonized	11.6	30.2
(65)	Belville, Cape Town (Mixed)	Cross-sectional (Urban)	563	50.9 ± 9.1	IDF	30.6	67.8
(75)	Soweto, Johannesburg (African)	Cross-sectional (Urban)	1251	18-84	Harmonized	N/A	42.1
(66)	Soweto, Johannesburg (African & Asian)	Cross-sectional (Urban)	724	18-70	Harmonized	Asian: 55.8 African: 19.3	Asian: 37.4 African: 39.2
(76)	Cape Town (African)	Cross-sectional (Urban)	1 099	25-74	Harmonized	16.5	43.0
(69)	Potchefstroom (African & European)	Cross-sectional (Urban)	907	43.0 ± 11.8	Harmonized	40.7	34.9
(70)	Cape Town (74.6% Mixed)	Cross-sectional (Urban)	489	46.3 ± 8.5	Harmonized	17.8	70.3
(71)	Boland, Western Cape (African)	Cross-sectional (Rural)	188	20-60	IDF	29	46
(77)	Durban (African, Mixed, Asian, & European)	Cross-sectional (Urban)	232	18-65	Harmonized	10	35.9
(72)	Mthatha, Eastern Cape (African)	Cross-sectional (Rural)	371	13-18	Harmonized	7	8

(62)	Eastern Cape (African)	Cross-sectional (Rural)	998	42.6 ± 16.5	Harmonized	15.6	24.8
(63)	Ellisras, Limpopo (African)	Cross-sectional (Rural)	627	18-30	Harmonized	9.8	30.8
(64)	Cape Town (African & Mixed)	Cross-sectional (Rural)	748	32-44	Harmonized	16.6	31.3

Ref: Reference; **Age:** Age range or mean age ± standard deviation as provided by the respective journal articles; **IDF:** International Diabetes Federation; **N:** Number of participants; **N/A:** Data not provided by the Journal article.

1.3.4. Ethnic disparities in components of the metabolic syndrome: why an interest in populations of African ancestry?

Indeed, studies that compared the ethnic differences in the prevalence of the metabolic syndrome are limited. However, the individual components of the metabolic syndrome have been extensively studied in populations of African ancestry and compared to other ethnic groups. Many of the reported studies compared populations of African ancestry to their European ancestry counterparts and suggested ethnic-specific differences in the associations of the metabolic syndrome components between populations of African and European ancestry (9,78–80).

Centralization of body fat

Studies conducted predominantly in populations of European ancestry demonstrated strong associations between increased centralized body fat, particularly visceral adipose tissue (VAT), and insulin resistance (the proposed primary link in the metabolic syndrome) (81). Hence, positive associations between elevated waist circumference or VAT, insulin resistance and the other components of the metabolic syndrome are traditionally acknowledged (82). On the other hand, increased accumulation of subcutaneous adipose tissue (SAT) in the peripheral (gluteal-femoral) body regions has been traditionally

associated with improved insulin sensitivity, and consequently lower risk of developing the metabolic syndrome components (83).

In contrast, studies comparing populations of African and European ancestries do not support the hypothesis that the VAT-insulin resistance relationship is the primary link in the metabolic syndrome. Populations of African ancestry are more obese and more insulin resistant than their European ancestry counterparts (84–88). However, the differences in insulin sensitivity between these two ethnic groups are independent of body fat and its distribution (89). Dual energy X-ray absorptiometry studies demonstrated that for the same level of total body adiposity, populations of African ancestry have lower waist circumference compared to other ethnic groups (90,91). Furthermore, as waist circumference increases, populations of African ancestry accumulate less VAT than their European ancestry counterparts (89,91–95), and VAT does not correlate as strongly with insulin resistance as shown in the populations of European ancestry (89). Regardless of these ethnic differences, centralization of body fat (VAT and SAT) is positively associated with insulin resistance, while leg fat mass has the opposite association, in populations of African ancestry as shown in populations of European ancestry (91).

Notably, there are no agreed cut-offs for centralization of body fat (waist circumference) in populations of African ancestry, and thresholds derived from studies in populations of European ancestry are currently recommended (44). Nevertheless, several studies suggested that the current recommended waist cut-offs are not appropriate for diagnosis of the metabolic syndrome in populations of African ancestry (62,75,96). Indeed, more evidence is still warranted to understand the relationship between centralization of body fat and the metabolic syndrome components in populations of African ancestry.

Elevated blood pressure

It is well-acknowledged that populations of African ancestry are disproportionally affected by elevated blood pressure when compared to other ethnic groups (67). However, epidemiological studies comparing the prevalence of elevated blood pressure in populations of African ancestry and populations of European ancestry have been inconsistent. For example, studies from developed nations suggested a higher prevalence of elevated blood pressure in populations of African ancestry when compared to their European ancestry counterparts (97–99). On the contrary, the prevalence of elevated blood pressure was lower in populations of African ancestry from developing nations (14) and in larger global studies (100), reflecting the importance of considering environmental factors. In South Africa, the prevalence of elevated blood pressure was highest in populations of European ancestry (66% in men and 60% in women) and lowest in populations of African ancestry (41% in men and 44% in women) in 2016 (14). Regardless of these inconsistencies in elevated blood pressure prevalence, distinct ethnic disparities in the aetiology, control, and treatment of elevated blood pressure in the populations of African ancestry, when compared to populations of European ancestry, have been consistently suggested for decades (101).

Several studies demonstrated that in comparison to populations of European ancestry, populations of African ancestry are more resistant to elevated blood pressure treatment (102) and consequently blood pressure is more difficult to control in populations of African ancestry (103). These observations may be attributed to ethnic differences in the pathophysiology of elevated blood pressure. Indeed, the regulation of blood pressure is complex and various pathophysiological factors have been associated with elevated blood pressure (104–106). Studies conducted in populations of African ancestry investigated the following physiological mechanisms involved in the elevated blood pressure pathophysiology: renin-aldosterone-angiotensin system (RAAS) activation

(107), endothelial dysfunction (108,109), kidney injury and function (110), insulin resistance (111), impaired baroreflex (112), oxidative stress (113), adrenomedullary/cortical hormones (114), blood volume (115), and salt retention (116,117).

A comparison between populations of African and European ancestry revealed distinct differences in the RAAS regulation and the related biochemical profiles (103). In comparison to populations of European ancestry, sodium in populations of African ancestry is excreted more slowly and less completely (118), leading to a suppression of the RAAS (119), and ultimately a low renin state (120). Certainly, low renin hypertension is more common in populations of African ancestry when compared to populations of European ancestry (121). In general, populations of African ancestry with elevated blood pressure appear to have a distinct biochemical profile, which is characterized by low renin plasma activity or concentrations, low or high plasma aldosterone concentrations, and low angiotensin I and II (103).

As elevated blood pressure is a major risk factor for cardiovascular diseases, the observed ethnic differences may partly explain the higher death rates attributed to cardiovascular diseases (particularly cerebrovascular disease, hypertensive heart disease, ischaemic heart disease and cardiomyopathy; see Figure 1.1) in South African individuals of African ancestry when compared to other South African ethnic groups (7).

Dyslipidaemia

Another metabolic syndrome component that is highly linked to cardiovascular diseases is dyslipidaemia. Dyslipidaemia is basically characterised by elevated triglycerides, total cholesterol, low-density-lipoprotein (LDL) cholesterol, and reduced HDL cholesterol concentrations (122). In comparison to their European ancestry counterparts, populations of African ancestry have lower circulating triglycerides, total cholesterol, and LDL

cholesterol concentrations (123–129). However, HDL cholesterol concentrations in populations of African ancestry were higher in studies from developed nations such as the United State of America (123,125), but lower or similar in developing nations such as South Africa (124,126,127), when compared to their European ancestry counterparts, suggesting mediation by environmental factors.

Studies in predominantly populations of European ancestry demonstrated that dyslipidaemia is strongly influenced by VAT (130) and insulin resistance (122). VAT partly influences the serum lipid profile by increasing the liver fat content, which subsequently increases the production of very low-density lipoprotein, and circulating triglycerides (131). Therefore, the favourable lipid profile observed in populations of African ancestry may be related to the lower VAT in this population when compared to populations of European ancestry (132). In support of this notion, populations of African ancestry were found to have less fat in the peritoneal cavity (VAT) and liver, after adjusting for age and total body fat (133). Further, the prevalence of fatty liver was lower in populations of African ancestry when compared to other ethnic groups (134). However, populations of African ancestry had lower circulating lipid concentrations even after adjusting for fat liver content, suggesting the involvement of other mechanisms (133). Certainly, serum lipids are influenced by several physiological factors, including the activities of lipid metabolizing enzymes such as lipoprotein lipase (LPL) which hydrolysis circulating triglycerides, and cholesteryl ester transfer protein (CETP) which catalyses the transfer of triglycerides and cholesteryl esters between lipoproteins (135). In a South African study that compared women of African ancestry to their European ancestry counterparts, SNPs in genes encoding these two enzymes (LPL and CETP) were associated with a favourable lipid profile in women of African ancestry, but not in their European ancestry counterparts, suggesting the involvement of a genetic component in these ethnic disparities (128).

1.4. Glucocorticoids

1.4.1 The role of glucocorticoids in the metabolic syndrome

Chronic exposure to excess glucocorticoids, from either endogenous cortisol or exogenous pharmacological sources, leads to a pathological condition known as Cushing's syndrome (136). The striking similarities between Cushing's syndrome and the metabolic syndrome have led to the hypothesis that glucocorticoids may be involved in the development of the metabolic syndrome (137,138).

Cushing's syndrome is known to affect the central nervous system, eyes, gastrointestinal tract, musculoskeletal tissue, skin, immune system, endocrine system, and metabolism (see Figure 1.4) (136). The metabolic features exhibited by individuals with Cushing's syndrome include elevated waist circumference, fasting glucose, blood pressure and the presence of dyslipidaemia, which are the primary components of the metabolic syndrome.

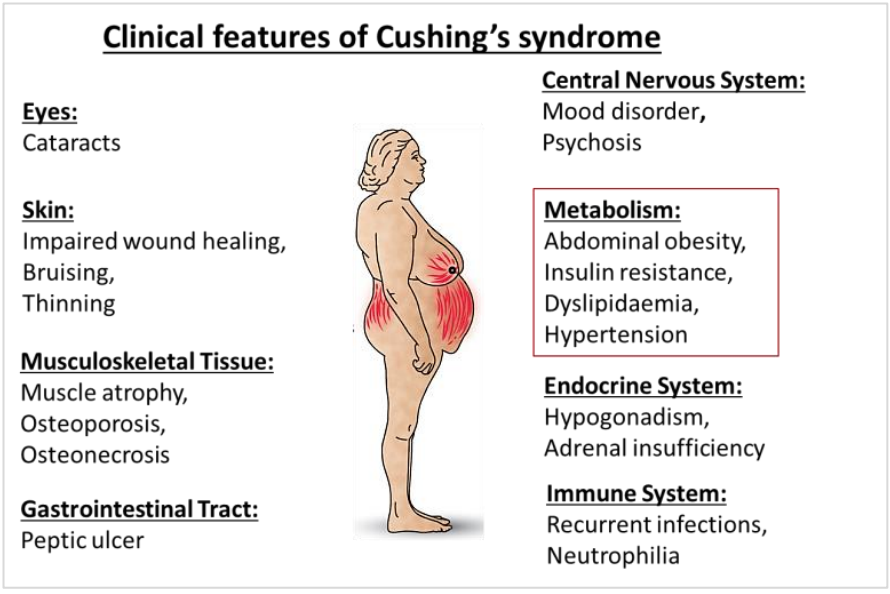


Figure 1.4: Clinical features of Cushing's syndrome (136)**.** ***Figure redrawn by me.***

Several clinical changes occur in Cushing's syndrome, and these may include the primary components of the metabolic syndrome**.**

1.4.2 What are glucocorticoids?

Glucocorticoids are a class of steroid hormones (Figure 1.5), that are naturally produced by all vertebrae (139), but are also synthesized and widely used in drugs for medical conditions such as adrenal insufficiency (140), allergies (141), chronic inflammation (142), autoimmune diseases (143), asthma (144), organ transplant (145), and cancer (146). Humans produce two glucocorticoids: cortisol and corticosterone (Figure 1.5), and they are distinguished from mineralocorticoids and sex hormones by their specific receptor binding, target cells, and physiological functions.

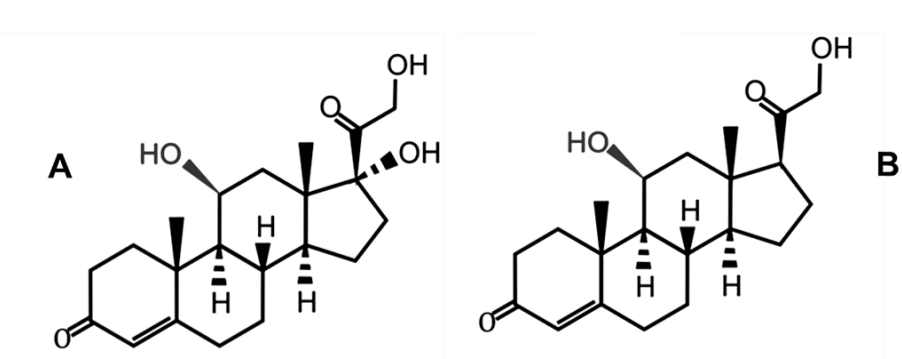


Figure 1.5: Chemical structures of the human glucocorticoids: cortisol (A) and corticosterone (B) (147).

Figure redrawn by me.

1.4.3 Glucocorticoid production and regulation

The hypothalamic pituitary adrenal axis

Glucocorticoids are produced via the hypothalamic pituitary adrenal (HPA) axis (see Figure 1.6). The HPA axis is activated by stress which triggers the hypothalamus to secret corticotropin-releasing hormone (CRH). Subsequently, CRH elicits the production of adrenocorticotropin hormone (ACTH) from the pituitary gland. Finally, ACTH stimulates the adrenal cortex to produce glucocorticoids (148). The produced glucocorticoids are involved in a negative feedback loop to inhibit both CRH and ACTH (148).

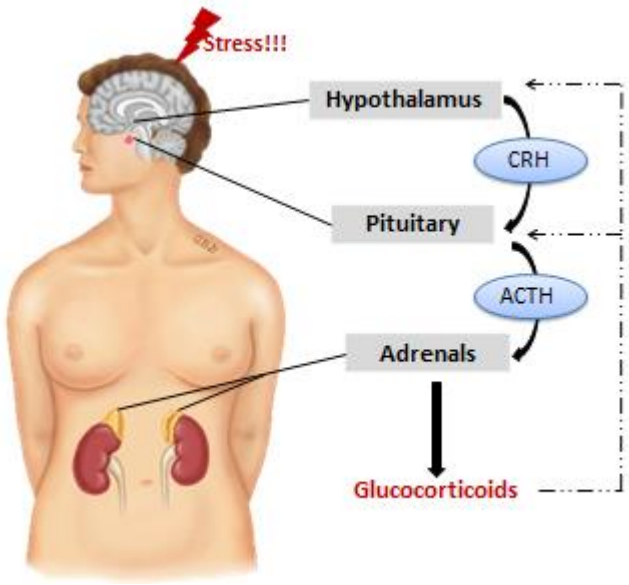


Figure 1.6: The production of glucocorticoids via the HPA axis. ***Figure modified with permission*** **(149)**.

Biosynthetic pathways

In the adrenal cortex, glucocorticoids are produced via steroidogenesis, a biosynthetic pathway that utilizes cholesterol to produce a wide range of steroid hormones (mineralocorticoids, progestogens, glucocorticoids, androgens, and oestrogens), through a series of enzymatic steps (Figure 1.7). Although cholesterol can be synthesized by the human adrenal tissue (150), most of the cholesterol originates from circulating LDLs (151). ACTH stimulates both *de novo* synthesis of cholesterol and uptake of LDL cholesterol into the steroidogenic cell via the LDL receptor (152). Once inside the target cell, cholesterol can either be directly used for *de novo* synthesis of steroid hormones or stored into lipid droplets where it can be retrieved by hormone-sensitive lipase (HSL). HSL is stimulated by ACTH in order to increase available free cholesterol for steroidogenesis (153).

The higher production of cortisol over corticosterone is primarily dependent on the relative function of the 17α-hydroxylase enzyme, as its converts precursors for corticosterone synthesis (pregnenolone and progesterone) to precursors for cortisol synthesis (17α-hydroxy-pregnenolone and 17α-hydroxy-progesterone, respectively). Hence, the enzyme

plays a key role in determining the ratio of corticosterone/cortisol in circulation (154). A different class of enzymes called 11β-Hydroxysteroid dehydrogenase (11β-HSD) determines the levels of active glucocorticoids within target tissues (155). This enzyme has two isoforms in humans, the 11β-HSD2 (type 2) which converts the active glucocorticoids (corticosterone and cortisol) to their inactive forms (11-dehydrocorticosterone and cortisone, respectively), and the 11β-HSD1 (type 1) which catalyses the reverse reaction (156). Certainly, the 11β-HSD enzymes are highly expressed in glucocorticoid sensitive tissues such as the adipose tissue, liver, and central nervous system (CNS) (157).

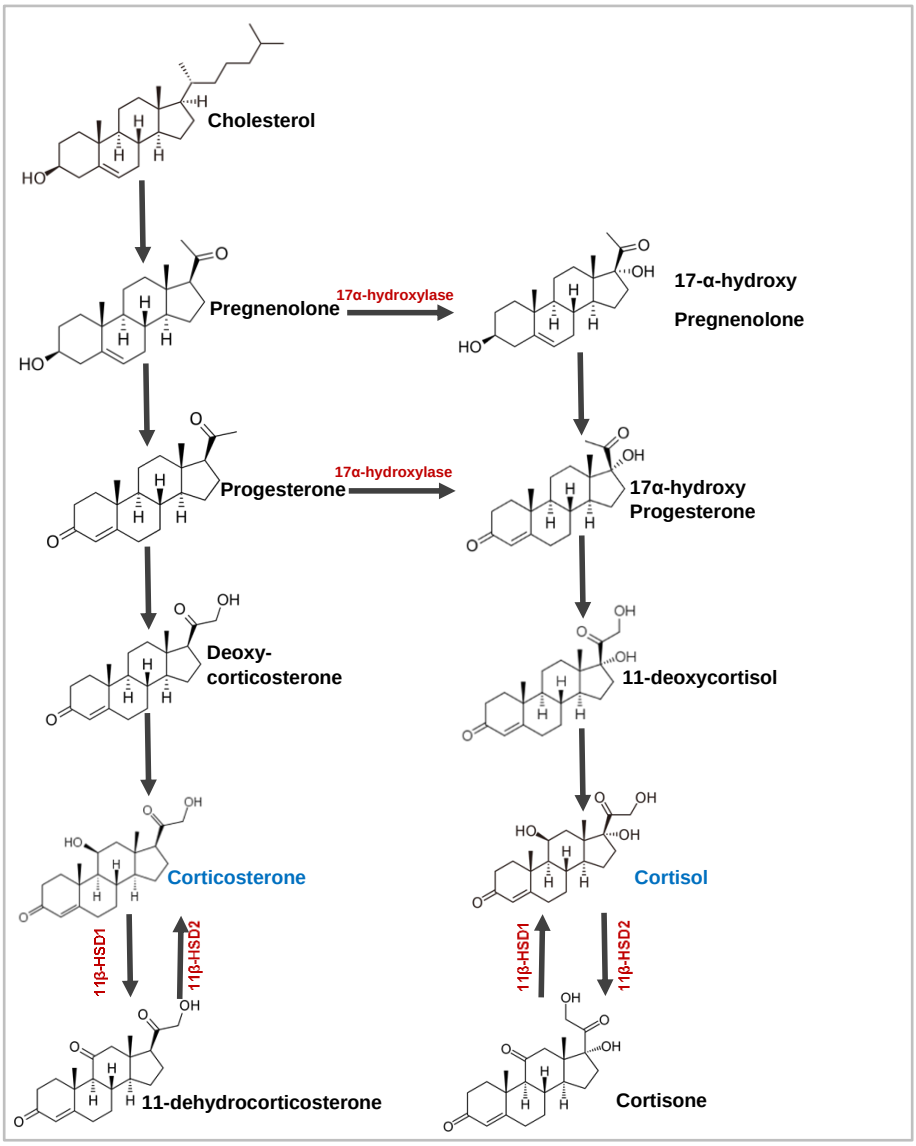


Figure 1.7: The biosynthesis of glucocorticoid hormones in humans. ***Figure redrawn by me.***

The pathway synthesizes the two **glucocorticoids (corticosterone and cortisol)** from cholesterol in the adrenal cortex. The ratio of corticosterone/cortisol is determined by the relative activity of **17α-hydroxylase**. The two glucocorticoids are active and bind to GRs, but they can be converted to their inactive forms (11-dehydrocorticosterone and cortisone, respectively) within the target tissues, by the action of **11β-HSD2**.

Glucocorticoid binding and release

There are many other physiological factors that determine the level of circulating glucocorticoids, and their actions within the target tissues. One of the key factors is their modulation by binding proteins (158). Notably, most circulating glucocorticoids are not free to activate their target receptors, instead they are bound to carrier proteins. Studies suggest that only about 5% of circulating glucocorticoids are unbound, whilst more than

85% are tightly bound to a carrier protein called corticosteroid binding globulin (CBG), and the remaining 10% are loosely bound to other carrier proteins, primarily albumin (159).

The CBG carrier protein serves not only as the main transport protein for glucocorticoids in the blood, but also plays a significant role in modulating their release into the target tissues (160). CBG binds glucocorticoids within its surface pocket which underlies the main β-sheet (161). The glucocorticoid-CBG complex possesses a fully exposed reactive centre loop (RCL) which is cleaved by elastase (derived from activated neutrophils) during inflammation. Cleavage of the RCL results to structural transition of CBG from a stressed high affinity glucocorticoid binding state to a relaxed low affinity glucocorticoid binding state. The conformational change is due to insertion of the loop into the CBG body (160). Such a conformational change allows the release of glucocorticoids to target tissues (see Figure 1.8). While this seems like a simple mechanism of glucocorticoid release; it is not just an on-off switch system but rather depends on several factors (e.g. genetics, body temperature, and glycosylation state) that influence CBG function and the plasticity of its binding site (162,163). Notably, alpha-1-antitrypsin (AAT) is a known inhibitor of neutrophil elastase, the enzyme that cleaves RCL of CBG (164). Thus, circulating AAT levels may be important in the binding and release of glucocorticoids by CBG (163).

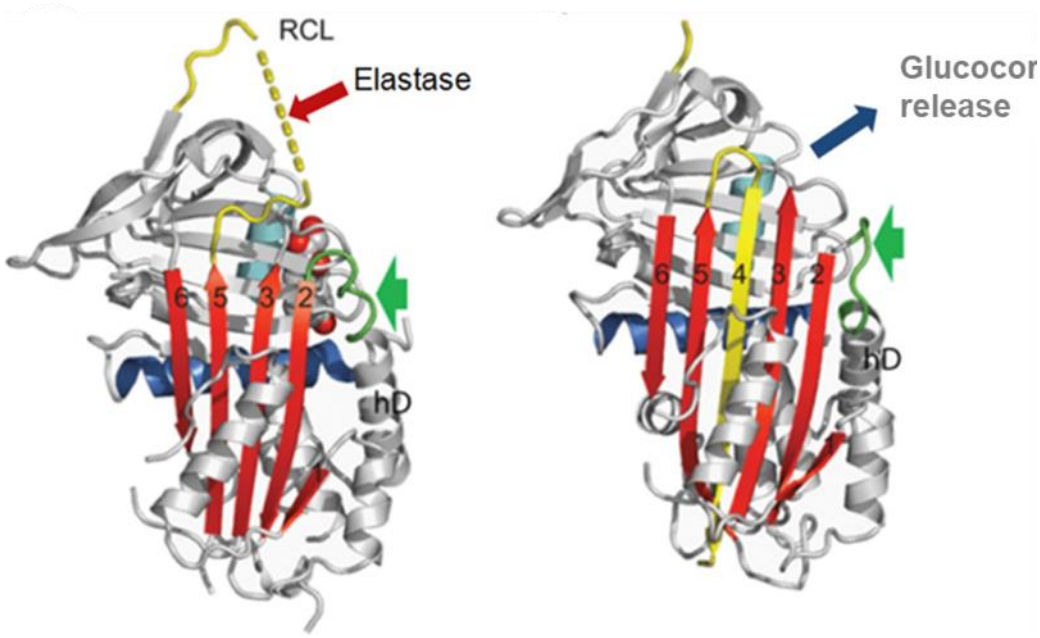


Figure 1.8: The binding and release of glucocorticoids by CBG. *Figure modified with permission* (160).

The cleavage of the glucocorticoid-CBG complex by elastase at target tissues (sites of inflammation) results to a conformational change of the complex, from a tightly bound (left) to a more relax form (right) which allows the release of glucocorticoids.

Glucocorticoid clearance

Only less than 1% of glucocorticoids are excreted directly by the renal tissue (165). Most of the glucocorticoid clearance occurs through an A-ring reduction process in the hepatic tissue (165). Key enzymes involved in the A-ring reduction pathway include 5α- and 5β-reductases, which convert the glucocorticoids to 5α- and 5β-dihydro-metabolites, respectively; and 3α-hydroxysteroid dehydrogenase (3α-HSD) which catalyses the conversion of the dihydro-metabolites to tetrahydro-corticosteroids (Figure 1.9) (166). The tetrahydro-corticosteroids are subsequently conjugated with either glucuronic acid or sulphate, which enhance their water solubility, and are excreted in the urine (167).

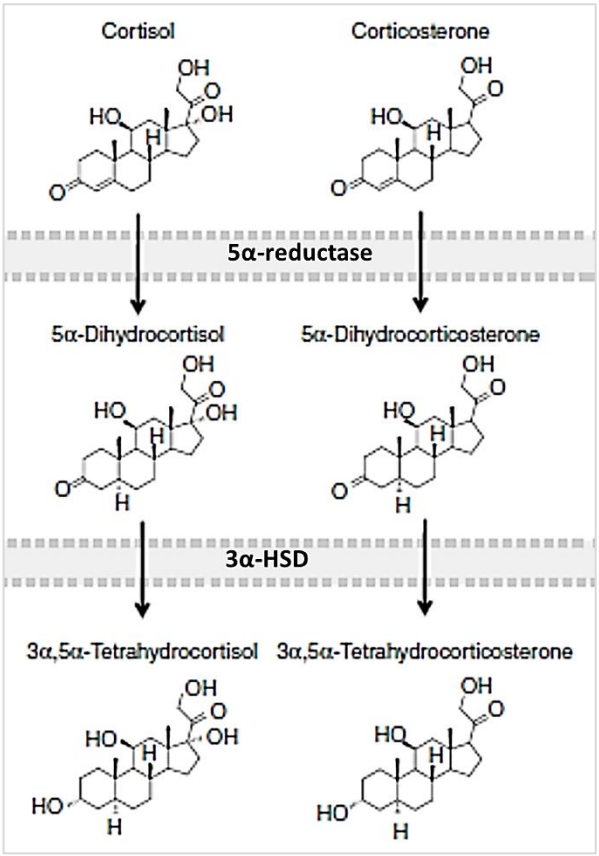


Figure 1.9: Metabolic clearance of glucocorticoids by 5α-reductase and 3α-HSD, to generate tetrahydro-glucocorticoids*. Figure used with permission* (166).

1.4.4 Physiological functions of glucocorticoids

Glucocorticoids have a wide range of physiological functions in humans. As they are produced in response to stress, their primary functions include the regulation of many physiological processes to maintain homeostasis (168). Glucocorticoids exert their intracellular actions by binding to either glucocorticoid receptors (GRs) or mineralocorticoid receptors (MRs). However, the distribution of MR is restricted to specific tissues, whilst GRs are widely expressed at higher levels in most tissues (169). The GR and MR reside in the cytoplasm where they are bound to chaperone proteins (e.g., the heat shock protein 90). However, the binding of glucocorticoids to these receptors forms a complex that trans-locates to the nucleus. In the nucleus the GR binds to glucocorticoid receptor elements in the promoter region of the target genes, in order to regulate their expression (170). This mechanism of regulation of expression is called transactivation.

Alternatively, glucocorticoids can also regulate genetic expression via trans-repression, whereby the GR binds to transcription factors to inhibit the upregulation of their target genes (171). The key glucocorticoid functions can be classified into either anti-inflammatory or metabolic, but glucocorticoids are also involved in other functions such as in body fluid homeostasis (172) and foetal development (173).

Anti-inflammatory effects

Glucocorticoids are well acknowledged for their anti-inflammatory functions in humans. For example, glucocorticoids inhibit the inflammatory response by down-regulating the expression of proinflammatory proteins, such as interleukin-1β, interleukin-6, and tumour necrosis factor-α (155), (174). Glucocorticoids are also known to suppress the genetic expression of chemokines (e.g. chemokine (C-C motif) ligand 2, interleukin-8, and macrophage-inflammatory protein-1α) and adhesion molecules (e.g. E-selectin and adhesion molecule-1) required for the recruitment of inflammatory cells to inflammation sites (175). In contrast, glucocorticoids also exhibit anti-inflammatory effects by up-regulating the genetic expression of key anti-inflammatory proteins (e.g. annexin-1, interleukin-4, and interleukin-10) (176).

Metabolic functions

As glucocorticoids are produced in response to perceived stress, metabolism is directed towards short-term benefits, and functions such as bone formation (177) and reproduction are inhibited (178). One of the key metabolic functions of glucocorticoids is promoting gluconeogenesis in the liver (179). The process occurs primarily through up-regulation of genetic expression of the key gluconeogenic enzymes, including glucose-6-phosphatase (180), phosphoenolpyruvate carboxykinase (181), and tyrosine aminotransferase (182), but also through mobilization of essential gluconeogenesis substrates to the liver (179). Further, glucocorticoids also promote glycogenolysis through up-regulation of glycogen synthase genetic expression (183).

The presence of glucocorticoids in peripheral tissues, such as the skeletal muscle and the adipose tissue, also leads to key metabolic changes. For example, in the skeletal muscle tissue, the uptake of glucose is significantly reduced (184) (179) and proteolysis is promoted to increase amino acid concentrations required as substrates for gluconeogenesis (185). Likewise, in the adipose tissue, the uptake of glucose is significantly reduced (184) (179), but the effects of glucocorticoids on lipid metabolism are largely dependent on the presence of other hormones such as insulin. For example, when the concentration of insulin is low, glucocorticoids promote lipolysis, but when the concentration of insulin is high, glucocorticoids promote lipid storage (183,186).

1.5. A separate role of corticosterone in human health and disease

Since it was previously assumed by many that the roles of corticosterone are identical to those of cortisol in humans (187), studies investigating the role of corticosterone in human diseases are greatly lacking. Nevertheless, accumulating evidence suggests that corticosterone may play a separate role in human health and disease.

1.5.1 Lower circulating corticosterone concentrations when compared to cortisol

The first evidence that suggested a separate role of corticosterone in human health and disease came from a comparison of its circulating concentrations with those of cortisol in humans. Under normal physiological conditions, corticosterone circulates at concentrations of about 5-10% of those of cortisol. The early corticosterone studies were predominantly conducted in populations of European ancestry and the concentrations varied widely (see Table 1.3). The variability in the reported values may have been due to differences in the specificities of the assay methods used. For instance, early studies used methods with poor specificity such as chromatographic purification methods combined with fluorescence detection (188,189). Subsequently, more specific techniques

such as radioimmunoassay and liquid chromatography-mass spectrometry (LCMS) were introduced for measuring circulating glucocorticoids (190,191). Although, both radioimmunoassay and LCMS methods are commonly used in clinical research, the LCMS method is preferred due to its higher specificity and sensitivity compared to radioimmunoassay. Although the methods for detecting glucocorticoid levels have been available for decades, only a limited number of studies reported circulating corticosterone concentrations, and these are shown in Table 1.3. Furthermore, the number of participants in most of these studies was relatively small. Nevertheless, the circulating corticosterone levels were in the range of 10-20 nmol/L in healthy subjects, but in subjects undergoing an induced stress response or sickness, circulating corticosterone was elevated to a maximum of 67.6 nmol/L (192). Since cortisol circulates in the blood at 10- to 20-fold higher concentrations than corticosterone, the reported ratio of corticosterone/cortisol ranged between 0.03-0.42 nmol/L in these studies.

Table 1.3: Studies describing circulating corticosterone (± cortisol) in populations of European ancestry.

Ref	N	Age (years)	Corticosterone (nmol/L)	Cortisol (nmol/L)	Corticosterone/cortisol	Sampling time	Method
(188)	21	NS	124.1 ± 14.4	298.0 ± 16.6	0.416	NS	Fluorescence
(190)	30 (18 Males; 12 Females	NS	31.7 ± 11.5	386.2 ± 33.1	0.082	NS	Isotope dilution & fluorescence
(189)	20	NS	86.6 ± 5.8	300.7 ± 15.1	0.288	NS	Fluorescence
(191)	20	NS	28.9 (23.1-51.9)	Not measured	N/A	NS	Isotope dilution & fluorescence
(193)	29	NS	19.0 (3.8-66.4)	270.3 (85.5-557.0)	0.067	NS	Double isotope
(194)	9 Males 10 Females	26.8 ± 2.6 31.3 ± 4.4	47.2 ± 6.0 49.7 ± 6.5	403.0 ± 37.5 422.4 ± 67.8	0.124 0.118	10:45-11:45	Fluorescence
(195)	10 (8 Males; 2 Females)	21-34	26.3 ± 3.2	634.5 ± 55.2	0.041	09:00	Radioimmunoassay
(196)	8	NS	11.5 ± 0.9	339.3 ± 22.1	0.034	NS	Competitive-protein binding
(197)	18	NS	12.1 ± 2.6	383.4 ± 4.0	0.030	09:00	Double isotope
(198)	15 Males 9 Females[1] 9 Females[2]	19-50	11.4 ± 1.7 20.4 ± 2.0 16.7 ± 2.1	386 ± 36 386 ± 36 359 ± 21	0.030 0.053 0.047	08:00	Radioimmunoassay
(199)	11	20-55	13.7 ± 2.6	Not measured	N/A	08:00	Radioimmunoassay
(200)	5 Female[1] 5 Female[2]	NS NS	10.4 ± 3.7 15.9 ± 6.8	Not measured	N/A	08:00-10:00	Radioimmunoassay
(201)	10	NS	23.0 ± 3.0	419.6 ± 28.7	0.055	09:00	Radioimmunoassay
(202)	31 Males 16 Females[1] 15 Females[2]	NS NS NS	12.2 ± 1.1 7.0 ± 1.1 12.7 ± 1.7	Not measured	N/A	08:00-11:00	Radioimmunoassay

Data presented as mean (±SEM) or range (). N: Number of participants; [1]Follicular phase; [2]Luteal phase; **NS:** Not specified; **N/A**: Not applicable.

Table 1.3 (continued): Studies describing circulating corticosterone (± cortisol) in populations of European ancestry

Ref	N	Age (years)	Corticosterone (nmol/L)	Cortisol (nmol/L)	Corticosterone/ cortisol	Sampling time	Method
(203)	20	NS	6.9 ± 2.6	Not measured	N/A	09:30	Enzyme Immunoassay
(204)	10	NS	11.7 ± 0.9	Not measured	N/A	08:00	Radioimmunoassay
	10	NS	4.4 ± 0.6			16:00	
(205)	11 Males	57 ± 1.9	16.4 ± 3.1	350.0 ± 81.5	0.047	NS	LCMS
(192)	16 Males	39.2 (23-70)	58.4 ± 9.2	830.4 ± 68.4	0.069	08:00-09:00	Radioimmunoassay
(154)	2018 (794 Males; 1224 Females)	53.3 ± 0.3	50.7 ± 1.2	724 ± 7	NS	07:30-11:00	Radioimmunoassay
	279 (189 Males; 90 Females) *	71.3 ± 0.2	20.2 ± 1.0	188 ± 6	NS	08:00-10:20	Radioimmunoassay
	903 (476 Males; 427 Females)	67.9 ± 0.1	22.3 ± 0.5	732 ± 6	NS	08:00-08:30	Radioimmunoassay
	2098 (838 Males; 1260 Females)	49.8 ± 0.3	20.3 ± 1.4	293 ± 4	NS	NS	Radioimmunoassay

Data presented as mean (±SEM) or range (). N: Number of participants; **NS:** Not specified; **N/A**: Not applicable; **LCMS:** Liquid chromatography mass spectrometry. *****Participants were subjected to dexamethasone suppression.

1.5.2 Corticosterone and cortisol may be differently regulated by the HPA axis

Although both corticosterone and cortisol are produced as end products of the HPA axis, the two glucocorticoids may respond differently to the HPA axis regulatory system. For example, corticosterone showed a much higher response to ACTH stimulation compared to cortisol in healthy participants (190). A higher corticosterone response was also observed in patients that were undergoing surgical stress (206) and hypoglycaemia (193). The differences in the response when comparing corticosterone to cortisol may have been partly related to differences in the susceptibility to trans-membrane transport by the adenosine triphosphate (ATP)-binding cassette (ABC) transporters.

1.5.3 Corticosterone and cortisol interact differently with ABC transporters

Additional evidence of a separate role of corticosterone in human health and disease was also suggested when ABC transporters were shown to differentially transport the two glucocorticoids (corticosterone and cortisol) across the cell membrane. ABC transporters are a class of active membrane pumps that utilize ATP hydrolysis to transport substrates across the cell membrane. This class of membrane pumps includes both importers and exporters.

ABCB1 is also known as P-glycoprotein or multidrug resistance protein 1 (MDR1) and was the first ABC protein to be characterized after the discovery of its role in cancer drug resistance. Early animal studies suggested that ABCB1 transporter also plays a role in the transport of glucocorticoids (207). However, when compared to corticosterone, the retention of cortisol was found to be lower in the rodent brain (208). Using knockout mouse models and radiolabelled substrates, several studies reported an increased uptake of cortisol and synthetic glucocorticoids, but not corticosterone by the CNS (205,209).

The findings that cortisol is selectively exported by ABCB1 in the CNS were also supported by data from *in vitro* and human studies. For example, *in vitro* studies demonstrated that the 17-hydroxyl group, which is present in cortisol but not in corticosterone, was required for glucocorticoids to serve as an ABCB1 substrate (210). Human studies suggested that the selective exportation of cortisol over corticosterone by ABCB1 occurred at the blood-brain barrier, and this may account for the major differences in the corticosterone/cortisol ratio when comparing the cerebrospinal fluid to the blood (192,211).

While ABCB1 exports cortisol over corticosterone in the CNS, another ABC transporter called ABCC1 exports corticosterone over cortisol in tissues outside the CNS. Contrary to ABCB1, the ABCC1 membrane transporter is not expressed in the CNS but is highly expressed in the adipose tissue (140). As the CNS and adipose tissue play key roles in glucocorticoid production and the metabolic syndrome, respectively, the expression of ABC transporters may be important in determining circulating glucocorticoid levels and the metabolic syndrome components. Indeed, it was demonstrated that ABCC1 *in vitro* inhibition increased intracellular corticosterone levels, but not cortisol (140).

As the HPA axis is regulated by a negative feedback loop by the two glucocorticoids (see Figure 1.6), the activity of ABCB1 may cause corticosterone to contribute disproportionally to this negative feedback regulation. Therefore, lower circulating corticosterone concentrations may lead to dysregulation of the HPA axis and an increase in circulating cortisol. Indeed, low circulating corticosterone concentrations were associated with components of the metabolic syndrome in populations of European ancestry (140,154). In support of these findings, the negative feedback control of the HPA axis was shown to be impaired in individuals with the metabolic syndrome (212). Therefore, these recent findings in populations of European ancestry suggest that corticosterone may be a more favourable glucocorticoid in humans and that the ratio of

corticosterone/cortisol may be important in determining components of the metabolic syndrome. However, cross-sectional studies investigating the associations between glucocorticoids and the metabolic syndrome components in humans exclusively focused on cortisol, with the assumptions that corticosterone has similar associations.

1.6. Cross-sectional studies investigating associations between cortisol and the metabolic syndrome and its components

1.6.1 Potential confounders in cross-sectional studies of cortisol

Although the role of cortisol in the metabolic syndrome had been proposed for decades (137), there were many inconsistencies in findings from the cross-sectional studies. The inconsistencies may be attributed to the fact that glucocorticoids are dynamic hormones that are affected by many confounders (213). In a statistical model, a confounder is defined as a variable that affects both the dependent variable and the independent variable (214). Common confounders that affect the associations between cortisol concentrations and components of the metabolic syndrome may include cortisol sampling time, age, sex, genetics, smoking, alcohol, diet, physical activity, and illness. As studies in humans exclusively focused on cortisol, whether these confounders similarly confound associations between corticosterone and components of the metabolic syndrome in humans remains unclear.

Cortisol sampling time

One of the well acknowledged key confounders in cortisol cross-sectional studies is the cortisol sampling time. Cortisol sampling time is the time when the samples from which cortisol was measured were taken from the study participants. Sampling time is particularly important when cortisol is measure in circulation or saliva, as cortisol concentrations in such samples have a diurnal nature (215). The peaks of circulating and salivary cortisol concentrations are generally observed 30-45 minutes after awakening

and the concentrations decrease throughout the day (216), (217). Figure 1.10 A and B show typical diurnal curves for morning serum and salivary cortisol concentrations from two independent studies in populations of European ancestry (218,219). As circulating and salivary cortisol concentrations change drastically after awakening, it is strongly recommended to consider sampling time when designing studies involving circulating or salivary cortisol concentrations, respectively (220).

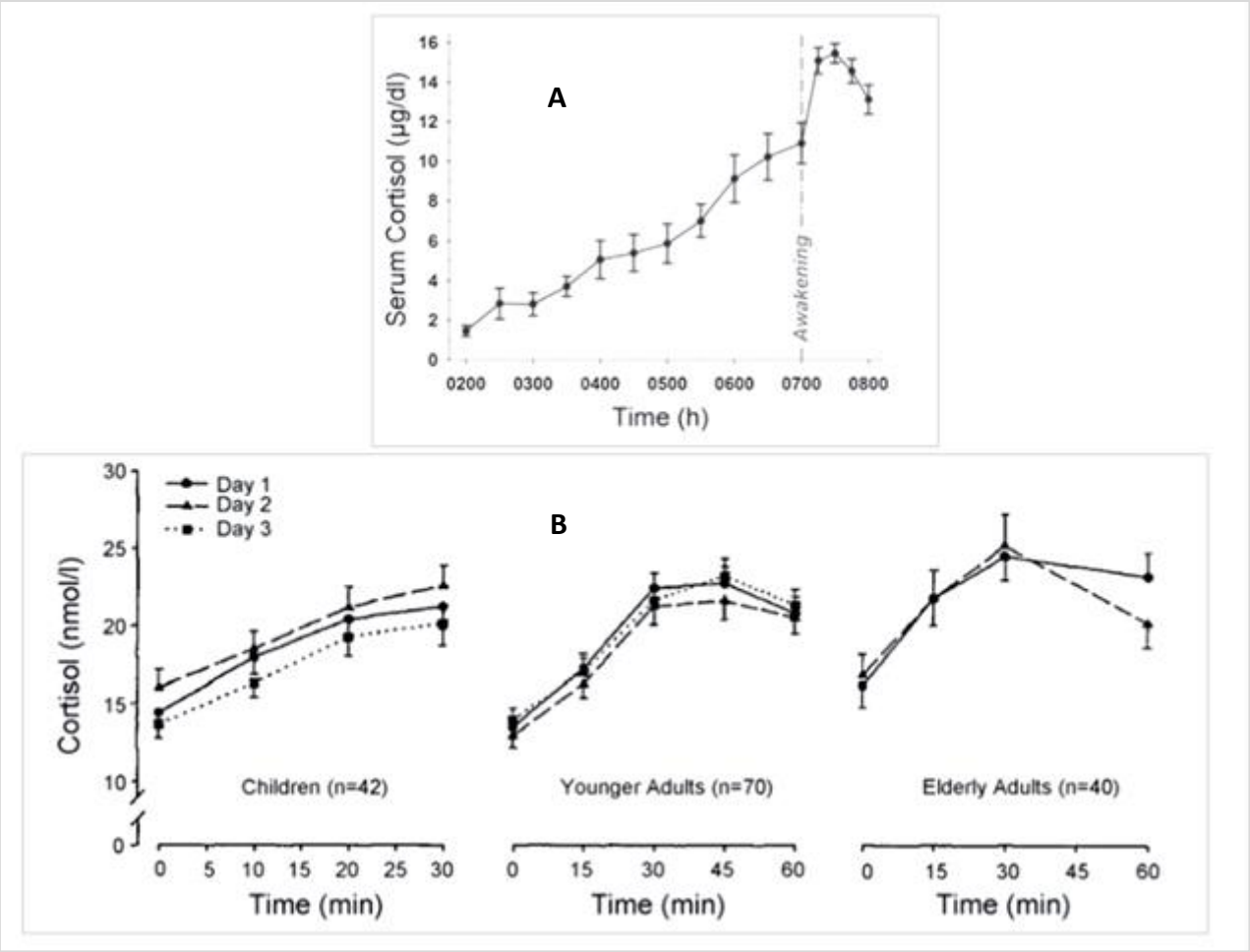


Figure 1.10: Serum cortisol concentrations minutes before and after awakening (A) (218) **and salivary cortisol concentrations minutes after awakening from different age groups (B)** (219)**.** ***Figures used with permission*****.**

Data presented as mean (±SEM)

One approach used to limit confounding due to variation in sampling times is to standardize the sampling time (often 8:00 am or 9:00 am is used) for the study participants (221). However, this approach assumes that the participants woke up at around the same time. A more effect approach is sampling at the same minutes after

awakening (e.g. at 45 minutes after awakening) (222). While this approach works well for self-sampled salivary samples, it is not practical for determining circulatory concentrations. Yet, a more convenient approach is to record the sampling times and adjust for sampling time in the statistical models (223). Unfortunately, many cross-sectional studies that assessed the relationship between cortisol concentrations and components of the metabolic syndrome failed to consider sampling time. This has contributed to contradicting findings in reported studies involving cortisol concentrations.

Age and sex

Age is a well-acknowledged risk factor for components of the metabolic syndrome. The risk of developing the metabolic syndrome increases with age globally, irrespective of sex or ethnicity (224). Findings from populations of European and Asian ancestries demonstrated an increase of circulating cortisol concentrations with age, regardless of the participant health statuses (225,226). Whether circulating cortisol levels also increase with age in populations of African ancestry remains to be tested.

In accordance with the observed sex disparities in the metabolic syndrome, sex differences in cortisol concentrations were shown in populations of European ancestry. Morning salivary cortisol concentrations were higher in women compared to men (227), and men demonstrated a more robust circulating cortisol response when subjected to an induced stressor (228). To my knowledge, studies examining sex differences in glucocorticoid concentrations in populations of African ancestry were not reported.

Smoking and alcohol

Both smoking and alcohol are well acknowledged behavioural risk factors for the metabolic syndrome (4). Studies in populations of non-African ancestry demonstrated that these two behavioural risk factors also associate with higher morning cortisol concentrations. For example, morning salivary cortisol concentrations were higher in

smokers compared to non-smokers (229). Similarly, heavy alcohol drinkers had higher salivary cortisol concentrations compared to moderate drinkers (230).

Although these associations have not been examined in South Africans of African ancestry, the rate of tobacco smoking and alcohol abuse are relatively high in this population, and this significantly differs by sex. More than 17.6% of the African ancestry adults (≥18 years old) were estimated to be daily smokers, but the prevalence in men was four times that of women (29.2% vs 7.3%) (231). Similarly, the rate of excessive alcohol consumption was also relatively high (16.3%) among the African ancestry adults (232). Remarkably, up to 57.2% of the adult African ancestry population men were classified as alcohol users (25). Moreover, South African men of African ancestry have been consistently shown to drink more than their female counterparts (25). Similarly, studies in developed countries such as the USA also suggested that there was a higher prevalence of tobacco and alcohol use in individuals of African ancestry in comparison to their European ancestry counterparts, and in women when compared to their male counterparts (4).

Other potential confounders

Findings in populations of non-African ancestry suggested that factors such as diet, physical activity, and illness may also influence cortisol concentrations in humans. The influence of diet on circulating cortisol concentrations was dependent on the type of diet consumed (233). For example, higher circulating cortisol concentrations were associated with a high carbohydrate-containing diet in populations of European ancestry (234). On the other hand, the majority of studies in populations of European ancestry suggested that physical exercise reduces circulating cortisol concentrations (235,236). Further, patients that were critically ill had significantly higher circulating cortisol concentrations than the general population, and this was attributed to reduced glucocorticoid metabolism in these patients (237).

South Africa is currently undergoing rapid urbanization which strongly influences both diet and physical activity. This was evident from observational studies which demonstrated that urban-dwelling South Africans of African ancestry have shifted to a Westernized diet and a more sedentary lifestyle when compared to populations of African ancestry in the rural areas (238).

Certainly, there are many factors that confound the association between cortisol and components of the metabolic syndrome. Unfortunately, many of the confounding factors were not considered and adjusted for in previous cross-sectional studies.

1.6.2 Cross-sectional studies of cortisol in populations of non-African ancestry

Much of the findings that suggested an association between glucocorticoid concentrations and the metabolic syndrome came from cross-sectional studies of cortisol in populations of non-African ancestry (187).

Notably, cortisol concentrations can be determined from the blood (serum/plasma) as circulating estimates, but also from saliva, hair, or urine samples (239). Each method of measurement has its own advantages and limitations. For example, circulating cortisol often represents total cortisol, most of which is bound to CBG (163). Conversely, salivary estimates represent free and bioactive cortisol (215). Additionally, collecting saliva is non-invasive, and reduces stress response during sampling (240). Hair cortisol is often used as a measurement of chronic exposure, whilst urinary cortisol is useful for daily estimates (215).

The findings from populations of non-African ancestry that assessed the associations between cortisol concentrations and the metabolic syndrome are briefly described below.

Associations between cortisol concentrations and the metabolic syndrome

Most observational studies investigated the role of cortisol in the metabolic syndrome by assessing the relationships with the individual components (adiposity, insulin resistance, blood pressure, and lipids) (241–263). However, several studies also tested the relationship between cortisol and the diagnosed metabolic syndrome. These cross-sectional studies were conducted either by comparing the cortisol concentrations in individuals with the metabolic syndrome to their respective control groups (264–276), or using ordinal regression models to assess the linear relationship between cortisol concentrations and the number of metabolic syndrome components present in subjects (277–279).

In most of the reported studies individuals with the metabolic syndrome had higher concentrations of cortisol measured in morning serum (270,274–278,280), saliva (morning or midnight) (264,266,267,271,273), hair (268,273), or 24-hour urine (270) samples, when compared to individuals without the metabolic syndrome. On the contrary, some studies reported no association between the metabolic syndrome and morning salivary (279) and serum cortisol concentrations (269). Additionally, individuals with the metabolic syndrome were also reported to have lower concentrations of morning salivary cortisol (265) when compared to individuals without the metabolic syndrome.

The contradictions in the above-mentioned studies may be partly due to differences in age, sex, smoking and alcohol statuses, health status, time of cortisol sampling, and the methods of cortisol measurement used in each study. In several studies, these factors were not considered as confounders when assessing the associations between cortisol concentrations and the metabolic syndrome.

Furthermore, the mechanism by which cortisol concentrations associate with the metabolic syndrome is thought to be mediated via one of its key individual components,

possibly obesity and/or insulin resistance. For example, chronic exposure to cortisol may result in unfavourable body fat distribution (260), which predisposes individuals to insulin resistance and the other components of the metabolic syndrome, as exemplified in Cushing's syndrome (136). Thus, differences in obesity and/or insulin sensitivity may have also contributed to the differences in the direction of association. Notably, the study that showed a negative association between salivary cortisol concentrations and the metabolic syndrome was composed of diverse ethnic groups which included 26% African ancestry (265).

Cortisol concentrations and measures of adiposity

The majority of the non-African (European and Asian) studies demonstrated a negative association (241,244,245,257,258,261,276,281–283) or no significant association (242,251,263,269,277,278,284,285) between circulating cortisol levels and obesity measures. Only a few studies showed a positive association between circulating cortisol and obesity (246,270,272).

Further studies have demonstrated that it is not increase circulating cortisol concentrations that often associate with obesity, but rather increased cortisol activity within the adipose tissue (156). For example, in obesity, the expression of 11BHSD1 is upregulated in the adipose tissue and downregulated in the hepatic tissue (259,286). Findings from animal models (287) and human urinary metabolic profiles (253,259,270,283,288) suggested that the increased metabolic clearance of cortisol in obesity was due to increased 5α-reductase activity in hepatic tissues of obese individuals. Thus, there is increased local cortisol activity within the adipose tissue during obesity, even when circulating cortisol concentrations are normal or lower.

In support of the role of cortisol in obesity, hair and salivary cortisol concentrations, which are used as an estimate of chronic cortisol exposure and free bioactive concentrations,

respectively, are often elevated in obesity (247,252,255,260,266–268,272,273,289,290). Although the exact mechanisms by which cortisol associates with obesity are still under investigation, glucocorticoids are known to promote human adiposity by increasing adipocyte differentiation and proliferation (291). Additionally, the GR is more highly expressed in the VAT than SAT and this may contribute to the role of glucocorticoids in promoting waist circumference and insulin resistance in individuals with elevated cortisol activity (292)

Therefore, it is hypothesized that increased glucocorticoid activity, particularly within the VAT, may promote insulin resistance, and ultimately the development of the other components of the metabolic syndrome (293).

Cortisol concentrations and measures of insulin resistance and glycaemia

Indeed, many cross-sectional studies have investigated the associations between cortisol concentrations and measures of insulin resistance (HOMA-IR) and glycaemia (241,243,245–247,251,254,256–258,260,261,263,265–270,273,276,277,281,284,294–296). Moreover, cortisol concentrations were compared between individuals with type 2 diabetes and their healthy controls (297–299).

Most studies demonstrated a positive association between circulating cortisol concentrations and glucose concentrations (fasting or 2-hour oral glucose tolarence test (OGTT) area under curve) (243,245,246,251,257,260,261,266–268,270,276,277,281) fasting insulin (257,260), HbA1c (243,254,268,270), and HOMA-IR (261,266,270,295), regardless of the medium in which cortisol concentrations were measured. However, like the associations with adiposity measures, there were a few studies that reported negative associations (256,296) and no significant associations (241,247,258,263,265,269,273,284,294) with the measures of insulin resistance and glycaemia.

Indeed, the mechanisms by which elevated cortisol concentrations associate with increased insulin resistance and glycaemia are thought to be largely mediated via adiposity (292). Hence, increased 11β-HSD1 activity or expresion, accumulation of cortisol concentrations within the adipose tissue, and increased metabolic clearance of cortisol were also shown in individuals with type 2 diabetes when compared to healthy individuals (300). Nevertheless, other mechanisms such as promotion of gluconeogenesis by glucocorticoids may be involved in the associations between cortisol and insulin and glucose concentrations in humans (179).

Cortisol concentrations and measures of elevated blood pressure

Similar to the associations with obesity and insulin resistance measures, the associations between cortisol and blood pressure were not always consistent. Although several studies reported no associations between cortisol concentrations and measures of elevated blood pressure (247,250,253,263,265,266,274), most studies showed positive associations between blood pressure (mostly systolic blood pressure) and cortisol concentrations in circulation (242,246,251,257,258,261,270,272,273,276,277,281,296), saliva (243,247,267,272,301,302), and urine (270). Additionally, some studies reported a negative correlation between salivary cortisol concentrations and blood pressure (both systolic and diastolic) (273,294). However, higher salivary cortisol concentrations were reported in individuals with elevated blood pressure when compared to their healthy counterparts (243,271).

Certainly, glucocorticoids are well acknowledged to influence blood pressure via several mechanisms in humans. These mechanisms occur through binding of the GR or MR in the kidneys or vascular system. In the kidneys, glucocorticoids impair nitric oxide-mediated renal vasodilation (303) possibly by suppressing nitric oxide synthase, but also influence salt and water retention, ultimately increasing blood pressure (304). Notably, the MR has a 10-folder higher affinity for glucocorticoids when compared to the GR, and

it is more ubiquitously distributed in the kidneys (305). Hence, under normal physiological conditions, access to the MR is strictly controlled by the activity of 11β-HSD2, which inactivates the glucocorticoids (156). Indeed, the relative functioning of the 11β-HSD enzymes were associated with the risk of elevated blood pressure in populations of non-African ancestry (306).

Additionally, in the vascular smooth muscle tissue, glucocorticoids are known to induce vasoconstriction of vessels by suppressing the calcium-activated potassium channel (307) and by up-regulating angiotensinogen and angiotensin-converting enzymes (308).

Therefore, the association between cortisol concentrations and measures of elevated blood pressure may occur via several mechanisms. These include direct influence of glucocorticoids on blood pressure, but also the influence of glucocorticoids on adiposity and ultimately blood pressure as a secondary metabolic syndrome component (293).

Cortisol concentrations and circulating lipid concentrations

Many studies have been conducted to assess the relationship between cortisol concentrations and the serum lipid profile (241,245–247,251,256–258,260,261,265–268,270,272,273,276–278,281,290,296) but the associations remain unclear. Several studies demonstrated a positive association between serum triglyceride and cortisol concentrations measured in circulation (257,258,261,272), hair (273), urine (270,272), and saliva (260). However, in most studies, no associations were shown (241,246,247,251,256,263,265,266,269,276–278,281,296).

Similarly, although many studies showed a negative association between HDL cholesterol concentrations and cortisol concentrations measured in saliva (267,290), hair (268), circulation (276,278), and urine (270); most studies reported no association (245,246,251,256,258,260,263,265,266,269,277,281,296); and a positive association was also shown (241).

Certainly, the role of cortisol in dyslipidaemia is thought to be complex. For example, chronic glucocorticoid exposure increases adiposity (BMI and waist circumference) which positively associates with dyslipidaemia (309). However, elevated cortisol activity in adipose tissue also induces hormone-sensitive lipase activity (310), increases whole body lipolysis (311), and stimulates lipoprotein lipase (312). These physiological changes have contradicting effects on the serum lipid profiles.

1.6.3 Cortisol in populations of African ancestry

Certainly, the above cross-sectional studies in populations of non-African ancestry suggested cortisol concentrations associate with components of the metabolic syndrome. However, whether these associations can be replicated in populations of African ancestry who show major differences in pathophysiology of the metabolic syndrome when compared to populations of European ancestry, remains unclear. Notably, several studies compared cortisol concentrations between populations of African ancestry and their European ancestry counterparts, and demonstrated lower circulating (73,124,313), salivary (314–316), and urinary (317) cortisol levels in populations of African ancestry, irrespective of age and sex, when compared to their European ancestry counterparts. However, whether these differences in cortisol concentrations are associated with differences in the pathophysiology of the metabolic syndrome is not yet known.

Notably, it is unlikely that the lower circulating cortisol concentrations in populations of African ancestry when compared to populations of European ancestry were due to increased glucocorticoid activity, as adipose tissue 11β-HSD1 activity and mRNA did not differ between women of African ancestry and their European ancestry counterparts (318). Nonetheless, women of African ancestry had lower expression of the GR in the SAT in comparison to their European ancestry counterparts, and the differences were associated with the lower VAT, higher adipose tissue inflammation, and lower insulin sensitivity observed in populations of African ancestry (318). Moreover, although 11β-

HSD1 activity in the SAT of European ancestry women were significantly associated with VAT and insulin resistance, these associations were not seen in women of African ancestry (318). Therefore, the observed ethnic differences in populations of African and European ancestry, in the association of insulin sensitivity and body fat distribution, may be partly related to glucocorticoid metabolism. Certainly, a limited number of cross-sectional studies investigated the associations between cortisol concentrations and some key components of the metabolic syndrome, and these are discussed below (Table 1.4).

Notably, the majority of studies in populations of African ancestry were aimed at assessing the relationship between cortisol concentrations and measures of elevated blood pressure (221,222,313,319–322). However, some of these studies also reported associations with glycaemia (HbA1c) (322) and adiposity (waist and BMI) (221) measurements. I am not aware of any study that has investigated the relationship between cortisol concentrations and the serum lipid profile in populations of African ancestry.

The only study, to my knowledge, that directly assessed the relationship between morning serum cortisol and the metabolic syndrome in a population African ancestry was conducted in 102 adult South African women, and found no association between morning serum cortisol concentrations and the metabolic syndrome (73). Most, but not all (73,222,320) studies in populations of African ancestry demonstrated a positive correlation between cortisol concentrations (measured in serum/saliva/urine) and measures of elevated blood pressure, irrespective of sex (221,313,319,321,322). On the contrary, no association was found between morning salivary cortisol concentrations and positive family history of elevated blood pressure in female and male adolescents of African ancestry (320). Moreover, no differences were found in morning salivary cortisol concentrations between individuals with elevated blood pressure and their controls in South African men and women of African ancestry (222). The lack of association in this

study may be attributable to lack of adjusting for all potential confounders, as only age and BMI were used as covariates (222).

Moreover, two studies in populations of African ancestry demonstrated that circulating cortisol concentrations were not only positively associated with measures of elevated blood pressure, but also with HbA1c (322) and adiposity measures (waist and BMI) (221), in populations of African ancestry as suggested by studies in populations of non-African ancestry. Hence, it is hypothesized that cortisol concentrations associate with all components of the metabolic syndrome in populations of African ancestry, as suggested by studies in populations of non-African ancestry. However, the associations of cortisol concentrations have not been tested against all primary components of the metabolic syndrome, particularly circulating lipid, glucose, and insulin concentrations, in populations of African ancestry. Furthermore, circulating corticosterone concentrations were shown to dissimilarly associate with components of the metabolic syndrome when compared to circulating cortisol concentrations, in populations of European concentrations (154). However, the associations between corticosterone concentrations and components of the metabolic syndrome have not been investigated in populations of African ancestry.

Table 1.4: Cross-sectional studies that investigated the associations between glucocorticoids and NCD-risk in populations of African ancestry.

Ref	N (F/M)	Age (years)	Health status	Cortisol medium	Sampling time	Assay	Adiposity	Insulin resistance & glycaemia	Elevated blood pressure	Dyslipidemia	Metabolic Syndrome
(319)	30 (30/0)	26-51	Healthy	Salivary	Immediately, 30 min and 60 min after awakening	EIA	NT	NT	Higher salivary cortisol levels (waking and 30 min) in night dippers	NT	NT
(320)	48 (16/32)	14-17	Family history of elevated blood pressure versus controls	Salivary	<30 min after awaking	RIA	NT	NT	No association		NT
(321)	397 (198/199)	18-55	Elevated blood pressure versus controls	Salivary; Urine-free	Salivary: 11pm and 7am; 24-hour urine	Salivary: EIA; Urinary: LCMS	NT	NT	Higher salivary cortisol levels (both pm and am) in elevated blood pressure	NT	NT
(313)	94 (0/94)	43.0 ± 8.2	Low testosterone	Serum	7:30-9:00am	ECLIA	NT	NT	Positively associated with blood pressure (Systolic and Diastolic)	NT	NT

(222)	200 (99/101)	21-62	Elevated blood pressure versus controls	Salivary	45min after awakening	ELISA	NT	NT	No association	NT	NT
(73)	102 (102/0)	19-56	Cohort	Serum	6:00-8:00am	RIA	NT	NT	NT	NT	No association
(322)	197 (98/99)	44.4 ± 8.2	Cohort	Serum	before 9:00am	ECLIA	NT	Positively associated with HbA1c	Positively associated with 24-hour blood pressure (Systolic and Diastolic)	NT	NT
(221)	102 (102/0)	31.12 (29.43; 32.81) 95%CI	Elevated blood pressure versus controls	Serum	8:00am	RIA	Positively associated with waist circumference and BMI only in controls	NT	Positively associated with systolic blood pressure	NT	NT

N: Number of participants; **F/M:** Female/Male; **EIA:** Enzyme Immunoassay; **LCMS:** Liquid Chromatography Mass Spectrometry; **ECLIA:** Electro-chemiluminescence immunoassay; **ELISA:** Enzyme-linked immunosorbent assay; **RIA:** Radioimmunoassay; **NT:** Association not tested; **HbA1c:** Glycated hemoglobin; **BMI:** Body Mass Index.

1.7 Genetics of glucocorticoid concentrations

Although the associations between cortisol concentrations and the metabolic syndrome and its components were previously shown, most of the evidence was from cross-sectional studies from which causality could not be inferred. As the genetic sequence of an individual is not altered by the environment, genetic association studies provide more reliable evidence of association. Indeed, circulating cortisol concentrations are highly heritable (323,324), and in candidate gene studies, many genes have been associated with circulating cortisol concentrations (325). However, candidate gene studies are limited by reliance on biological knowledge of the glucocorticoids and are subject to bias. On the other hand, GWAS provide a more powerful, hypothesis-free, and unbiased approach to identify genes associated with disease traits (326).

1.7.1 The *SERPINA6/A1* locus

Bolton et al. used a GWAS meta-analysis approach to identify SNPs that influenced morning plasma cortisol concentrations in 12 597 participants of European ancestry (163). The findings were also replicated in 2 795 participants of the same ancestry (163). The GWAS hit was found in chromosome 14 (see Figure 1.11), and was attributed to three SNPs, namely: rs12589136, rs11621961, and rs2749527. The minor allele (T allele) of rs12589136 was associated with higher morning plasma cortisol concentrations whilst minor alleles (both T alleles) of rs11621961 and rs2749527 were associated with lower morning cortisol concentrations. However, these SNPs (rs12589136, rs11621961, and rs2749527) only accounted for less than 1% of the variation in morning plasma cortisol, suggesting the contribution of other factors.

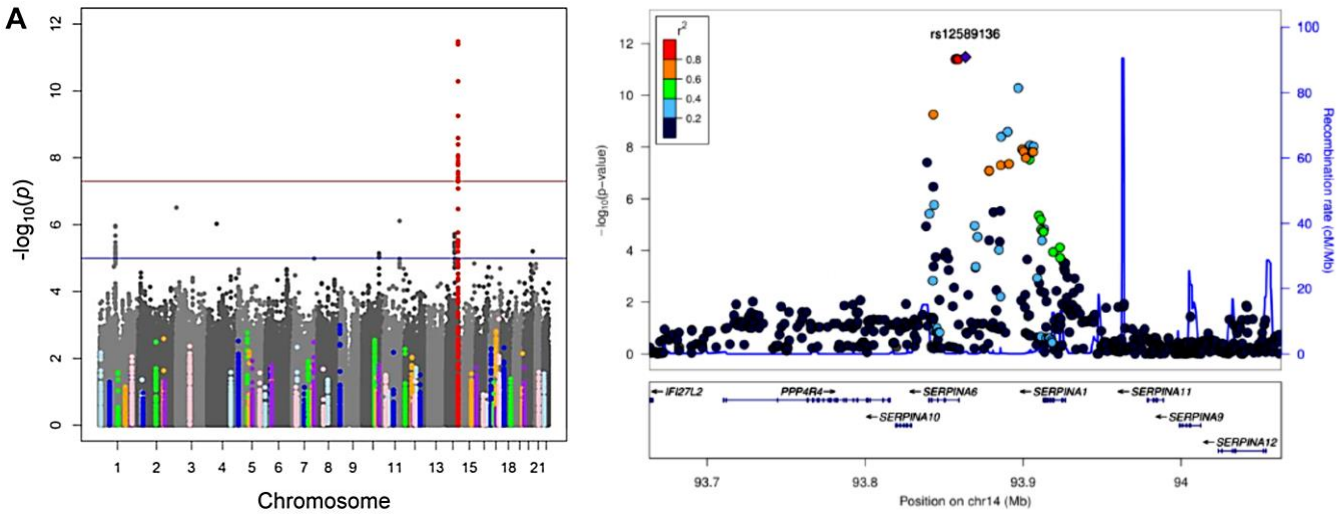


Figure 1.11: Association results from the meta-analysis of GWAS for morning plasma cortisol in populations of European ancestry. *Figures used with permission* (163).

(A) The Manhattan plot showed that the SNPs associated with morning plasma cortisol were in chromosome 14. The blue and red horizontal lines indicate moderate significance ($P < 5 \times 10^{-5}$) and GWAS significance ($P < 5 \times 10^{-8}$). **(B)** LocusZoom plots for the association of rs12589136 (the lead SNP) with morning plasma cortisol, and the degree of correlation with nearby SNPs.

The locus was found to span *SERPINA6* and *SERPINA1*, two important genes in glucocorticoid binding and release (163). *SERPINA6* encodes CBG, the major cortisol binding protein in the blood (159). *SERPINA1* encodes AAT which inhibits the release of glucocorticoids from CBG (327). Hence, these findings in populations of European ancestry suggested that cortisol binding and release may play a significant role in determining circulating cortisol concentrations, and thus the metabolic syndrome and its components in humans.

Indeed, the three SNPs in the GWAS for morning cortisol concentrations (rs11621961, rs12589136 and, rs2749527) were strongly associated with CBG's binding capacity. The rs12589136 SNP was also strongly associated with RCL/total CBG ratio; whilst rs2749527 was additionally associated with total plasma CBG concentrations (163). The relative function of AAT may also play a key role in determining free glucocorticoid

concentrations in circulation. Although the relationship between AAT function and the metabolic syndrome remains unclear, Bolton et al. (163) demonstrated an inverse relationship between plasma AAT and cortisol concentrations in populations of European ancestry. Notably, a recent Mendelian randomization analysis also used the three above-mentioned SNPs (rs12589136, rs11621961, and rs2749527), and suggested a causal effect of cortisol metabolism in cardiovascular disease in populations of European ancestry (223). However, whether these associations can also be replicated in other populations remains unknown.

1.7.2 The *CYP17A1* gene

Additional genetic evidence on the role of glucocorticoids in the metabolic syndrome came from GWAS studies that suggested associations between the *CYP17A1* gene and measures of elevated blood pressure and adiposity. The *CYP17A1* gene encodes an enzyme that has both 17α-hydroxylase and 17,20-lyase activities, which are key steps in glucocorticoid biosynthesis. The 17α-hydroxylase activity catalyses the conversion of corticosterone precursors (pregnenolone and progesterone) to cortisol precursors (17α-hydroxy-pregnenolone and 17α-hydroxyprogesterone, respectively), and thus determines the corticosterone/cortisol ratio. On the other hand, 17,20-lyase activity catalyses the conversion of the cortisol precursors to sex hormone precursors (328). The GWAS that suggested an association of the *CYP17A1* SNPs with some components of the metabolic syndrome were mostly conducted in non-Africans (predominantly including populations of European and Asian ancestry) and are summarized in Table 1.5, but also briefly described below.

In many GWAS, rs1004467 was consistently associated with measures of elevated blood pressure in populations of predominantly European and Asian ancestry (329–334). The A allele (major allele) of rs1004467 was associated with increased systolic blood pressure

(329–331), diastolic blood pressure (333), mean arterial pressure (332), pulse (330), risk of elevated blood pressure (330), and risk of myocardial infarction (335). Similarly, the minor allele of rs3824755 (C allele) was associated with higher mean arterial pressure in East and South-East Asian populations (334). Additionally, the minor allele of rs6163 (A allele) was associated with elevated waist circumference in populations of European ancestry (336).

The GWAS findings were supported by candidate gene studies that demonstrated that the risk allele of rs1004467 was associated with measures of adiposity (BMI and VAT) and increased risk for elevated blood pressure in East Asians (337–339). As the *CYP17A1* gene encodes the enzyme that determines the corticosterone/cortisol ratio, these findings support the hypothesis that corticosterone plays a separate role in human health and disease.

Table 1.5: Genome-wide association studies that reported associations between *CYP17A1* SNPs and components/outcome related to the metabolic syndrome.

SNP	CHR_POS	Context	Risk Allele	Risk Allele Frequency	Associated Trait	Initial Sample	Replication Sample	Ref
rs1004467	104594507	Non-coding transcript exon	A	0.87	Myocardial infarction	166 459 Mixed ethnicities	NA	(335)
			A	0.90	Systolic blood pressure	29 136 Europeans	34 433 Europeans	(329)
			A	0.68	Systolic blood pressure	130 777 Japanese	158 261 East Asians and Europeans	(330)
			A	0.83	Systolic blood pressure	38 450 Hispanics and African- Americans	NA	(331)
			C	0.11	Mean arterial pressure	146 562 Europeans, African Americans, and Hispanics	NA	(332)
			A	NR	Diastolic blood pressure	321 262 Mixed ethnicities	NA	(333)
			A	NR	Systolic blood pressure	321 262 Mixed ethnicities	NA	(333)
			A	NR	Pulse	321 262 Mixed ethnicities	NA	(333)
			A	0.68	Mean arterial pressure	130 777 Japanese	158 261 East Asians and Europeans	(330)
			A	0.68	Pulse	130 777 Japanese	158 261 East Asians and Europeans	(330)
			A	0.68	Elevated blood pressure	50 792 Japanese	127 725 East Asians and Europeans	(330)
rs3824755	104595849	Intron	C	0.32	Mean arterial pressure	26 600 East Asians and South East Asians	28 783 East Asians	(334)
rs6163	104596924	Synonymous	A	0.39	Waist circumference	143 480 Europeans	80 253 different ethnicities	(336)
			A	0.39	Hip circumference	143 480 Europeans	80 253 different ethnicities	(336)

The table shows all the *CYP17A1* SNPs that were associated with human traits in genome-wide association studies. The data are derived from GWAS-catalog (https://www.ebi.ac.uk/gwas/genes/*CYP17A1*). All genomic locations are reported using GRCh37.p13. **SNP:** Single nucleotide polymorphism; **CHR_POS:** Chromosome 10 position; **3'-UTR:** 3 prime untranslated region; **NR:** Not reported; **NA:** Replication not conducted.

Since the relative function of the CYP17A1 enzyme (17α-hydroxylase function) determines the corticosterone/cortisol ratio, it was hypothesized that glucocorticoids mediate the associations between *CYP17A1* SNPs and metabolic syndrome components(154). Certainly, SNPs located at 2kb upstream region of the transcription start site (rs138009835, rs2150927 and rs2486758) were found to have significant functional effects on the 17α-hydroxylase activity in participants of European ancestry with elevated blood pressure (340). The findings were also supported by a recent study that demonstrated associations between rs2486758 of *CYP17A1* and the ratio of circulating corticosterone/cortisol in two independent cohorts of European ancestry (154).

To my knowledge, none of these genetic association studies have been tested in populations of African ancestry, who are not only disproportionally affected by the metabolic syndrome, but have a distinct genetic architecture when compared to populations of European ancestry (341).

1.8 Genetic architecture in populations of African ancestry

Although the influence of ethnicity in determining genetic factors of human diseases has been known for many years (342), most of the reported genetic association studies were conducted in populations of European ancestry. For example, more than 49% of GWAS were conducted in population of European ancestry, whilst less than 3% were conducted in populations of African ancestry (343).

According to the “out of Africa” theory, the dominant model for the origin and migration of anatomically modern humans, populations of African ancestry are the oldest ethnic group in the world (344). Consequently, the long evolutionary history of anatomically modern humans has led to a more diverse genetic architecture in populations of African ancestry compared to other populations (341). For example, projects involving genomic research

in populations of African ancestry such as the African Genome Variation Project (AGVP) (345), the 1000 Genomes Project (346), the International HapMap (347), and the AWI-gen project (348) have confirmed that populations of African ancestry have a greater number of haplotype blocks due to lower levels of linkage disequilibrium (LD). Populations of African ancestry also have more complex LD structures and more complex population substructures compared to other populations (341).

The genetic diversity in populations of African ancestry has important ramifications for translating the discoveries from GWAS studies, which were predominantly conducted in populations of European ancestry. Such ramifications include the use of pharmacogenetics and risk scores based on GWAS discoveries (37). On the other hand, the shorter haplotype blocks in populations of African ancestry allow for more effective localization of the casual SNPs, through fine mapping of the loci identified in other populations (349). Although, a large number of loci were associated with disease risk in populations of European ancestry, only a limited proportion have been replicated and fine-mapped in populations of African ancestry (341).

1.9 Literature review summary

The literature review highlighted key aspects of the metabolic syndrome in populations of African ancestry. Comparing populations of African ancestry to those of European ancestry clearly showed ethnic differences in components of the metabolic syndrome, particularly the relationship between insulin sensitivity and body fat distribution (85,86,127–129,87,88,102,103,123–126). For example, although populations of African ancestry are more obese (84) and more insulin resistant (85–88) than populations of European ancestry in similar environmental settings, the differences in insulin sensitivity are independent of body fat and its distribution (89).

The pathophysiology of the metabolic syndrome in populations of African ancestry remains understudied, and therefore, the exact mechanisms underlying these ethnic differences are not yet known. However, some evidence in South African women of African ancestry suggested that glucocorticoids may be partly involved (318). Indeed, excess glucocorticoid exposure is known to detrimentally influence several components of the metabolic syndrome including body fat distribution, insulin resistance, blood pressure, and serum lipid profile, as exemplified in Cushing's syndrome (136). The involvement of cortisol in the metabolic syndrome was also suggested by many studies in populations of European ancestry that demonstrated associations between circulating cortisol concentrations and components of the metabolic syndrome, including insulin resistance and adiposity, measures of elevated blood pressure, the metabolic syndrome, and dyslipidaemia (242,245,246,251,257,258,261,270,272,276,277,280,281,295,296). These were further supported by genetic association studies which demonstrated that SNPs at the *SERPINA6/A1* locus, which were associated with circulating cortisol concentrations, were also associated with incidents of cardiovascular disease in populations of European ancestry (163,223). This genetic locus spans two genes that play key roles in glucocorticoid binding and release. *SERPINA6* encodes CBG, the major glucocorticoid binding protein, while *SERPINA1* encodes AAT, which inhibits the release of glucocorticoids from CBG (163).

In addition, another gene, *CYP17A1*, which encodes an enzyme that plays a key role in the glucocorticoid biosynthetic pathway was consistently associated with measures of elevated blood pressure and adiposity in populations of European and Asian ancestry (329,337–339,350–354). Humans secrete two glucocorticoids: cortisol and corticosterone, and the *CYP17A1* enzyme converts precursors for corticosterone to precursors for cortisol. Hence the relative function of the *CYP17A1* enzyme determines

the ratio of the two human glucocorticoids (154). Notably, recent studies have supported the hypothesis that corticosterone has a physiological role distinct from cortisol (140). Furthermore, in a study comprised of populations of European ancestry, corticosterone and the corticosterone/cortisol ratio were associated with *CYP17A1* SNPs, as well as components of the metabolic syndrome (154).

1.10 Study rationale and research questions

The association between circulating glucocorticoids, particularly cortisol, and the metabolic syndrome has been suggested by many studies. However, whether similar associations exist in populations of African ancestry, who display differences in components of the metabolic syndrome when compared to populations of European ancestry, remains uncertain. Studies investigating the role of circulating glucocorticoid concentrations in populations of African ancestry are limited to small cross-sectional studies that tested associations between cortisol concentrations and measures of elevated blood pressure (221,313,322), HbA1c (322), adiposity (BMI and waist circumference) (221), and the metabolic syndrome (73). However, these were small cross-sectional studies and most failed to adjust for some of the key confounders (e.g., BMI). Furthermore, whether circulating corticosterone concentrations and the corticosterone/cortisol ratio also associate with the components of the metabolic syndrome in populations of African ancestry, as recently shown in populations of European ancestry, is unknown. Moreover, I am not aware of any study that has assessed the genetic bases of these associations in populations of African ancestry, who also display differences in genetic architecture when compared to populations of European ancestry.

Hence, the primary aim of the study was to use epidemiological and genetic methods to investigate the associations between circulating glucocorticoid concentrations and components of the metabolic syndrome in South African men and women of African ancestry.

To address this aim, the study was divided into the following three research questions as follows:

Research question 1: Are circulating glucocorticoids associated with the metabolic syndrome and its components in men and women of African ancestry?

Hypothesis: Circulating glucocorticoid concentrations similarly associate with components of the metabolic syndrome in South African men and women of African ancestry, as observed in populations of non-African ancestries.

Objectives:

1. Determine serum cortisol and corticosterone concentrations in a population of 1104 African South African men and women, using a liquid chromatography mass spectrometry method.
2. Use linear or logistic regression models to investigate whether serum cortisol and corticosterone concentrations and the corticosterone/cortisol ratio associate with the following components of the metabolic syndrome:
    I. BMI and waist circumference
    II. Systolic and diastolic blood pressure
    III. Serum lipid (triglycerides, total-, HDL-, and LDL-cholesterol) concentrations
    IV. Fasting glucose, 2-hour OGTT glucose, fasting insulin, Matsuda index (OGTT derived insulin sensitivity), HOMA2-IR

V. The metabolic syndrome (diagnosed using the 2009 harmonized guidelines)

3. Assess whether sex-based interactions exist in the investigated regression models.

Research question 2: Are SNPs at *CYP17A1* and *SERPINA6/A1* that have been previously associated with components of the metabolic syndrome and/or circulating glucocorticoids in Europeans, also associated with the metabolic syndrome and its components in women of African ancestry?

Hypothesis: *CYP17A1* and *SERPINA6/A1* SNPs, that were previously associated with components of the metabolic syndrome and/or circulating glucocorticoid concentrations in populations of non-African ancestries, are also associated with the metabolic syndrome and its components in women of African ancestry.

Objectives:

1. Select African-specific tag SNPs (n = 12) at *CYP17A1* and *SERPINA6/A1* loci that were previously associated with components of the metabolic syndrome and/or circulating glucocorticoid concentration in population of non-African ancestries.
2. Genotype 858 South African women of African ancestry for the selected tag SNPs.
3. Perform association analysis of the genotyped SNPs with cardiometabolic risk factors including:
   I. BMI and waist circumference
   II. Systolic and diastolic blood pressure
   III. Serum lipid (triglycerides and HDL-cholesterol) concentrations
   IV. Fasting glucose

V. The metabolic syndrome (diagnosed using the 2009 harmonized guidelines)

Research question 3: Are *CYP17A1* and *SERPINA6/A1* SNPs, including those not previously reported in Europeans, associated with the metabolic syndrome and its components and serum glucocorticoid concentrations in men and women of African ancestry?

Hypothesis: SNPs at *CYP17A1* and *SERPINA6/A1* are associated with components of the metabolic syndrome and serum glucocorticoid concentrations in men and women of African ancestry.

Objectives:

1. Extract and clean data of SNPs (both genotyped and imputed) at *CYP17A1* (n= 39) and *SERPINA6/A1* (n = 590) for 4431 unrelated South African men and women of African ancestry from an African GWAS dataset.
2. Perform analysis of associations between *CYP17A1* and *SERPINA6/A1* SNPs, and cardiometabolic risk factors including:
    I. BMI and waist circumference
    II. Systolic and diastolic blood pressure
    III. Serum lipid (triglycerides, total-, HDL-, and LDL-cholesterol) concentrations
    IV. Fasting glucose and insulin, HOMA2-IR
    V. The metabolic syndrome (diagnosed using the 2009 harmonized guidelines)
3. Test whether the SNPs at *CYP17A1* and *SERPINA6/A1* associate with serum glucocorticoid (cortisol and corticosterone) concentrations and/or the corticosterone/cortisol ratio, in a sub-population of 877 South African men and women of African ancestry.

Chapter 2 Research paper 1

Associations between fasting serum glucocorticoid concentrations and the metabolic syndrome in ageing black South African adults

2.1 Abstract

Background: Associations between glucocorticoids and the metabolic syndrome and its components have been reported in non-Africans. The present study investigated these associations in African men and women, whose metabolic phenotype differs compared to Europeans.

Methods: In this cross-sectional study comprising men (n = 316) and women (n = 788) of African ancestry, metabolic syndrome components, including measures of adiposity, blood pressure, glycaemia, insulin resistance, and lipid profile were measured. The 2009 harmonized criteria were used to define the presence of the metabolic syndrome. Circulating serum glucocorticoid concentrations were measured using a liquid chromatography-mass spectrometry.

Results: Fasting serum cortisol concentrations were associated with greater odds of having the metabolic syndrome (odds ratio (OR): 95% confidence interval (CI) = 1.50: 1.04, 2.17, p = 0.029) and higher systolic (p = 0.018) and diastolic (p = 0.002) blood pressure, but lower LDL (p = 0.009) and higher HDL (p = 0.017) cholesterol concentrations, in both men and women. In contrast, fasting corticosterone concentrations were only associated with higher insulin sensitivity (Matsuda index; p = 0.021), but not independently of BMI, in men and women. Sex-specific associations were observed, such that both fasting serum cortisol and corticosterone concentrations were

associated with higher fasting plasma glucose ($p < 0.001$ and $p = 0.040$, respectively) and HbA1c concentrations ($p = 0.030$ and $p = 0.035$, respectively) in men, but lower HbA1c concentrations ($p = 0.030$ and $p = 0.042$) in women.

Conclusions: The associations between circulating glucocorticoid concentrations and components of the metabolic syndrome exhibit both glucocorticoid- and sex-specificity in Africans.

2.2 Introduction

Non-communicable disease risk factors including elevated waist circumference, blood pressure, fasting blood glucose and triglyceride concentrations, and reduced circulating HDL cholesterol concentrations, often cluster together (44). These risk factors are commonly referred to as components of the metabolic syndrome, as when three or more are present in any individual, the condition is clinically defined as the metabolic syndrome (44). The mechanisms underlying the metabolic syndrome are unclear, but some evidence suggests the involvement of glucocorticoids. For example, chronic exposure to excess cortisol leads to Cushing's syndrome, which exhibits metabolic features that are similar to those of the metabolic syndrome (136). Accordingly, previous cross-sectional studies suggested associations between circulating cortisol concentrations and measures of glucose intolerance (281), HbA1c (355), insulin resistance (257), blood pressure (251,276), and serum lipid concentrations (241,278,356), and the metabolic syndrome (274,277). In contrast, the majority of studies demonstrated an inverse (241,257,258) or no association (251,277,284) of circulating cortisol concentrations with BMI and waist circumference, due to increased metabolic clearance of glucocorticoids within the adipose tissue and liver (259).

Cortisol may not be the only glucocorticoid involved in the metabolic syndrome, as humans also produce corticosterone. However, cortisol production is almost ten times that of corticosterone (194). Hence, previous studies had exclusively focused on cortisol, with the assumption that these two glucocorticoids have identical functions (187). This assumption has been challenged by evidence which demonstrated tissue-specific export of these two glucocorticoids by ABC transporters (140,154). In accordance with these findings, a cross-sectional study in a population of European ancestry demonstrated distinct associations between cortisol and corticosterone with components of the metabolic syndrome (154).

Most of the reported cross-sectional studies of cortisol and the metabolic syndrome have been conducted in populations of non-African ancestry. People of African ancestry have a different metabolic phenotype to that of their European counterparts. In urban settings, populations of African ancestry have higher prevalence of obesity and insulin resistance, yet have less VAT than populations of European ancestry (85,89,357). Although the mechanisms underlying these ethnic differences are not yet known, a recent study suggested that glucocorticoids may be involved. South African women of African ancestry demonstrated lower gene expression of the GR in SAT than South African women of European ancestry (318). The lower GR expression in the women of African ancestry was associated with less VAT, higher adipose tissue inflammation, and lower insulin sensitivity. Furthermore, several South African studies have shown lower circulating cortisol concentrations in populations of African ancestry when compared to those of European ancestry (73,124).

Whether circulating glucocorticoids are associated with the metabolic syndrome and its components in populations of African ancestry, as demonstrated in populations of European ancestry, remains uncertain. The few small studies conducted in populations

of African ancestry reported associations between circulating cortisol concentrations and measures of elevated blood pressure (221,313,322), as well as HbA1c (322) and adiposity (BMI and waist circumference) (221). These studies did not test the other key components of the metabolic syndrome (measures of insulin resistance and serum lipid concentrations). In addition, none of the studies in populations of African ancestry considered corticosterone. Accordingly, the aim of this study was to investigate the associations between fasting serum glucocorticoid (corticosterone and cortisol) concentrations and the metabolic syndrome and its components in South African men and women of African ancestry.

2.3 Methods

2.3.1 Study population and sampling procedure

The study sample consisted of adult male and female participants recruited from various studies at the SAMRC/Wits DPHRU, based at the Chris Hani Baragwanath Academic Hospital in Soweto, Johannesburg. Study sample 1 (ethical clearance M090620) included women recruited in Soweto, Johannesburg as part of the Africa Wits-INDEPTH partnership for Genomic Research (AWI-Gen) study (348). These women (n = 1007) who were randomly recruited between 2011 and 2013 were the caregivers of the Birth to twenty plus (Bt20+) cohort. Bt20+ is an ongoing longitudinal study, and its profile has been described elsewhere (358). The majority of the caregivers of the Bt20+ participants are South African men and women of African ancestry who were self-identified as South-Eastern Bantu language speakers, and only this ethnic group was included in the present study. Details of data collection protocols for the AWI-Gen study have been described elsewhere (359). Study sample 2 included participants (n = 501 men; n = 529 women) recruited as part of a longitudinal study designed to identify the determinants of type 2

diabetes risk in middle-aged South African men and women of African ancestry (ethical clearance M160604). Although these participants were a sub-sample of the AWI-Gen study, for the present study only the follow-up data collected between January 2017 and August 2018 were included.

The selection of the participants for the present study was primarily based on the availability of serum samples for glucocorticoid determination. Participants who were pregnant or using corticosteroid medication were excluded from the study (Figure 2.1). A total of 1 104 participants were selected for the present study, with the selection process described in Figure 2.1. Further ethical clearance for all analyses conducted as part of the present study was also obtained (ethical clearance no: M160225) from the University of the Witwatersrand Human Research Ethics Committee (Medical).

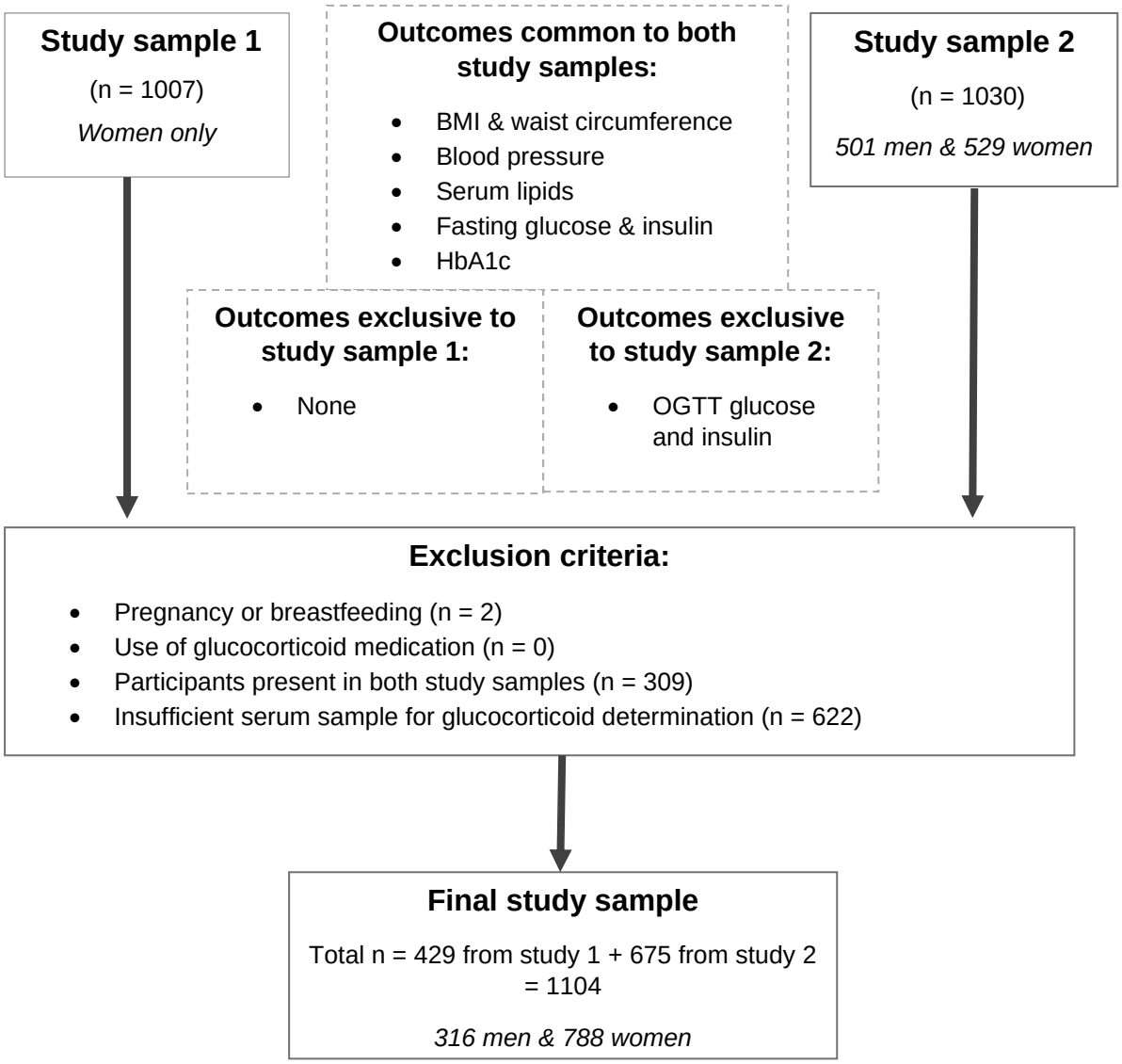


Figure 2.1: Selection of the study sample used in the present study.

2.3.2 Participant testing procedures

Participants were asked to remove heavy clothes and shoes prior to weight and height measurements. Weight was measured using a calibrated digital scale (Dismed, Halfway House, South Africa) to the nearest 0.1 kg. Standing height was measured using a fixed-wall stadiometer (Holtain, Crymych, United Kingdom (UK)) to the nearest 0.1 cm. The BMI was then calculated as weight (kg) divided by height squared (m^2). Waist circumference was determined at the level of the umbilicus when the participants were standing and measured using a soft measuring tape to the nearest 0.5 cm. Systolic and diastolic blood pressure were measured on the left arm using a digital blood pressure reader (Omron M6, Kyoto, Japan) and appropriate cuffs. After the participant had been seated for at least five minutes, three blood pressure readings were taken at two-minute intervals. For each participant, the average of the second and third readings was recorded and used in the analyses. Participants who self-reported as smoking any form of tobacco and/or drinking any form of alcohol were classified as drinkers and/or smokers, respectively. Participants were asked to bring their chronic medication to the interview sessions to confirm and record the type of medication used.

2.3.3 Blood sampling and biochemical analyses

Standard venepuncture techniques were used to collect blood samples after 10-12 hours of fasting for the subsequent determination of serum lipids, HbA1c, serum insulin, plasma glucose, and serum glucocorticoid concentrations. Blood sampling time for the fasting samples was recorded and ranged from 07:00-11:59 am. Subsequently, a standard OGTT was conducted on a sub-sample of participants (Study 2 in Figure 2.1, n = 636). During the OGTT, the participants ingested 75 g anhydrous glucose dissolved in 250 ml water. Blood samples were then collected at 30-minute intervals for 120 minutes for the subsequent determination of the Matsuda Index (OGTT-derived insulin sensitivity)

(http://mmatsuda.diabetes-smc.jp/english.html) and two-hour OGTT glucose concentrations.

Serum lipid concentrations, including triglycerides and total- and HDL-cholesterol, were measured using prescribed enzymatic methods with the RX Daytona Chemistry Analyzer (Randox, Crulim, UK). The inter- and intra-assay coefficients of variation (CVs) for the serum lipids were 1.4% and 1.5% for triglycerides, 1.3% and 1.3% for total cholesterol, and 1.7% and 1.8% for HDL cholesterol. Serum LDL cholesterol concentrations were then calculated using the Friedewald equation (LDL cholesterol (mg/dL) = total cholesterol - HDL cholesterol - (triglycerides/5)) (360). The HbA1c concentrations were measured in whole blood samples using the D-10 Haemoglobin testing system (BioRad, Hercules, United States of America (USA)), and the inter- and intra-assay CVs were both 1.8%. Plasma glucose concentrations were measured using the RX Daytona Chemistry Analyzer (Randox, Crulim, UK), and the Advia 1800 Chemistry Analyser (Siemens Healthcare Diagnostics, Tarrytown, USA) was used to measure serum insulin concentrations. The inter- and intra-assay CVs were 3.3% and 1.3% for glucose, and 2.9% and 2.7% for insulin. The HOMA2-IR calculator version 2.2.3 was used to estimate insulin resistance levels (https://www.dtu.ox.ac.uk/homacalculator) from fasting serum insulin and plasma glucose concentrations.

A liquid-liquid extraction technique (Agilent Technologies, California, USA) was used to extract all steroids. The fasting serum cortisol and corticosterone concentrations were subsequently analysed via ultra-high-pressure liquid chromatography-mass spectrometry (UPLC-MS) (Waters Corp, Milford, USA). The inter- and intra-assay CVs for serum corticosterone and cortisol concentrations were 7.3% and 2.9% and 13.6% and 9.6%, respectively.

2.3.4 Definition of the metabolic syndrome

The 2009 harmonized guidelines were used for the diagnosis of the metabolic syndrome (44). The harmonized diagnosis requires the presence of three or more of the following: elevated waist circumference (≥ 94 cm in men, ≥ 80 cm in women), elevated triglyceride concentrations (≥ 1.7 mmol/L), reduced HDL cholesterol concentrations (< 1.0 mmol/L in males, < 1.3 mmol/L in females), elevated blood pressure (systolic ≥ 130 and/or diastolic ≥ 85 mmHg), and elevated fasting glucose concentrations (≥ 5.6 mmol/L). The use of prescribed medications for elevated blood pressure and diabetes were also considered as relevant indicators of the presence of elevated blood pressure and fasting blood glucose concentrations, respectively.

2.3.5 Statistical analyses

All data exploration and statistical analyses were conducted in R version 3.6.2 (361). Prior to the statistical analyses, the normality of the continuous variables was assessed using distribution graphs (QQ-plots, histograms, and box plots) as well as the Shapiro-Wilk test. All the continuous variables shown in Table 2.1 were not normally distributed and are thus presented as medians (25th-75th percentiles). Therefore, the Mann-Whitney U test was used to assess the significant differences in the continuous variables between men and women (Table 2.1). Differences in the categorical variables (prescribed medication, prevalence of the metabolic syndrome, and lifestyle factors) were compared between men and women using a Chi-square test (Table 2.1).

Logistic or linear regression models were used to determine associations between serum glucocorticoid concentrations and the metabolic syndrome and its components, respectively. Blood sampling time, age, sex, alcohol, and smoking were used as covariates in all regression models. Prescribed medications for blood pressure, diabetes

and lipids were also used as covariates in regression models that involved measures of blood pressure and measures of glycaemia and insulin resistance, and lipid profile. The associations between circulating glucocorticoids and adiposity are often confounded by the increased metabolic clearance of glucocorticoids within the adipose and liver tissues in obesity (259). Hence, BMI was included as an additional covariate in all models (except where BMI, waist circumference and the metabolic syndrome were the outcomes). For the regression models, all continuous dependent variables were log transformed to achieve normal distributions prior to inclusion in the models. The regression models were first conducted in the whole study sample comprising both men and women. In these models, sex interactions were also tested, and the sex interaction p values were recorded as “Sex Int”. All models showing significant sex interactions (Sex Int: $p < 0.05$) were subsequently stratified by sex and each regression model was repeated in men and women.

To test the validity of the linear regression models, the presence of a linear relationship between the serum glucocorticoid concentrations and the relevant outcome variable were first confirmed in each model, using scatter plots. The absence of multicollinearity within each model was confirmed by assessing the variance inflation factor (VIF) values (all VIF < 3.0). Finally, the validity of each linear regression model was confirmed by testing the normality of the residuals.

The tested outcome variables included the metabolic syndrome, BMI, waist circumference, systolic and diastolic blood pressure, fasting plasma glucose and serum insulin, two-hour plasma glucose concentrations, HOMA2-IR, Matsuda Index, and serum lipid (triglyceride, total-, LDL- and HDL-cholesterol) concentrations.

Data from the logistic models are presented as the odds ratio for the metabolic syndrome, for both corticosterone and cortisol, with adjustment for potential confounders (age, sex, blood sampling time, smoking and alcohol) (Figure 2.2). For the linear models, the beta coefficients (effect sizes) for the components of the metabolic syndrome that were adjusted for the potential confounders only (referred as Model 1) and for the potential confounders plus BMI (referred as Model 2) are presented in Table 2.2.

2.4 Results

2.4.1 Participant characteristics

The characteristics of the men and women are presented in Table 2.1, with a comparison of the categorized components of the metabolic syndrome between the sexes shown in Supplementary Table S2.1. Women were younger than men ($p < 0.001$) and had higher BMI ($p < 0.001$) and waist circumference ($p = 0.029$) (Table 2.1). Accordingly, a greater proportion of women were classified with obesity (65.2% vs 25.3%; $p < 0.001$) and had elevated waist circumference (88.3% vs 48.4%; $p < 0.001$) (Table S1).

Despite differences in obesity, fasting plasma glucose concentrations were similar between men and women. However, compared to men, women had greater fasting serum insulin concentrations, HOMA2-IR, two-hour plasma glucose concentrations, HbA1c, and lower insulin sensitivity (Matsuda index) (all $p < 0.001$). Accordingly, a larger proportion of women were using diabetes medication (11.2% vs 6.1%; $p = 0.022$) than men (Table 2.1). In contrast, the proportion of men and women with elevated blood glucose (fasting blood glucose ≥ 5.6 mmol/l and/or using diabetes medication) did not differ (Table S2.1).

Although serum HDL-cholesterol and triglyceride concentrations did not differ by sex, total cholesterol and LDL-cholesterol concentrations were higher in women compared to men ($p < 0.001$ for both) (Table 2.1). Moreover, a greater proportion of women had reduced

HDL cholesterol (57.0% vs 18.0%), but the prevalence of elevated triglycerides was greater in men (15.2 vs 8.2%) ($p < 0.001$ for both) (Table S1). There were no observed differences in the use of cholesterol-lowering medication between the sexes.

Systolic and diastolic blood pressure were both higher in men ($p = 0.017$ and $p = 0.003$, respectively) (Table 2.1), and this finding was accompanied by a greater proportion of men having elevated systolic (≥ 130 mmHg and/or using blood pressure medication, 53.0% vs 46.3%; $p = 0.017$), diastolic (≥ 85 mmHg and/or using blood pressure medication, 61.3 vs 52.8%; $p = 0.003$), and overall blood pressure (73.4% vs 66.5%; $p = 0.030$) (Table S1). However, a greater proportion of women used blood pressure medication (37.9% vs 27.1%; $p = 0.002$).

Table 2.1: Participant characteristics

	n	All (n =1104)	Men (n = 316)	Women (n = 788)	p
Age and adiposity					
Age (years)	1096	51.9 (46.8-57.0)	53.0 (48.1-59.0)	51.0 (46.0-56.3)	**<0.001**
BMI (kg/m^2)	1094	30.7 (25.2-35.8)	25.2 (21.0-30.1)	32.8 (28.1-37.3)	**<0.001**
Waist circumference (cm)	1066	95.0 (86.0-104.5)	93.0 (81.8-103.8)	96.0 (87.2-105.0)	**0.029**
Blood pressure					
Systolic blood pressure (mmHg)	1076	128.2 (115.5-142.8)	131.0 (118.2-145.0)	127.0 (114.0-142.5)	**0.017**
Diastolic blood pressure (mmHg)	1076	86.0 (78.0-94.7)	87.5 (80.5-96.0)	85.5 (76.5-94.0)	**0.003**
Glycaemia and insulin resistance					
Fasting glucose (mmol/L)	1038	5.0 (4.5-5.5)	5.0 (4.4-5.4)	5.0 (4.5-5.6)	0.147
Fasting insulin (µIU/ml)	964	8.0 (4.4-13.9)	5.8 (2.3-11.7)	9.1 (5.2-14.6)	**<0.001**
Two-hour glucose (mmol/L)	636	5.9 (4.7-7.1)	5.7 (4.3-6.7)	6.0 (5.0-7.3)	**<0.001**
HbA1c (%)	873	5.8 (5.4-6.2)	5.5 (5.2-6.1)	5.8 (5.5-6.3)	**<0.001**
HOMA2-IR	938	1.0 (0.6-1.8)	0.7 (0.3-1.5)	1.2 (0.7-1.9)	**<0.001**
Matsuda index (OGTT-derived insulin sensitivity)	555	5.7 (3.1-9.4)	6.7 (3.4-12.4)	4.8 (2.8-7.5)	**<0.001**
Serum lipids					
Total cholesterol (mmol/L)	1012	4.3 (3.7-5.0)	4.1 (3.5-4.8)	4.4 (3.8-5.1)	**<0.001**
LDL cholesterol (mmol/L)	1012	2.7 (2.1-3.3)	2.4 (1.8-3.0)	2.8 (2.2-3.4)	**<0.001**
HDL cholesterol (mmol/L)	1013	1.2 (1.0-1.4)	1.2 (1.0-1.5)	1.2 (1.0-1.4)	0.148
Triglycerides (mmol/L)	1012	0.9 (0.7-1.2)	0.9 (0.7-1.3)	0.9 (0.7-1.2)	0.253
Glucocorticoids					
Blood sampling time (min)	1027	90.0 (60.0-140.0)	54.0 (40.0-74.8)	113.0 (75.0-165.0)	**<0.001**
Corticosterone (nmol/L)	924	6.3 (3.5-13.6)	5.0 (3.3-9.0)	6.8 (3.6-17.9)	**<0.001**
Cortisol (nmol/L)	972	182.5 (79.5-342.5)	153.8 (76.3-295.8)	201.2 (82.5-371.9)	**0.002**
Medication					
Blood pressure med (n (%))	854	290 (34.0)	84 (27.1)	206 (37.9)	**0.002**
Diabetes med (n (%))	792	73 (9.2)	19 (6.1)	54 (11.2)	**0.022**
Cholesterol-lowering med (n (%))	895	61 (6.8)	21 (6.8)	40 (6.8)	1.000
Metabolic syndrome					

Prevalence (2009 harmonized definition) (n (%))	1066	440 (40.7)	83 (26.3)	357 (47.6)	**<0.001**
Lifestyle					
Smoking (n (%))	1084	224 (20.7)	160 (50.8)	64 (8.3)	**<0.001**
Alcohol (n (%))	674	329 (48.8)	226 (71.7)	103 (28.7)	**<0.001**

Continuous data presented as median (25th-75th percentiles) and categorical data presented as n (%). A Wilcoxon Rank Sum test was used to statistically compare the continuous variables, whilst a chi-square test was used to statistically compare the categorical variables. **n:** Number of observations; **min:** Minutes from the earliest sampling time (07h00); **Med:** Medication; **p:** P value of the Wilcoxon Rank Sum statistical or Chi-square test.

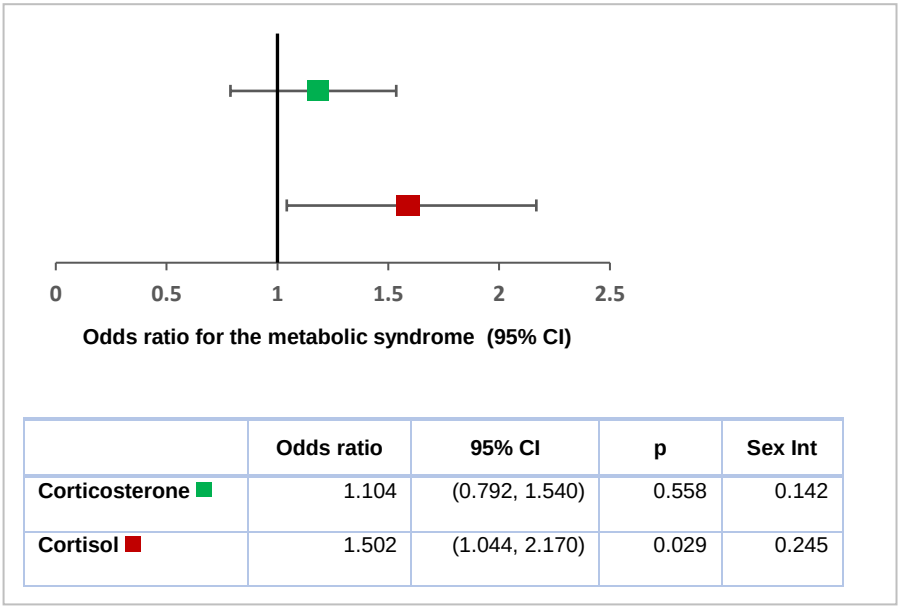
A greater proportion of women than men were classified as having the metabolic syndrome (47.6% vs 26.3%; $p < 0.001$). Meanwhile, a greater proportion of men smoked (50.8% vs 8.3%; $p < 0.001$) and drank alcohol (71.7% vs 28.7%; $p < 0.001$) compared to women.

When comparing the serum glucocorticoid concentrations (Table 2.1), women had significantly higher serum concentrations of corticosterone ($p < 0.001$) and cortisol ($p < 0.002$). These differences were accompanied by later average blood sampling times in women compared to men ($p < 0.001$). Notably, the differences in serum glucocorticoid concentrations were observed even after adjusting for sampling time, measures of adiposity (BMI and waist circumference), and/or other potential confounders (age, smoking, alcohol) (results not shown).

2.4.2 Associations with the metabolic syndrome and its components

Only adjusted regression models that either showed significant associations ($p < 0.05$) or a significant sex-interaction (Sex Int: $p < 0.05$) are presented in Figure 2.2 and Table 2.2. The unadjusted models and all other models that were tested are presented in Table S2.2. Figure 2.2 shows that whilst serum corticosterone concentrations were not associated with the odds of having the metabolic syndrome, serum cortisol

concentrations were associated with greater odds of presenting with the metabolic syndrome. The association between circulating glucocorticoids and the metabolic syndrome did not differ by sex (Sex Int: p = 0.142 for corticosterone and p = 0.245 for cortisol).



	Odds ratio	95% CI	p	Sex Int
Corticosterone ■	1.104	(0.792, 1.540)	0.558	0.142
Cortisol ■	1.502	(1.044, 2.170)	0.029	0.245

Figure 2.2: Associations between fasting serum glucocorticoid concentrations and odds of developing the metabolic syndrome.

95% CI: 95% confidence intervals; p: p value for the logistic regression model; Sex Int: p value for sex interaction. Models adjusted for age, blood sampling time, sex, smoking, and alcohol.

There were no significant associations between serum glucocorticoid (corticosterone and cortisol) concentrations and measures of adiposity (BMI and waist circumference) (Table 2.2). However, there was a significant sex-interaction in the associations between serum cortisol concentration and BMI (Table 2.2), but the sex-stratified analyses revealed no significant sex-specific associations (Figure 2.3). There were no significant sex interactions in the associations between either of the serum glucocorticoid measures and waist circumference.

Only serum cortisol concentrations were associated with higher systolic (p = 0.028) and diastolic (p = 0.004) blood pressure, even after adjusting for BMI (p = 0.018 and p = 0.002, respectively). These associations did not differ by sex (Table 2.2).

Table 2.2 :Associations of serum glucocorticoid concentrations with components of the metabolic syndrome.

	Glucocorticoid	Model 1 (Adjusted for confounders)			Model 2 (Adjusted for confounders and BMI)		
		Beta (95% CI)	p	Sex Int	Beta (95% CI)	p	Sex Int
BMI	Corticosterone	-0.046 (-0.096, 0.004)	0.071	0.063			
	Cortisol	-0.011 (-0.064, 0.041)	0.679	**0.040**			
Systolic BP	Corticosterone	0.007 (-0.029, 0.043)	0.714	0.656	0.014 (-0.021, 0.049)	0.430	0.956
	Cortisol	0.042 (0.005, 0.079)	**0.028**	0.955	0.043 (0.007, 0.080)	**0.018**	0.634
Diastolic BP	Corticosterone	0.009 (-0.023, 0.041)	0.572	0.897	0.016 (-0.016, 0.047)	0.324	0.775
	Cortisol	0.051 (0.017, 0.085)	**0.004**	0.918	0.052 (0.019, 0.085)	**0.002**	0.546
Fasting Glucose	Corticosterone	-0.008 (-0.055, 0.039)	0.739	**0.009**	-0.001 (-0.048, 0.046)	0.953	**0.008**
	Cortisol	0.042 (-0.010, 0.094)	0.110	**<0.001**	0.044 (-0.008, 0.095)	0.097	**<0.001**
Two-hour Glucose	Corticosterone	-0.017 (-0.099, 0.065)	0.683	0.971	-0.001 (-0.082, 0.079)	0.972	0.824
	Cortisol	-0.013 (-0.099, 0.074)	0.775	0.090	-0.010 (-0.095, 0.075)	0.810	**0.047**
HbA1c	Corticosterone	-0.013 (-0.045, 0.019)	0.435	**0.003**	-0.010 (-0.042, 0.022)	0.543	**0.002**
	Cortisol	-0.001 (-0.036, 0.035)	0.965	**0.003**	-0.001 (-0.036, 0.035)	0.974	**0.001**
Matsuda Index	Corticosterone	0.220 (0.034, 0.407)	**0.021**	0.444	0.134 (-0.036, 0.303)	0.122	0.921
	Cortisol	0.083 (-0.117, 0.284)	0.415	0.572	0.048 (-0.133, 0.229)	0.603	0.848
LDL Cholesterol	Corticosterone	-0.095 (-0.193, 0.003)	0.057	**0.019**	-0.086 (-0.184, 0.012)	0.085	**0.029**
	Cortisol	-0.138 (-0.241, -0.034)	**0.009**	0.565	-0.136 (-0.239, -0.034)	**0.009**	0.768
HDL Cholesterol	Corticosterone	0.018 (-0.066, 0.102)	0.678	0.263	-0.009 (-0.088, 0.071)	0.830	0.377
	Cortisol	0.105 (0.017, 0.193)	**0.020**	**0.038**	0.102 (0.019, 0.186)	**0.017**	0.083

Beta: Beta coefficient; **95% CI:** 95% confidence intervals; **p:** p value for the linear regression model; **Sex Int:** p value for sex interaction. **Confounders:** Age, blood sampling time, sex, smoking, alcohol, and relevant medication.

No significant associations of serum corticosterone and cortisol concentrations were observed with measures of glycaemia in the combined sample of men and women. However, there were significant sex-interactions for associations with fasting blood glucose (Sex Int: p = 0.009 and p < 0.001, respectively) and HbA1c (Sex Int: p = 0.003 for both) concentrations (Table 2.2, Figure 2.3). These sex-interactions remained

significant after adjusting for BMI. Subsequent sex-stratified analyses revealed that both serum corticosterone and cortisol concentrations were associated with higher fasting blood glucose ($p = 0.040$ and $p < 0.001$, respectively) and HbA1c ($p = 0.035$ and $p = 0.030$, respectively) concentrations in men, but lower HbA1c concentrations ($p = 0.042$ and $p = 0.030$, respectively) in women (Figure 2.3). Although the association between serum cortisol and two-hour OGTT glucose concentrations showed a significant sex-interaction after adjusting for BMI (Sex Int: $p = 0.047$), sex-stratified analyses revealed no significant associations in either sex (Table 2.2, Figure 2.3).



Figure 2.3: Sex-stratified associations of fasting serum corticosterone and cortisol concentrations with the metabolic syndrome components

95% CI: 95% confidence intervals. Models adjusted for age, blood sampling time, smoking, alcohol, relevant medication, and BMI. * p value < 0.05; # p value < 0.001.

Serum corticosterone, but not serum cortisol, was associated with higher Matsuda index (p = 0.021), but this was no longer significant after adjusting for BMI (p = 0.122) (Table

2.2). Serum glucocorticoid concentrations were not associated with fasting insulin concentrations or HOMA-IR.

Serum cortisol concentrations were associated with lower LDL (p = 0.009) and higher HDL (p = 0.020) cholesterol concentrations (Table 2.2). These associations remained significant after adjusting for BMI (p = 0.009 and 0.017, respectively). While serum corticosterone concentrations were not associated with any of the serum lipid concentrations, there was a significant sex-interaction for LDL cholesterol concentrations (Sex Int: p = 0.019) which were not altered by inclusion of BMI (Sex Int: p = 0.029) (Table 2.2). However, sex-stratified analyses revealed no significant sex-specific associations for LDL cholesterol concentrations (Figure 2.3). In contrast, although there was evidence of sex interaction for HDL cholesterol concentrations (Sex Int: p = 0.038), this was not significant after including BMI as a covariate.

2.5 Discussion

In this study, we have shown for the first-time glucocorticoid- and sex-specific associations between fasting serum glucocorticoid concentrations and the metabolic syndrome and its components in South African men and women of African ancestry. Fasting serum cortisol concentrations were associated with greater odds of having the metabolic syndrome, as well as higher systolic and diastolic blood pressure, but were also associated with a favourable lipid profile (lower LDL and higher HDL concentrations). In contrast, none of these associations were observed for serum corticosterone concentrations, which were instead associated with higher insulin sensitivity, as estimated using the Matsuda index. We have also shown that although women had higher concentrations of both cortisol and corticosterone, even after adjusting for adiposity, sex-specific associations of both glucocorticoids with measures of glycaemia were similar.

Specifically, both fasting serum cortisol and corticosterone concentrations were associated with higher fasting plasma glucose and HbA1c concentrations in men, but lower HbA1c concentrations in women.

The observation that women of the present study had a higher prevalence of the metabolic syndrome compared to their male counterparts (45.8% vs 27.8%) was in accordance with previous studies in populations of African ancestry (59). However, this is the first study to show that fasting serum cortisol concentrations were associated with greater odds of having the metabolic syndrome in both men and women of African ancestry. Nevertheless, cross-sectional studies comprising predominantly populations of Asian and European ancestries, have previously reported that individuals with the metabolic syndrome had higher circulating cortisol concentrations compared to individuals without the metabolic syndrome (274,277). The relationship between circulating cortisol concentrations and greater odds of having the metabolic syndrome may be attributed to underlying associations with the components used in the clinical definition of the metabolic syndrome. However, in the present study, the relationship between serum cortisol concentrations and the metabolic syndrome may have been largely driven by blood pressure as serum cortisol was not associated with elevated waist circumference or serum triglyceride concentrations, while the association with HDL cholesterol concentration was positive.

The finding that fasting serum cortisol, but not corticosterone, concentrations were associated with higher systolic and diastolic blood pressure, were also in accordance with previous studies in populations of both African and non-African ancestries. Circulating cortisol concentrations have been consistently associated with both systolic and diastolic blood pressure in populations of African, Asian and European ancestries

(221,251,276,313,322). However, the only study that has investigated corticosterone suggested that the associations between circulating corticosterone concentrations and systolic blood pressure were weaker when compared to cortisol, and were not present in other independent European cohorts (154). Glucocorticoids influence blood pressure via several mechanisms, but primarily through binding of the GR or mineralocorticoid receptor (MR) in the kidneys or vascular system. In the kidneys, glucocorticoids impair nitric oxide-mediated renal vasodilation (303) possibly by suppressing nitric oxide synthase, but also by influencing salt and water retention, ultimately increasing both systolic and diastolic blood pressure (304). There is however, no strong evidence to suggest that corticosterone and cortisol bind differently to the GR or MR and that these mechanisms are directly involved in the different associations of the two glucocorticoids with blood pressure (323,362). Recent studies suggest that the differences in the associations of the two glucocorticoids with components of the metabolic syndrome may be partly related to differences in hypothalamic pituitary adrenal (HPA) axis regulation (140). This hypothesis originates from the fact that cortisol and corticosterone differ in their susceptibility to transmembrane transport by ABC transporters. The ABC transporters are transmembrane proteins that transport a wide variety of substrates, including glucocorticoids, across cellular membranes (363). Animal studies suggested that ABCB1, an ABC transporter expressed in the central nervous system (CNS), selectively exports cortisol but not corticosterone across the blood-brain barrier (205). Thus, corticosterone may contribute disproportionately to the negative feedback control of the HPA axis within the CNS. As both glucocorticoids similarly bind to the GR and MR to increase blood pressure (303), higher circulating concentrations of corticosterone are accompanied by a more robust HPA axis regulatory response, in comparison to cortisol, to inhibit overall excess glucocorticoid production (205).

In addition to the above direct mechanisms, it has been suggested that the association between glucocorticoids and elevated blood pressure may be partly mediated by adiposity (258), which is associated with insulin resistance, dyslipidaemia, dysglycaemia, and the metabolic syndrome (44). However, there was no strong evidence from the present study to support mediation by adiposity, as adjusting for either BMI or waist circumference did not alter the relationship between serum glucocorticoid concentrations and measures of blood pressure. Further, there were no significant associations between serum glucocorticoid concentrations and measures of adiposity. However, the association between serum cortisol concentrations and BMI differed by sex (p value for sex interaction = 0.040). We are not aware of any previous study that suggested sexual dimorphism in the associations between circulating cortisol concentrations and measures of adiposity in any population. Thus, further studies that include other population groups and larger samples of men and women are required to explore these sex-specific interactions.

There was no association between serum cortisol concentrations and insulin resistance in our study. However, we have shown for the first time that fasting serum corticosterone concentrations were associated with higher insulin sensitivity, as assessed using the Matsuda index. As this relationship appeared to be confounded by BMI, it is possible that this glucocorticoid-specific association between serum corticosterone concentrations and insulin sensitivity is also linked to ABC transporters. While ABCB1 exports cortisol over corticosterone in the CNS, another ABC transporter called ABCC1 exports corticosterone over cortisol in the adipose tissue (140). Consequently, only elevated circulating cortisol concentrations, but not corticosterone, may increase cortisol activity within the adipose tissue, subsequently promoting insulin resistance. As elevated circulating corticosterone concentrations inhibit overall glucocorticoid production via HPA axis regulation,

corticosterone may subsequently reduce cortisol activity within the adipose tissue, and ultimately suppress cortisol-induced insulin resistance. However, further studies that investigate the role of the two glucocorticoids within human adipose tissue and HPA axis regulation are required to explore this hypothesis.

In addition to the above observations, we also showed that serum cortisol concentrations were associated with a favourable lipid profile (lower serum LDL and higher HDL cholesterol concentrations). Several studies have investigated the relationship between circulating cortisol and serum lipid concentrations, but the findings are inconsistent (241,251,276–278,356). Although most studies have not found an association with HDL cholesterol (251,277), some studies reported an inverse association between circulating cortisol and HDL cholesterol concentrations (276,278), whilst another reported a positive association (241). Likewise, while some studies have demonstrated an association between circulating cortisol and higher LDL cholesterol concentrations (356), other studies showed no evidence of this association (251,277) in populations of European ancestry. These contradicting findings were thought to be related to differences in age and BMI. However, in the present study, the associations between serum cortisol and HDL and LDL cholesterol concentrations were independent of BMI, negating the involvement of adiposity-related mechanisms. It is possible that the associations between circulating cortisol concentrations and the lipid profile are mediated via body fat distribution in both men and women. However, further studies are necessary to understand the exact mechanisms involved.

In contrast to the associations discussed above which exhibited glucocorticoid-specificity, findings from the present study also suggested for the first-time that both serum corticosterone and cortisol concentrations showed similar sex-specific associations with measures of glycaemia. As these sex-specific associations were similar for both

corticosterone and cortisol, mechanisms related to ABC transporters are unlikely to be involved. Rather, these sex-specific associations may be related to differences in glucocorticoid signaling, as demonstrated in rodent models. Mice treated with dexamethasone (a synthetic glucocorticoid) demonstrated major sex-specific transcriptional responses in the hepatic tissue (364,365). Moreover, another study showed that chronic exposure to excess glucocorticoid concentrations increased circulating glucose concentrations in male mice, but female mice were protected from these effects (366). Indeed, both corticosterone and cortisol are known to increase circulating glucose concentrations by promoting gluconeogenesis in the liver (179). Hence, sex disparities in glucocorticoid signaling within the liver may explain the observed sex-specific associations between serum glucocorticoid concentrations and measures of glycaemia, which in turn may be mediated by sex hormones and body fat distribution (367). Further human studies are required to investigate the role of hepatic glucocorticoid signaling and interactions with sex hormones and body fat distribution, to understand the observed sex-specific associations.

This study was limited by the lack of measurements for body fat distribution which are likely to explain the observed associations. For example, cortisol activity and clearance are much higher in VAT due to a higher number the glucocorticoid receptors compared to SAT (368). Moreover, the use of a cross-sectional study design was also a limitation as causality could not be inferred. Women were overrepresented in the present study, and this is common in many epidemiological studies in populations of African ancestry (9,369). Although associations with several outcome variables were tested, adjustment for multiple testing was not conducted. However, as the outcomes are all thought to be interrelated and are components of the metabolic syndrome, adjusting for multiple testing may be too conservative and limit the probability of showing true effects. Furthermore,

circulating glucocorticoids are often measured at a set time (e.g., 8:00 or 9:00 am) due to their diurnal nature. Although blood sample times in the present study varied widely, all samples were taken in the morning and blood sampling time was adjusted for in all models. However, this study would have benefitted additionally from obtaining data on time of awaking, which is also a well-known confounder of fasting cortisol concentrations.

Regardless of these limitations, this is the largest study to date that has compared the associations of both corticosterone and cortisol concentrations with components of the metabolic syndrome in a population of African ancestry. This study used a UPLC-MS method for measuring serum glucocorticoid concentrations which is preferred due to its high specificity and sensitivity. In addition, all known confounders including sampling time, age, sex, relevant medication, smoking, alcohol, and adiposity, were adjusted for in the statistical analyses.

In conclusion, the present study suggests that circulating glucocorticoid concentrations are associated with the metabolic syndrome and its individual components in South African men and women of African ancestry. Although we are yet to investigate the mechanisms involved, the observed associations exhibit both glucocorticoid- and sex-specificity in this population. Therefore, future studies investigating relationships between glucocorticoids and the metabolic syndrome should include the measurement of both cortisol and corticosterone and assessment of sex-based interactions.

Chapter 3 Research paper 2

Associations of common variants at *CYP17A1* and *SERPINA6/A1* with the metabolic syndrome and its components in black South African women

3.1 Abstract

Background: Common genetic variants at *CYP17A1* and *SERPINA6/A1* have previously been associated with circulating glucocorticoids and/or components of the metabolic syndrome in populations of non-African ancestry. This pilot study explored the associations of these loci with the metabolic syndrome and its individual components in women of African ancestry.

Methods: A sub-sample (n= 858) of adult women of African ancestry, between the ages of 25 and 84 years, were selected on the basis of the availability of DNA samples, and excluded if they were pregnant, breastfeeding or using any corticosteroid medication. Definition of the metabolic syndrome and its individual components was based on the 2009 harmonized criteria. Single nucleotide polymorphism (SNP) selection was based on genetic variants at both *CYP17A1* and *SERPINA6/A1* loci. To select African-specific tagSNPs, we identified European haplotype blocks from which each previously identified European SNP resides. A total number of 12 SNPs were selected and genotyped using the Open Array Taqman assay in the QuantStudio 12K Flex platform.

Results: There was evidence of an association between the rs2749527 SNP at *SERPINA6/A1* and higher fasting plasma glucose concentrations (standardized beta: 95% confidence intervals (95% CI) = 0.11: 0.03 to 0.18, p = 0.004), and paradoxically, the same SNP was associated with lower odds of having the metabolic syndrome (Odds

ratio: 95% CI = 0.47: 0.27 to 0.82, p = 0.007), when assuming additive models. Similarly, in an additive model, rs7147098, another *SERPINA6/A1* SNP, was associated with higher diastolic blood pressure (standardized beta: 95% CI = 0.09: 0.02 to 0.16, p = 0.009).

Conclusions: These data show that common genetic variants which have been associated with circulating glucocorticoid concentrations in populations of European ancestry are also associated with the metabolic syndrome in women of African ancestry. Although we have still to confirm glucocorticoid mediation in these associations, our findings are consistent with the role of glucocorticoids in the metabolic syndrome.

3.2 Introduction

The metabolic syndrome is defined as the clustering of interrelated risk factors for type 2 diabetes and cardiovascular disease (CVD). Consequently, such risk factors are commonly referred to as components of the metabolic syndrome and include elevated waist circumference, blood pressure, fasting blood glucose and circulating triglyceride, and reduced HDL cholesterol concentrations (44). Although the exact mechanisms by which these components interrelate to form the metabolic syndrome are unknown, the prevailing hypothesis suggests that elevated waist circumference and insulin resistance are the primary links (54). Environmental factors, such as unhealthy eating, sedentary lifestyle, tobacco smoking and alcohol abuse, play an important role in the development of the metabolic syndrome (17). However, not all individuals exposed to these environmental factors are affected, suggesting the involvement of genetic components. Indeed, the degree of heritability for the metabolic syndrome has been estimated at 10-30% (370). Therefore, identifying new genetic risk factors associated with the metabolic syndrome and its components has the potential to improve our understanding of the relevant mechanisms involved.

SNPs at *CYP17A1* were associated with measures of elevated waist circumference (336–338) and blood pressure (329–334) in populations of predominantly European and Asian ancestry. As *CYP17A1* encodes an enzyme that catalyses the conversion of precursors for corticosterone to those for cortisol synthesis in the glucocorticoid biosynthetic pathway, glucocorticoids are thought to partly mediate these genetic associations (328). Accordingly, associations between circulating glucocorticoid concentrations and the components of the metabolic syndrome in populations of both African and non-African ancestry have been reported (242,245,251,257,258,261,272,276,277,280,281,295,296). Moreover, common variants that are significantly associated with circulating cortisol concentrations in a European GWAS were identified at a locus that spans *SERPINA6* and *SERPINA1* (163). The *SERPINA6* and *SERPINA1* genes encode CBG, the primary glucocorticoid binding protein in the blood, and AAT which inhibits the release of glucocorticoids from CBG, respectively (163).

All the above genetic association studies were conducted in populations of predominantly European and Asian ancestry. Compared to genomes of non-African ancestries, African genomes are generally characterized by a greater number of haplotype blocks due to lower levels of LD and more complex LD structures and population substructures (341,345). Consequently, conducting genetic association studies in populations of African ancestry allows for more effective localization of the effect SNPs.

Hence, the aim of this pilot study was to explore the associations of previously identified *CYP17A1* and *SERPINA6/A1* loci with the metabolic syndrome and its components in a sample of African ancestry. South African women of African ancestry were chosen for this pilot study because they have a higher prevalence of the metabolic syndrome compared to their male counterparts and women of other ethnic ancestries, in similar urban settings (59,66,76).

3.3 Methods

3.3.1 Study participants

The study participants comprised 858 adult women from various studies at the SAMRC/Wits DPHRU, at the Chris Hani Baragwanath Academic Hospital (Soweto, Johannesburg, South Africa). Selection of these participants was primarily based on availability of DNA or whole blood for DNA extraction, from 2003-2016 study time points. Participants who were pregnant or using corticosteroid medication during the time of sampling were excluded from the present study. All the selected women were caregivers of the Bt20+ cohort, which has been described elsewhere (358)**.** A subsample of these women (n = 246) were randomly recruited between 2003 and 2008, whilst another subsample (n = 585) were recruited as part of the AWI-Gen study (348) between 2011 and 2013. The remainder of the participants (n = 27) were recruited as part of a longitudinal study designed to delineate the pathophysiology of type 2 diabetes in middle-aged women of African ancestry (371) between 2015 and 2016.

Regarding ethnicity, the selected women were self-identified as Bantu-speakers. Bantu-speakers belong to the Niger-Kordofanian ethno-linguistic African ancestral sub-group and their genetic diversity and migration history were previously described (372).

Ethical clearance for the Bt20+ cohort study was obtained from the University of the Witwatersrand Human Research Ethics Committee (Medical) (ethical clearance number: M010556). Further ethical clearance for all analyses conducted in the present study was also obtained (ethical clearance number: M160225).

3.3.2 Participant testing procedures

Weight and standing height were measured in participants wearing light clothes and without shoes, using a calibrated digital scale (Dismed, Halfway House, SA) to the nearest 0.1 kg and a fixed-wall stadiometer (Holtain, Crymych, UK) to the nearest 0.1 cm,

respectively. BMI was subsequently calculated as weight (kg) divided by height squared (m^2). Waist circumference was identified as the level of umbilicus and measured using a soft measuring tape to the nearest 0.5 cm.

Systolic and diastolic blood pressures were measured after the participant had been seated for at least 5 minutes, on the left arm using a digital blood pressure reader (Omron M6, Kyoto, Japan). For each participant, the blood pressure was measured three times at two-minute intervals, and the average of the second and third readings was recorded and used in the analyses. During interviews, participants were asked if they smoke any form of tobacco, and subsequently classified as smokers and non-smokers. The participants were also asked to bring their medications to the interviews for confirmation of the type of chronic medication used.

3.3.3 Blood sampling and biochemical analyses

Standard venepuncture technique was used to collect blood samples from participants after fasting for 10-12 hours. The blood samples were subsequently used for DNA extraction and determination of blood biomarkers, including plasma glucose and serum lipid (triglycerides and HDL cholesterol) and insulin concentrations. DNA was extracted from whole blood samples using the salting out method (373). Subsequently, DNA was quantified using PicoGreen dsDNA quantification assay (Thermo Fisher Scientific, Wilmington, USA) and all samples were normalized to 50 ng/µl concentrations. Serum triglyceride, HDL cholesterol, and plasma glucose concentrations were measured using prescribed enzymatic methods on an automated chemistry analyzer (Randox, Crumlin, UK). The inter and intra-assay CVs were 1.4% and 0.5-1.6% for serum triglyceride, 1.7% and 1.0-2.3% for serum HDL cholesterol, and 3.3% and 0.4-0.5% for plasma glucose concentrations, respectively. Serum insulin concentrations were assessed on an immunoassay system (Siemens Healthcare Diagnostics, Tarrytown, USA). The inter and

intra-assay CV ranges were 3.3-4.6% and 4.8-5.9% for serum insulin concentrations, respectively.

3.3.4 Definition of the metabolic syndrome

Based on the 2009 harmonized guidelines (44), diagnosis of the metabolic syndrome required the presence of three or more of the following components: elevated waist circumference (≥ 80 cm), blood pressure (systolic ≥ 130 and/or diastolic ≥ 85 mm Hg), fasting blood glucose (≥ 5.6 mmol/L), serum triglyceride (≥ 1.7 mmol/L) and serum HDL cholesterol (< 1.3 mmol/L) concentrations. Use of prescribed medications for blood pressure and diabetes were also considered relevant indications of the presence of the above metabolic syndrome components.

3.3.5 Selection of tag SNPs

SNPs at *CYP17A1* (rs1004467 and rs2486758) and *SERPINA6/A1* (rs11621961, rs2749527, rs2749529, and rs12589136) loci, that were previously associated with circulating glucocorticoid concentrations and/or metabolic syndrome components (measures of elevated blood pressure and waist circumference), were identified from the literature (see Table 3.1). The European haplotype blocks where these SNPs reside were used to select further African-specific tag SNPs from these loci, to ensure that any indirect SNP effects could potentially also be captured. Whole genome sequence data from the 1000 Genomes project (346) was used to estimate the European haplotype block sizes. A multi-marker approach ($r^2 > 0.80$) was used in Haploview version 4.2 (374) to select tag SNPs with minor allele frequency (MAF) > 5% in the African Genome Variation Project (AGVP) Zulu dataset (345), as a proxy representative of an African population closely relevant to this study population. Twelve SNPs (four SNPs at *CYP17A1* and eight SNPs at *SERPINA/A1*) were selected for genotyping. Notably, two out of four of the selected *CYP17A1* SNPs (rs17115104 and rs743572) are African-specific and were not included

in the European GWAS of circulating cortisol concentrations. Additionally, there was no evidence of association between the other two SNPs (rs1004467 and rs2486758) and circulating cortisol in the European GWAS (163).The selected SNPs and their genomic location and alleles are shown in Table 3.1.

Table 3.1: Single Nucleotide Polymorphisms included in this study.

Locus	SNP	Location	Call rate (%)	Alleles		Genomic region	Previous associations
				Major	Minor		
CYP17A1	rs17115104	10:104594401	99.9	T	A	Intron	African-specific tag SNP
	rs1004467	10:104594507	99.9	A	G	Intron	Systolic (Europeans, East Asians, Hispanics and African Americans) (329–331) and diastolic blood pressure (Europeans) (333), pulse pressure (East Asians and Europeans) (330), mean arterial pressure (Europeans) (332) and hypertension (East Asians and Europeans) (330); BMI (337) and visceral fat and subcutaneous fat areas (East Asians) (337)
	rs743572	10:104597152	99.2	A	G	5' UTR	African-specific tag SNP
	rs2486758	10:104597480	100.0	T	C	Promoter	Circulating corticosterone and the corticosterone/cortisol ratio (Europeans) (154)
SERPINA6/A1	rs11621961	14:94769476	99.6	C	T	1.1 kb downstream of *SERPINA6*	Circulating cortisol (Europeans) (163)
	rs7147098	14:94769817	99.2	G	A	<1 kb downstream of *SERPINA6*	African-specific tag SNP
	rs12589136	14:94793686	99.8	G	T	4.0 kb upstream of *SERPINA6*	Circulating cortisol (Europeans) (163)
	rs1956173	14:94816240*	99.9	G	A	26.5 kb upstream of *SERPINA6*, 26.8 kb downstream of *SERPINA1*	African-specific tag SNP
	rs2749529	14:94820459*	98.2	T	A	30.7 kb upstream of *SERPINA6*, 22.6 kb downstream of *SERPINA1*	Circulating cortisol (Europeans) (163)
	rs2749527	14:94827068	99.8	C	T	16.0 kb downstream of *SERPINA1*	Circulating cortisol (Europeans) (163)
	rs11160172	14:94829998	99.8	T	C	13.1 kb downstream of *SERPINA1*	African-specific tag SNP
	rs4900230	14:94839102	99.9	A	G	4.0 kb downstream of *SERPINA1*	African-specific tag SNP

All SNPs and their locations are reported using Human Genome Assembly **GRCh37.p13**. **Location** denotes chromosome and base pair positions. *****SNP position is intergenic (located between *SERPINA6* and *SERPINA1* genes); **5' UTR**: 5 prime untranslated region; **kb:** Kilobase pair.

3.3.6 Genotyping

Genotyping was conducted using a customized Taqman OpenArray Genotyping platform on the QuantStudio 12K Flex real time PCR system (Applied Biosystems, Foster City, USA). The genotypes were called using the QuantStudio 12K Flex software (Applied Biosystems, Foster City, USA). Quality control of the genotypes was performed by a trained laboratory technician and confirmation of the cluster plots was performed by an additional senior laboratory technician.

3.3.7 Data quality control and missingness

Pre-analyses quality control steps were conducted using PLINK v.1.9 software (375) and included removal of participants and SNPs with high missingness (> 20% and > 10%, respectively). Moreover, SNPs with low MAF (< 5%) and those failing the Hardy-Weinberg Equilibrium threshold (p < 0.01) were also removed (Figure 3.1).

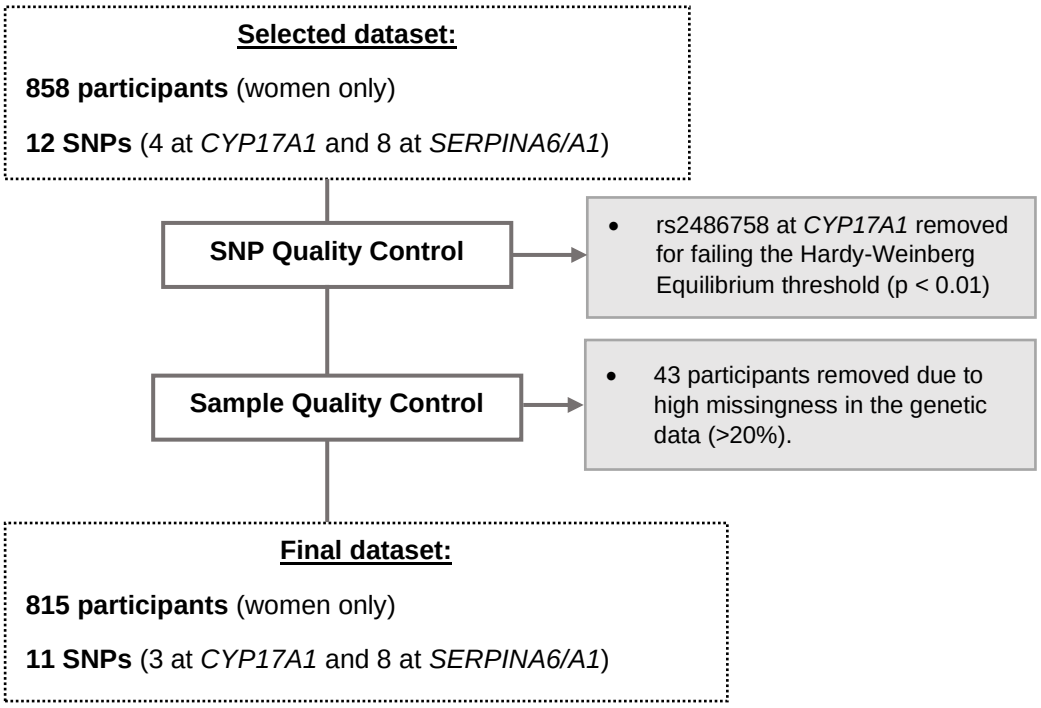


Figure 3.1: Selection of the dataset used in the analyses

3.3.8 Statistical analyses

Normality of the continuous variables was assessed using distribution graphs (histograms and box plots) and the Shapiro-Wilk test in R version 3.6.2 (361). Measurements of systolic and diastolic blood pressures, fasting plasma glucose, serum insulin, and lipid (triglycerides and HDL cholesterol) concentrations were all log-transformed prior to inclusion as outcome variables in the linear regression models. BMI and waist circumference were normally distributed, and these were not mathematically transformed. All genetic association tests were conducted in PLINK v.1.9 software (375) by comparing additive, dominant and recessive models for each SNP. The prevailing genetic model was then assumed in the associations between the selected SNPs and odds of having the metabolic syndrome. Logistic regression models were used to test the genetic associations with odds of having the metabolic syndrome, whilst linear regression models were used for the individual components of the metabolic syndrome. Age, smoking, BMI (except when the metabolic syndrome, BMI or waist circumference were the outcomes) and relevant chronic medication were included as potential confounders in all regression models. A Bonferroni correction was used to adjust for multiple testing. The number of independent SNPs were determined with 'indep-pairwise' method in PLINK using the following thresholds: window size = 50 kilobase pairs, variance inflation factor = 5 and r^2 = 0.5. Hence, the Bonferroni corrected p value threshold was determined as p = 0.05 / 5 (number of independent SNPs) = 0.01. Nevertheless, all genetic associations with p values less than 0.05 were presented in the results, to compare the directions of association with previous studies and to minimize possible introduction to type 2 errors.

3.4 Results

3.4.1 Characteristics of the study sample

The basic characteristics of the final 815 participants are presented in Table 2.

The majority of the participants were obese (63.5%) and presented with elevated waist circumference (87.1%). Similarly, the majority of the participants were classified as having elevated blood pressure (63.7%), but only 23.0% were using chronic blood pressure medication. The next most prevalent risk factors were reduced fasting HDL cholesterol concentrations (60.1%), followed by elevated blood glucose (24.5%), and fasting triglyceride (13.2%) concentrations. The use of chronic medications for diabetes was relatively low (5.0%). Overall, the majority of the participants were classified as having the metabolic syndrome (84.4%). Only 8.5% (n = 69) of the participants were smokers.

Table 3.2: Characteristics of the study sample.

Characteristic	N (out of 815)	median (IQR) or n (%)
Age and adiposity		
Age (years)	815	47.0 (42.0-53.0)
BMI (kg/m^2)	809	32.2 (27.5-37.3)
Obesity (≥ 30 kg/m^2)	809	514 (63.5)
Waist circumference (cm)	776	96.0 (86.5-106.0)
Waist circumference ≥ 80 cm	776	676 (87.1)
Blood pressure		
Systolic blood pressure (mmHg)	790	127.3 (115.0-142.0)
Systolic blood pressure ≥ 130 mmHg	790	357 (45.2)
Diastolic blood pressure (mmHg)	789	85.5 (76.7-94.5)
Diastolic blood pressure ≥ 85 mmHg	789	408 (51.7)
Using blood pressure medication	743	171 (23.0)
Elevated blood pressure (Elevated systolic and/or diastolic blood pressure and/or using blood pressure medication)	766	509 (63.7)
Insulin and Glucose		
Fasting insulin (uIU/ml)	521	9.9 (5.8-15.6)
Fasting glucose (mmol/L)	633	4.9 (4.5-5.4)
Fasting glucose ≥ 5.6 mmol/L	633	132 (20.9)
Using diabetes medication	746	37 (5.0)

Elevated fasting glucose and/or using diabetes medication	600	147 (24.5)
Serum lipids		
Fasting HDL cholesterol (mmol/L)	604	1.2 (0.9-1.4)
Fasting HDL-cholesterol < 1.3 mmol/L	604	363 (60.1)
Triglycerides (mmol/L)	604	1.0 (0.8-1.4)
Fasting triglycerides ≥ 1.7 mmol/L	604	80 (13.2)
Metabolic syndrome		
Prevalence (n (%))	678	572 (84.4)

Continuous data presented as median (25th-75th percentiles) and categorical data presented as n (%). **N:** Number of observations; **BMI:** Body Mass Index; **HDL:** High Density Lipoprotein.

3.4.2 Genetic associations with the metabolic syndrome and its individual components

Associations of the SNPs with the individual components of the metabolic syndrome, after adjusting for age, smoking, BMI (except when BMI or waist circumference were the outcomes) and relevant chronic medication, are presented in Table 3.3. Only models that showed significant associations ($p < 0.05$) are shown (Table 3.3).

At *CYP17A1*, rs1004467 was associated with lower BMI in the additive model ($p = 0.030$), whilst the same SNP along with rs17115104 were associated with lower waist circumference in the additive ($p = 0.017$ and $p = 0.013$, respectively) and dominant models ($p = 0.034$ and $p = 0.028$, respectively). However, none of these associations were significant after considering multiple testing (all $p > 0.01$). Moreover, there were no associations between any of the tested *CYP17A1* SNPs and measures of blood pressure, fasting glucose concentrations and serum lipid concentrations.

At *SERPINA6/A1*, rs7147098 was associated with higher systolic BP in the additive model ($p = 0.047$) and diastolic blood pressure in both the additive ($p = 0.009$) and dominant ($p = 0.019$) models. In contrast, rs12589136 was associated with lower systolic blood

pressure in the additive (p = 0.010) and dominant (p = 0.015) models. Further, rs2749527 at *SERPINA6/A1* was associated with higher fasting plasma glucose concentrations in all tested genetic models (p = 0.004 for additive, p = 0.011 for dominant, and p = 0.021 for recessive). However, only the additive models for the positive associations between rs7147098 and diastolic blood pressure, and between rs2749527 and fasting plasma glucose concentrations, were significant after considering multiple testing. There were no associations for any of the *SERPINA6/A1* SNPs with measures of adiposity or serum lipid concentrations.

Table 3.3: Genetic associations with the metabolic syndrome and its components.

SNP	Phenotype	Genetic Model	STD Beta/OR (95% CI)	P
rs1004467	BMI	Additive	-0.08 (-0.14, -0.01)	0.030
		Dominant	-0.07 (-0.13, 0.00)	0.059
		Recessive	-0.06 (-0.13, 0.01)	0.086
	Waist circumference	Additive	-0.08 (-0.15, -0.01)	0.017
		Dominant	-0.07 (-0.14, -0.01)	0.034
		Recessive	-0.06 (-0.13, 0.01)	0.087
rs17115104	Waist circumference	Additive	-0.09 (-0.15, -0.02)	0.013
		Dominant	-0.08 (-0.14, -0.01)	0.028
		Recessive	-0.07 (-0.13, 0.00)	0.058
rs7147098	Systolic blood pressure	Additive	0.07 (0.00, 0.13)	0.047
		Dominant	0.06 (-0.01, 0.13)	0.073
		Recessive	0.05 (-0.02, 0.11)	0.174
	Diastolic blood pressure	Additive	0.09 (0.02, 0.16)	0.009
		Dominant	0.08 (0.01, 0.15)	0.019
		Recessive	0.06 (-0.01, 0.13)	0.071
rs12589136	Systolic blood pressure	Additive	-0.09 (-0.15, -0.02)	0.010
		Dominant	-0.08 (-0.15, -0.02)	0.015
		Recessive	-0.05 (-0.12, 0.01)	0.126
rs2749527	Fasting plasma glucose	Additive	0.11 (0.03, 0.18)	0.004
		Dominant	0.10 (0.02, 0.17)	0.011
		Recessive	0.09 (0.01, 0.16)	0.021

Linear regression models adjusted for age, smoking, relevant medication, and BMI (except where BMI, waist circumference or odds of having the metabolic syndrome were the outcome variables). **Std Beta:** Standardized beta coefficient value; **95%CI:** 95% confident intervals; **p:** P value for the beta coefficient.

Based on the above observations, an additive model was assumed for the associations between the eleven selected SNPs and the odds of having the metabolic syndrome. The adjusted logistic regression models for these associations are shown in Figure 3.2. One SNP, rs2749527 at *CYP17A1*, was associated with lower odds of having the metabolic syndrome even after considering multiple testing (Odds ratio: 95% confidence intervals = 0.47: 0.27 to 0.82; p = 0.007).

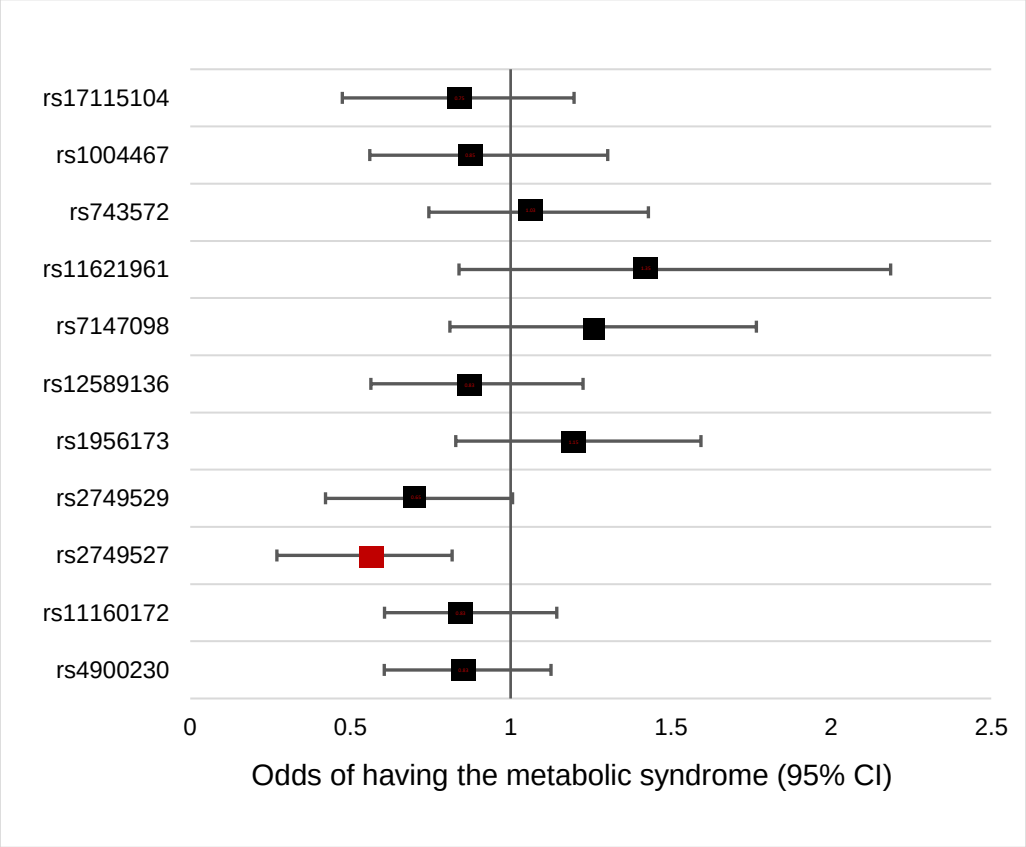


Figure 3.2: Genetic associations with the odds of having the metabolic syndrome.

Logistic models adjusted for age and smoking. The rs2749527 SNP at *CYP17A1* was associated with lower odds of having the metabolic syndrome, even after adjusting for multiple testing ($p < 0.01$).

3.4.3 Comparing the minor allele frequencies of the identified SNPs in other populations

The MAFs of the identified SNPs are shown and compared to those of other major population groups in Table 3.4. All of the identified SNPs have similar MAFs compared to other populations of African ancestry. In contrast, the MAF of rs17115104 is lower in

Europeans and Americans compared to Africans, and this SNP is monomorphic in Asians. Moreover, the minor allele of rs2749527 is present in much higher frequencies in all other populations compared to populations of African ancestry (31.4 to 68.3% in non-Africans versus 5.6 to 9.9% in Africans) (Table 3.4).

Table 3.4: Minor allele frequencies of the identified SNPs in the present study sample and other populations.

SNP	Minor allele	Minor allele frequencies by ancestry					
		Present study	Other Africans	Europeans	Americans	East Asians	South Asians
rs1004467	G	0.149	0.191	0.094	0.212	0.347	0.259
rs17115104	A	0.117	0.141	0.001	0.012	0.000	0.000
rs7147098	A	0.232	0.197	0.117	0.072	0.033	0.235
rs12589136	T	0.167	0.146	0.204	0.131	0.402	0.382
rs2749527	T	0.056	0.099	0.509	0.683	0.639	0.314

SNP: Single Nucleotide Polymorphism. Minor allele frequencies from the other populations were obtained from the **1000 genomes phase 3** dataset. **Other Africans** include Yoruba in Ibadan (Nigeria), Luhya in Webuye (Kenya), Gambian in Western Divisions (Gambia), Mende (Sierra Leone), Esan (Nigeria), Americans of African Ancestry in SW (USA) and African Caribbeans (Barbados). **Europeans** include Utah Residents (CEPH) with Northern and Western European Ancestry, Toscani (Italia), Finnish (Finland), British (England and Scotland) and Iberian Population (Spain). **Americans** include Mexican Ancestry from Los Angeles (USA), Puerto Ricans (Puerto Rico), Colombians from Medellin (Colombia) and Peruvians from Lima (Peru). **East Asians** include Han Chinese in Beijing (China), Japanese in Tokyo (Japan), Southern Han Chinese, Chinese Dai in Xishuangbanna (China) and Kinh in Ho Chi Minh City (Vietnam). **South Asians** include Gujarati Indian from Houston (Texas), Punjabi from Lahore (Pakistan), Bengali (Bangladesh), Sri Lankan Tamil (the United Kingdom) and Indian Telugu (the United Kingdom).

3.5 Discussion

In the present study, common variants at *CYP17A1* and *SERPINA6/A1*, two loci linked to the biology of glucocorticoids, were associated with key components of the metabolic syndrome in women of African ancestry. One of the key and novel findings in this study was that rs2749527 at *SERPINA6/A1* was associated with lower odds of having the metabolic syndrome, but paradoxically, higher fasting plasma glucose concentrations in

this sample of women of African ancestry. This study also demonstrated for the first time that rs7147098, another *SERPINA6/A1* SNP, was associated with higher diastolic blood pressure in these women. These novel genetic associations were significant even after considering multiple testing in the additive genetic models and are in accordance with the involvement of glucocorticoids in the metabolic syndrome.

The rs2749527 SNP at *SERPINA6/A1* was previously shown to be associated with lower cortisol concentrations in men and women of European ancestry (163). However, both circulating cortisol and corticosterone concentrations were associated with higher fasting glucose and HbA1c concentrations in men, but lower HbA1c concentrations in women, in our recent study in a population of African ancestry (376). It is therefore likely that while rs2749527 associates with lower circulating glucocorticoid concentrations in both men and women, the lower circulating glucocorticoid concentrations associate with higher fasting glucose concentrations in women only.

Although the mechanism remains to be investigated, rs2749527 may enhance the release of glucocorticoids from CBG into the liver, consequently, increasing gluconeogenesis, but reducing circulating glucocorticoid concentrations, due to increased metabolic clearance of glucocorticoids in the liver during obesity (287). In support of this hypothesis, the rs2749527 SNP was associated with higher cortisol binding activity in populations of European ancestry and the prevalence of obesity in the women of this study was relatively high (> 60%). Moreover, sex disparities in glucocorticoid signalling in the liver have been previously suggested. For example, chronic exposure to glucocorticoids increased fasting glucose concentrations in male mice but not in their female counterparts [57]. Certainly, the reduction in circulating glucocorticoid concentration as a result of rs2749527 would consequently reduce the risk of developing the components of the metabolic syndrome in women of the present study. Accordingly, circulating cortisol concentrations are generally higher in men and women with the

metabolic syndrome compared to their controls [19, 22, 24, 42, 44–47]. As the identified effect allele (minor allele T) of rs2749527 is much more common in populations of both European and Asian ancestry compared to Africans, the effect size in these non-African populations is likely to be higher. Hence, follow up studies in Europeans and Asians are recommended.

Similar to the above observations, the association between rs7147098 and higher diastolic blood pressure was also in accordance with mediation by circulating glucocorticoid concentrations and involvement of glucocorticoid binding and release from CBG. Circulating glucocorticoid concentrations are known to associate with higher blood pressure in populations of both African and non-African ancestry [42, 49–51]. Accordingly, rs7147098 resides within the same *SERPINA6/A1* haplotype block as rs12589136 (r^2 and D' > 0.80), which was associated with both higher circulating cortisol concentrations and higher cortisol binding activity in populations of European ancestry (163). In contrast to Europeans, the rs7147098 and rs12589136 SNPs were not strongly correlated in the present study (r^2= 0.05, D'= 0.84). Therefore, rs7147098 may be more strongly correlated with the effect SNP compared to rs12589136 in populations of African ancestry. In support of this hypothesis, the association between rs12589136 and systolic blood pressure was negative, contradicting the involvement of glucocorticoids, and also failed the multiple testing significance threshold. Certainly, further studies that include glucocorticoid measurements are required to confirm mediation by circulating glucocorticoids.

Another association that was in contrast to previous findings and failed the multiple testing significance threshold was the associations of rs17115104 and rs1004467 at *CYP17A1* with lower adiposity. This finding is in contrast to a previous study in East Asians which demonstrated that rs1004467 was associated with higher BMI and waist circumference [7]. Notably, in populations of European and Asian ancestries, rs17115104 generally

resides within the same *CYP17A1* haplotype block as rs1004467 (D' and r^2 > 0.80). However, in the present study, these two SNPs were only partially correlated (r^2 = 0.68, D' = 0.94). As the MAF of the lead SNP (rs1004467) appears to be lower in Africans when compared to Asians, the observed associations require confirmation in a large sample size in populations of African ancestry. Nevertheless, it is also possible that these observations are related to ethnic disparities in the biology of glucocorticoids and/or adiposity. The *CYP17A1* gene encodes an enzyme with key functions in steroidogenesis. In addition to the 17-alpha-hydroxylase activity that directly influences biosynthesis of glucocorticoids (both cortisol and corticosterone) (328), the enzyme also has 17,20-lyase activity, which catalyzes the conversion of the cortisol precursors to sex hormone precursors (377). While no studies have compared glucocorticoids between women of East Asian and African ancestry, women of African ancestry have been consistently reported to be more obese but with lower circulating cortisol concentrations compared to women of European ancestry [50, 53, 54]. However, whether these ethnic differences also exist when comparing women of African ancestry to their Asian ancestry counterparts remains to be investigated.

The present study has some limitations. For example, the reported investigations were conducted in women only and sex interactions could not be tested. As this was a pilot study, a *priori* power calculation was not conducted, and the sample size was based on availability of DNA samples for genotyping. For example, a post hoc power analysis suggested that the statistical power to detect associations between rs2749527 and the metabolic syndrome was approximately 58.3% (Appendix G). This low statistical power is likely to have been due to a high prevalence of the metabolic syndrome in the studied population (84.4%). Consequently, the lack of genetic associations with some of the key metabolic syndrome components, such as circulating lipid concentrations, may have reflected low statistical power rather than a true lack of association in women of African

ancestry. Hence, further studies that also include men of African ancestry and a larger sample size are necessary to confirm the observed findings. Regardless of the above limitations, the study also has several strengths. We focused on haplotype blocks that were previously identified in populations of European and Asian ancestry. Thus, the chances of finding false positive associations was minimized. Further, the study was analyzed in a sample of urban-dwelling women of African ancestry, who are known to be disproportionally affected by the metabolic syndrome but are largely understudied. Therefore, the reported findings add new knowledge to our understanding of the pathophysiology of the metabolic syndrome in African women.

In conclusion, the study demonstrated that common variants at *CYP17A1* and *SERPINA6/A1* were associated with key components of the metabolic syndrome in women of African ancestry. These findings support the hypothesis that glucocorticoids are involved in the metabolic syndrome in populations of African ancestry. Future functional studies are required to understand the mechanisms involved in the observed associations.

Chapter 4 Research Paper 3

CYP17A1 and *SERPINA6/A1* polymorphisms are associated with components of the metabolic syndrome in black South African men and women

4.1 Abstract

Background: Genetic associations between single nucleotide polymorphisms (SNPs) at *CYP17A1* and *SERPINA6/A1* and circulating glucocorticoid concentrations and key components of the metabolic syndrome have been reported in people of European and Asian ancestry. The aim of this study was to investigate these genetic associations in men and women of African ancestry, who are disproportionally affected by the metabolic syndrome and its components.

Methods: We selected 4431 (56.7% women) South African men and women from the AWI-Gen project, after excluding non-South Africans, and relatives. The AWI-Gen participants were genotyped using the H3A genotyping array and imputed with the African reference panel on the Sanger Imputation. From these genotype data, we extracted a total of 581 SNPs (31 from the *CYP17A1* and *550* from the *SERPINA6/A1* loci). The metabolic syndrome and its components were defined using the 2009 harmonized guidelines. Serum glucocorticoid concentrations were measured in a sub-sample of 877 participants (304 men and 573 women) using a liquid chromatography-mass spectrometry method. All genetic associations were conducted in PLINK and we used 10 000 permutations to address multiple testing.

Results: *CYP17A1* SNPs were associated with higher waist circumference in both men and women (P value adjusted for multiple testing (p2) = 0.032). Other *CYP17A1* SNPs were associated with fasting insulin and the homeostatic model assessment of insulin

resistance in men only (all p2 < 0.020), whilst other *CYP17A1* SNPs were associated with higher fasting cortisol concentrations in women only (p2 = 0.005). *SERPINA6/A1 SNPs* were associated with diastolic blood pressure in both men and women (p2 < 0.010), while in women only, different *SERPINA6/A1* SNPs were associated with lower high-density lipoprotein (HDL) cholesterol (p2 < 0.050), and other SNPs with higher HDL cholesterol (p2 = 0.005).

Conclusions: Our findings support the hypothesis that *CYP17A1* and *SERPINA6/A1* SNPs are associated with components of the metabolic syndrome in populations of African ancestry. As several of these novel genetic associations showed sex-specificity, future studies should also assess sex-interactions, and explore the mechanisms involved.

4.2 Introduction

Risk factors for type 2 diabetes and CVD, including elevated waist circumference, blood pressure and fasting blood glucose concentration and the presence of dyslipidaemia, cluster together to form the metabolic syndrome (44). The prevalence of the metabolic syndrome generally differs by both sex and ethnicity, with women of African ancestry exhibiting a higher prevalence compared to their male counterparts and women of other ancestries in similar urban settings (9,59,378). The underlying mechanisms through which these sex- and ethnic-based differences occur, are unknown. Therefore, identifying biomarkers and common genetic variants that are associated with components of the metabolic syndrome, has the potential to improve the biological understanding of this condition and consequently the risk for type 2 diabetes and CVD.

Population-based cross-sectional studies have suggested that circulating cortisol, the primary glucocorticoid in humans, is associated with a higher risk of having the metabolic syndrome and its components (242,245,251,257,261,272,276,277,281,295,296). Likewise, SNPs at genes involved in glucocorticoid biology have been implicated in key

components of the metabolic syndrome (340). *CYP17A1* SNPs are associated with measures of elevated blood pressure in populations of predominantly Asian and European ancestry (329,331,334,337–339,351), and with measures of adiposity in a population of East Asian ancestry (337). As the *CYP17A1* gene encodes an enzyme that converts precursors for corticosterone to precursors for cortisol (328), the associations between *CYP17A1* SNPs and these key metabolic syndrome components are thought to be mediated by glucocorticoids (340). Considering that the metabolic syndrome components are interrelated, the *CYP17A1* SNPs may also be associated with elevated fasting blood glucose and the presence of dyslipidaemia, but this hypothesis is yet to be tested.

Further, in a GWAS in populations of European ancestry, inter-individual variation in circulating cortisol concentrations was associated with SNPs at a locus that spans *SERPINA6* and *SERPINA1* genes (163). *SERPINA6* encodes CBG, the primary glucocorticoid-binding protein in circulation, whilst *SERPINA1* encodes AAT, which is involved in the regulation of the binding and release of glucocorticoids from CBG (379).

The above-mentioned genetic studies were limited to populations of non-African ancestries and only a few investigated sex-specific associations (163,223,337). Genetic association studies in populations of African ancestry provide more effective localization of the effect SNPs compared to studies in populations of European ancestry (345). Accordingly, our recent pilot study in women of African ancestry, demonstrated associations between *SERPINA6/A1* SNPs and fasting plasma glucose and diastolic blood pressure (unpublished). However, the relationships between *CYP17A1* SNPs and measures of adiposity were in the opposite direction compared those previously reported in East Asians (337). Moreover, our previous findings suggested sex-specific

associations between circulating glucocorticoid concentrations and metabolic syndrome components, which has not been shown in other populations (376).

Hence, the primary aim of this study was to investigate the associations between *CYP171A* and *SERPINA6/A1* SNPs and the metabolic syndrome and its components, in South African men and women of African ancestry. The study further investigated the hypothesis that the observed genetic associations were related by circulating glucocorticoid concentrations.

4.3 Methods

4.3.1 Study population

This study comprised of participants from the AWI-Gen project. Briefly, AWI-Gen is a population-based cross-sectional study that comprises of over 12 000 adult men and women of African ancestry from six research centres in the west, east and southern part of Africa (348). The Dikgale Health and Demographic Surveillance System (HDSS), MRC/Wits Agincourt HDSS and SAMRC/Wits DPHRU are the AWI-Gen research centres that are based in South Africa and only participants from these research centres were included in the present study. Hence, the present study included 5268 participants (2226 men and 3042 women) of African ancestry from these three South African research centres. The AWI-Gen data collection was approved by the University of Witwatersrand Human Research Ethics Committee (Medical) (Certificate number: M170880). Likewise, the committee approved all secondary analyses described in the present study (Certificate number: M160225).

4.3.2 Participant testing procedures

Measurements of all phenotypic data have been described in detail previously (359), and are only briefly described below.

Anthropometry, blood pressure, and lifestyle factors

Weight was measured to the nearest 0.1 kg using a digital scale (Kendon Medical, SA), and height was measured to the nearest millimeter using a Harpenden digital stadiometer (Holtain, Wales, UK). Weight and height were subsequently used to calculate body mass index (BMI = weight in kg/height in metres^2). Waist circumference was measured to the nearest millimeter using a stretch-resistant soft measuring tape (SECA, Hamburg, Germany), at the level of the narrowest part of the torso, halfway between the lowest rib and the iliac crest.

Blood pressure was measured on the right arm using a digital blood pressure reader (Omron M6, Kyoto, Japan) and appropriately sized cuffs. Each participant rested for at least five minutes prior to the blood pressure measurement in a seated position. The blood pressure was measured in triplicate at two-minute intervals, but only the average of the second and third readings were recorded and used in the analyses.

In terms of lifestyle factors, smoking and alcohol status were determined by asking the participants if they smoked any form of tobacco product (e.g., cigarettes, cigars, or pipes), or consumed any form of alcoholic beverage (e.g., beer, wine, spirits, fermented cider, or traditional beer), respectively. The participants were subsequently classified as smokers/non-smokers and drinkers/non-drinkers. The participants were asked to bring all their medications to the interview sessions for confirmation and recording of chronic medications used.

Blood sampling and biochemical analyses

Participants fasted overnight for 10-12 hours prior to collection of fasting blood samples. A standard venepuncture technique was used to collect the blood samples for determination of fasting serum glucose, insulin, lipid, and glucocorticoid concentrations, and to obtain buffy coat for DNA extraction. Fasting serum glucose and lipids were

measured using the Randox Plus chemistry analyzer (Crumlin, Country Antrim, UK) by colorimetric assays. The intra-assay CV for serum glucose concentration was 2.3%, whilst the intra-assay CV for serum lipid concentrations were all less than 1.5%. The concentrations of LDL cholesterol were subsequently estimated using the Friedewald Equation (360). Fasting serum insulin concentrations were determined by an enzyme-linked chemiluminescent immunometric assay on the Immulite 1000 immunoassay system (Siemens, Hamburg, Germany) and the intra-assay CV was less than 2.0%. HOMA-IR was subsequently computed from the fasting glucose and insulin values, using the HOMA2-IR calculator version 2.2.3 (https://www.dtu.ox.ac.uk/homacalculator).

Serum corticosterone and cortisol concentrations were determined on a sub-sample of 877 participants (304 men and 573 women) from the Soweto DHPRU cohort. Selection of these participants was based on availability of serum samples for glucocorticoid determination. The time of fasting blood sampling for these selected participants was recorded and ranged between 7:30 am to 11:59 am. A liquid-liquid extraction technique (Agilent Technologies, California, USA) was used to extract the glucocorticoids. The serum glucocorticoid concentrations were subsequently analysed by UPLC-MS (Waters Corp, Milford, USA). The inter and intra-assay CVs were 7.3% and 2.9% for serum corticosterone, and 13.6% and 9.6% for cortisol concentrations, respectively.

4.3.3 Definition of the Metabolic Syndrome

The presence of the metabolic syndrome was based on the 2009 Harmonized criteria (44). Briefly, the following cut-off points were used to define components of the metabolic syndrome: i) elevated waist circumference (≥ 94 cm in men and ≥ 80 cm in women); ii) elevated triglycerides (≥ 1.7 mmol/L); iii) reduced HDL cholesterol (< 1.0 mmol/L in men and < 1.3 mmol/L in women); iv) elevated blood pressure (≥ 130 mmHg for systolic and/or ≥ 85 mmHg for diastolic and/or using blood pressure medication); v) elevated glucose (≥

5.6 mmol/L and/or using diabetes medication). Participants with three or more of the above components were scored as metabolic syndrome cases, and the participants without the metabolic syndrome were used as controls.

4.3.4 Genotyping, imputation, and data quality control

DNA was extracted from buffy coats using a modified salting-out method (373)**.** The Human Heredity and Health in Africa (H3Africa) genotyping array (Illumina Inc., California, USA) was used to perform genome-wide genotyping of over 2.3 million SNPs in all AWI-Gen participants. The H3Africa genotyping array was specifically designed to account for the larger genetic diversity and smaller haplotype blocks observed in the genomes of populations of African ancestry. All samples were genotyped via the Illumina FastTrack service using the H3Africa genotyping array. Pre-imputation quality control (QC) steps included removal of participants and SNPs with high data missingness (> 5%), SNPs with low MAF (< 1%) and those failing the HWE test ($p < 1 \times 10^{-4}$). Mitochondrial, non-autosomal, and ambiguous SNPs that failed to match the GRCh37 reference alleles and strands were also removed.

The Sanger Imputation Server (380) was used to perform imputation on the cleaned dataset, which comprised of 1 729 661 SNPs and 10 903 participants from all AWI-Gen research centres. The African Genome Resources was used as the reference panel, EAGLE2 (381) for pre-phasing, and the default positional Burrows-Wheeler transform algorithm for the imputation (382). Post-imputation QC steps included the removal of poorly imputed SNPs (info score < 0.6), SNPs with low MAF (< 1%), and SNPs failing the HWE ($p < 1 \times 10^{-4}$). The final cleaned and imputed AWI-Gen dataset had 13.98 million SNPs.

From these data, SNPs at the *CYP17A1* gene were selected to include all available introns and exons. In addition, the 2 kb region upstream of the transcription start site of

CYP17A1 was also included, as SNPs in this region were previously associated with glucocorticoid concentrations and the metabolic syndrome in populations of European ancestry (154,340). Secondly, all SNPs starting from the *SERPINA6* gene to the *SERPINA1* gene and including the locus in-between the two genes were also selected. The locus in-between *SERPINA6* and *SERPINA1* was included because the SNPs identified from the GWAS of circulating cortisol were located in-between these two genes (163). After removing the non-South African samples from the dataset, related participants were also removed (PI_HAT > 0.1875) (n = 758 participants removed). Due to previously reported associations between glucocorticoid metabolism and critical illness (237), participants previously diagnosed with cancer were also removed from the dataset (n = 79 participants removed). The final selected dataset used in the present analyses comprised of 581 SNPs (31 from the *CYP17A1* and 550 from the *SERPINA6/A1* loci) in 4431 South African participants of African ancestry (1918 men and 2513 women).

4.3.5 Statistical analyses

The Shapiro-Wilk test and histogram plots were used to assess the distribution of continuous variables in STATA v.1.13.1 software (Stata Corp LLC, Texas, USA). Non-normally distributed outcome variables were first mathematically transformed (e.g., using either log or square root transformations) to obtain normality prior to inclusion in the linear regression models. The Mann-Whitney *U* and Chi-square tests were used to test statistical differences between men and women in the continuous and categorical variables, respectively

All genetic association tests were performed using PLINK v.1.9 software (375). Associations between the selected SNPs and the metabolic syndrome and its components were tested using logistic and linear regression models, respectively. Similarly, linear regression models were also used to examine the associations between

the SNPs and serum glucocorticoid concentrations in the sub-sample of the Soweto participants. Age, sex, smoking, alcohol, and BMI (except where BMI, waist circumference, or the metabolic syndrome were the outcome variables) were included as potential confounders in all regression models. Due to the diurnal nature of glucocorticoids, blood sampling time was also included as an additional covariate in models where serum glucocorticoid concentrations were the outcome variables. To minimise confounding due to prescribed medication, participants taking prescribed diabetes-, blood pressure-, and lipid-medications (see Table 4.1 for numbers) were excluded from linear regression analyses involving glucose or insulin, blood pressure, and serum lipids, respectively. Notably, while this method was used to address confounding due to prescribed medication, adjusting for the medication in the regression models was also explored and yielded similar results. In all regression models, 10 000 permutations were used to address multiple testing and the p values (adjusted for multiple testing) were shown as p2. Given the low statistical power, a 10 000 permutation was more suitable for the study. A Bonferroni correction would have reduced the statistical power, and consequently increase the probability of producing false negatives. Sex interactions were also tested, and the sex interaction p values were recorded as “Sex Int”. All models showing significant sex interactions (Sex Int < 0.05) were subsequently stratified by sex.

The outcomes investigated in this study included the metabolic syndrome and its individual components (waist circumference, systolic and diastolic blood pressure, fasting glucose, triglycerides, and HDL), as well as BMI, fasting LDL and total cholesterol, insulin, HOMA-IR, corticosterone, cortisol, and the corticosterone/cortisol ratio. However, only models that showed significant associations after adjusting for multiple testing (p2 < 0.05) and models that showed significant sex interactions (Sex Int < 0.05) are presented in the results.

4.4 Results

4.4.1 Study sample characteristics

Table 4.1 shows the characteristics of the study participants and a comparison by sex. Likewise, the prevalence of the categorized individual components of the metabolic syndrome are compared by sex in Figure 4.1. Although age did not differ by sex, women had higher BMI and waist circumference than men ($p < 0.001$ for both) (Table 4.1). Accordingly, a greater proportion of women were obese and had elevated waist circumference compared to men ($p < 0.001$ for both) (Figure 4.1). In contrast, men had higher diastolic blood pressure compared to women ($p = 0.027$) (Table 4.1), and this was accompanied by a greater proportion of men with elevated diastolic blood pressure compared to women ($p = 0.013$) (Figure 4.1). There were no differences in systolic blood pressure, the use of blood pressure medication and overall elevated blood pressure (which considered both systolic and diastolic blood pressures and medication) between sexes.

Women had higher concentrations of fasting serum glucose ($p < 0.001$) (Table 4.1) and were a greater proportion of the participants with elevated blood glucose ($p < 0.020$) (Figure 4.1) compared to men. In contrast, there were no differences in fasting serum insulin concentrations, HOMA2-IR, or the use of diabetes medication, between men and women. For circulating lipids, women had higher fasting serum concentrations of total and LDL cholesterol ($p < 0.001$ for both), and triglycerides ($p = 0.003$) (Table 4.1), and this was accompanied by a greater proportion of women with low serum HDL cholesterol ($p < 0.001$) (Figure 4.1). In contrast, there were no sex differences in elevated serum triglycerides and the use of lipid-lowering medication.

Corresponding to the above observations, a greater proportion of women had the metabolic syndrome compared to men ($p < 0.001$) (Figure 4.1). Conversely, a greater

proportion of men smoked and consumed alcohol ($p < 0.001$ for both). In the sub-sample, women had higher fasting serum concentrations of corticosterone and cortisol compared to men ($p = 0.003$ for both) (Table 4.1).

Table 4.1: Characteristics of the study sample.

	N	All (N= 4431)	Men (N=1918)	Women (N=2513)	p value
Age and adiposity					
Age *(years)*	4431	52 (46-58)	52 (46-58)	52 (46-58)	0.185
BMI *(kg/m^2)*	4147	27.2 (22.2-32.7)	23.2 (20.1-27.4)	30.5 (25.8-35.5)	**<0.001**
Waist circumference *(cm)*	4148	95.8 (89.4-101.5)	88.0 (78.0-89.8)	98.0 (92.3-103.2)	**<0.001**
Blood pressure					
Systolic blood pressure *(mmHg)*	3496	128.5 (116.0-144.0)	129.0 (116.5-144.0)	128.0 (115.5-144.0)	0.424
Diastolic blood pressure *(mmHg)*	3499	83.0 (74.5-91.5)	84.0 (75.0-92.5)	82.5 (74.0-91.5)	**0.027**
Glycaemia and insulin resistance					
Fasting glucose *(mmol/L)*	3406	4.9 (4.6-5.4)	4.8 (4.4-5.3)	5.0 (4.6-5.5)	**<0.001**
Fasting insulin *(uIU/ml)*	3417	7.2 (4.3-13.1)	7.0 (4.3-13.3)	7.4 (4.3-12.9)	0.327
HOMA-IR	2985	1.6 (0.9-3.1)	1.6 (0.9-3.2)	1.7 (0.9-3.0)	0.768
Serum lipids					
Total cholesterol *(mmol/L)*	4345	4.1 (3.5-4.9)	4.0 (3.3-4.7)	4.3 (3.6-5.0)	**<0.001**
LDL cholesterol *(mmol/L)*	4309	2.5 (1.9-3.1)	2.3 (1.8-2.9)	2.6 (2.0-3.2)	**<0.001**
HDL cholesterol *(mmol/L)*	4347	1.2 (1.0-1.4)	1.1 (0.9-1.4)	1.2 (1.0-1.4)	0.111
Triglycerides *(mmol/L)*	4344	0.9 (0.7-1.3)	0.9 (0.6-1.3)	0.9 (0.7-1.3)	**0.003**
Medication*					
Blood pressure med *(n/N (%))*	3450	991 / 3450 (28.7)	329 / 1350 (24.3)	662 / 2100 (31.5)	0.869
Diabetes med *(n/N (%))*	3450	366 / 3450 (10.6)	134 / 1362 (9.8)	232 / 2088 (11.1)	0.259
Cholesterol med *(n/N (%))*	2030	6 / 2030 (0.3)	2 / 1337 (0.1)	6 / 693 (0.9)	0.211
Lifestyle*					
Smoking *(n/N (%))*	3108	597 / 3108 (19.2)	538 / 1078 (49.9)	59 / 2030 (2.9)	**<0.001**
Alcohol *(n/N (%))*	2502	1292 / 2502 (51.6)	954 / 1168 (81.7)	338 / 1334 (25.3)	**<0.001**
Glucocorticoids					
Corticosterone *(nmol/L)*	650	6.0 (3.5-12.2)	5.0 (3.4-8.8)	6.4 (3.6-14.6)	**0.003**
Cortisol *(nmol/L)*	673	183.3 (96.6-322.6)	143.0 (74.1-295.7)	208.3 (84.2-343.8)	**0.003**
Corticosterone/cortisol	548	0.05 (0.02-0.09)	0.04 (0.02-0.07)	0.05 (0.02-0.09)	**0.050**

Continuous data presented as **median (IQR: Interquartile range)** and categorical data presented as number of prevalent / total number of observations **(n/N)** and percentage **(%)**. **Wilcoxon Rank Sum** and C**hi-square test** were used to statistically compare the continuous and categorical variables, respectively, between the sex groups. **p value**: p value for the statistical difference between men and women; **BMI:** Body Mass Index; **HOMA-IR:** Homeostatic Model Assessment of Insulin Resistance; **LDL:** Low Density Lipoprotein; **HDL:** High Density Lipoprotein; **Med:** Medication. *****All medication and lifestyle factors (smoking and alcohol) were self-reported during interview sessions.

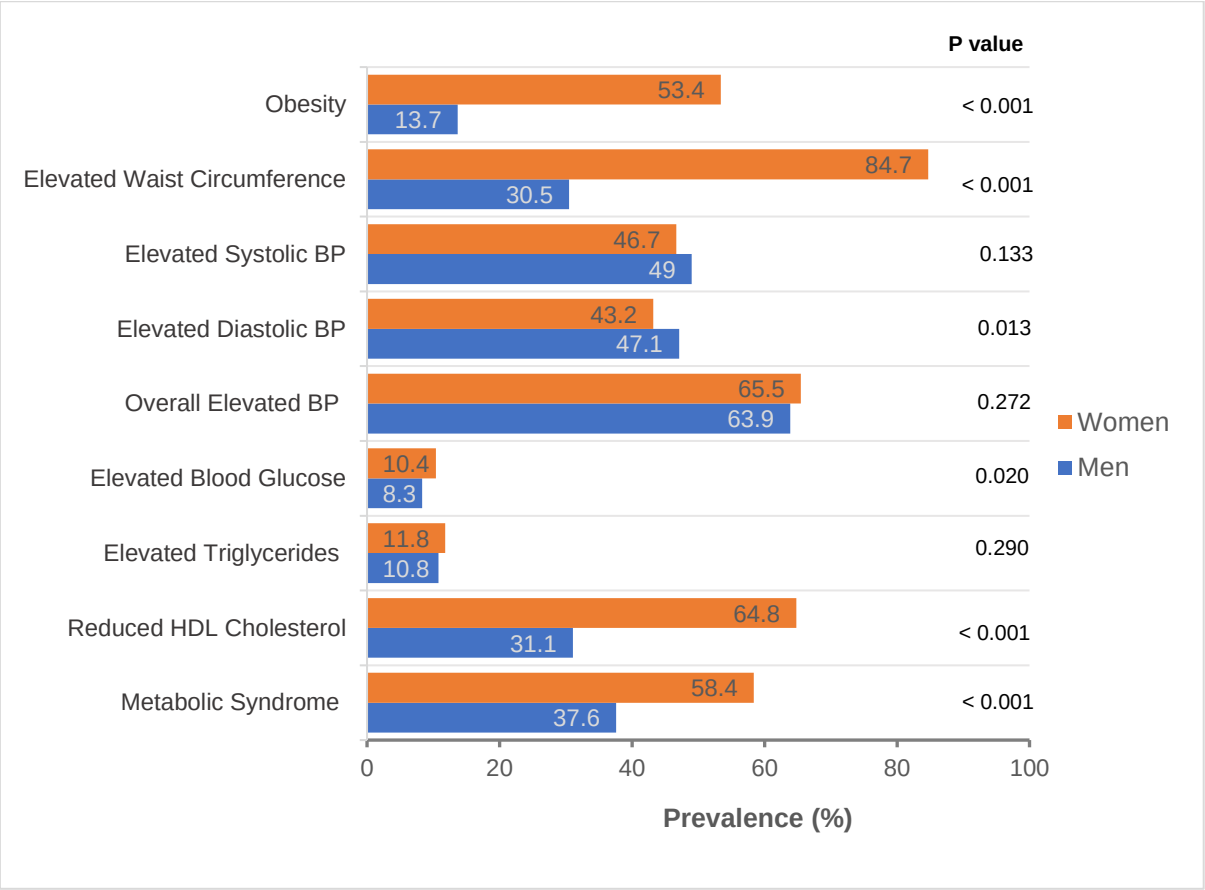


Figure 4.1: A comparison of the prevalence of the metabolic syndrome and its components between men and women of African ancestry.

p: P value for the statistical difference between men and women, using a chi-square test. **BP:** Blood pressure. **Obesity:** BMI ≥ 30 kg/m^2; **Elevated Waist Circumference:** ≥ 94 cm in men and ≥ 80 cm in women; **Elevated Systolic BP:** ≥ 130 mmHg; **Elevated Diastolic BP:** ≥ 85 mmHg; **Overall Elevated BP:** Elevated Systolic BP and/or Diastolic BP and/or using BP medication; **Elevated Blood Glucose:** Elevated fasting glucose (≥ 5.6 mmol/L) and/or using diabetes medication; **Elevated Triglycerides:** Elevated fasting triglycerides (≥ 1.7 mmol/L); **Reduced HDL Cholesterol:** Reduced fasting HDL-cholesterol (< 1 .0 mmol/L in men, < 1.3 mmol/L in women); **Metabolic Syndrome:** 2009 Harmonized Criteria (44).

4.4.2 *CYP17A1* SNPs and the metabolic syndrome components

There were no significant associations between any of the tested *CYP17A1* SNPs and the odds of having the metabolic syndrome. However, rs17115144 and rs114190228 at *CYP17A1,* were associated with higher waist circumference in the men and women combined (Table 4.2). Figure 4.2 shows the LocusZoom and the box and whisker plots that summarize the observed association between the lead SNP (rs17115144) and waist circumference in the study sample comprising both sexes. The LocusZoom plot in Figure 4.2 (a) suggested that rs17115144 and rs114190228 are in strong LD ($r^2 > 0.8$) in populations of African ancestry and may represent the same signal in the observed associations. There were no sex interactions between any of the tested SNPs and waist circumference (Table 4.2).

Table 4.2 Associations of *CYP17A1* SNPs in men and women of African ancestry

Phenotype	SNP	Genotypes			Linear regression model adjusted for confounders			
		MM	Mm	mm	Beta (95% CI)	p	p2	Sex Int
Waist circumference	rs17115144	GG	GA	AA	0.182 (0.068, 0.297)	0.002	0.032	0.175
	rs114190228	GG	GA	AA	0.182 (0.068, 0.297)	0.002	0.032	0.232
Fasting insulin	rs10883783	TT	TA	AA	-0.073 (-0.141, -0.005)	0.035	0.389	**0.011**
	rs743575	TT	TG	GG	-0.072 (-0.140, -0.005)	0.036	0.398	**0.009**
	rs4919687	GG	GA	AA	-0.072 (-0.139, -0.004)	0.037	0.409	**0.010**
	rs10883784	CC	CT	TT	-0.067 (-0.134, 0.001)	0.053	0.524	**0.006**
	rs10786714	GG	GC	CC	-0.067 (-0.135, 0.000)	0.051	0.512	**0.005**
HOMA-IR	rs10883783	TT	TA	AA	-0.085 (-0.157, -0.013)	0.021	0.261	**0.033**
	rs743575	TT	TG	GG	-0.085 (-0.157, -0.014)	0.020	0.252	**0.029**
	rs4919687	GG	GA	AA	-0.085 (-0.157, -0.013)	0.020	0.257	**0.030**

	rs10883784	CC	CT	TT	-0.080 (-0.152, -0.008)	0.029	0.336	**0.021**
	rs10786714	GG	GC	CC	-0.081 (-0.153, -0.009)	0.028	0.328	**0.020**
Fasting cortisol	rs115978957	AA	AG	GG	0.132 (0.032, 0.232)	0.010	0.134	**0.047**
	rs116032963	CC	CA	AA	0.132 (0.032, 0.232)	0.010	0.134	**0.047**

Linear regression models adjusted for age, sex, smoking, alcohol, and BMI (except where waist circumference was the outcome); **M:** Major allele; **m:** Minor allele; **Beta:** Unstandardized beta coefficient for the linear regression model; **95% CI:** 95% confident intervals; **p:** P value for the unstandardized beta coefficient; **p2:** P value after adjusting for multiple testing using 10 000 permutations; **Sex Int:** P value for sex interaction.

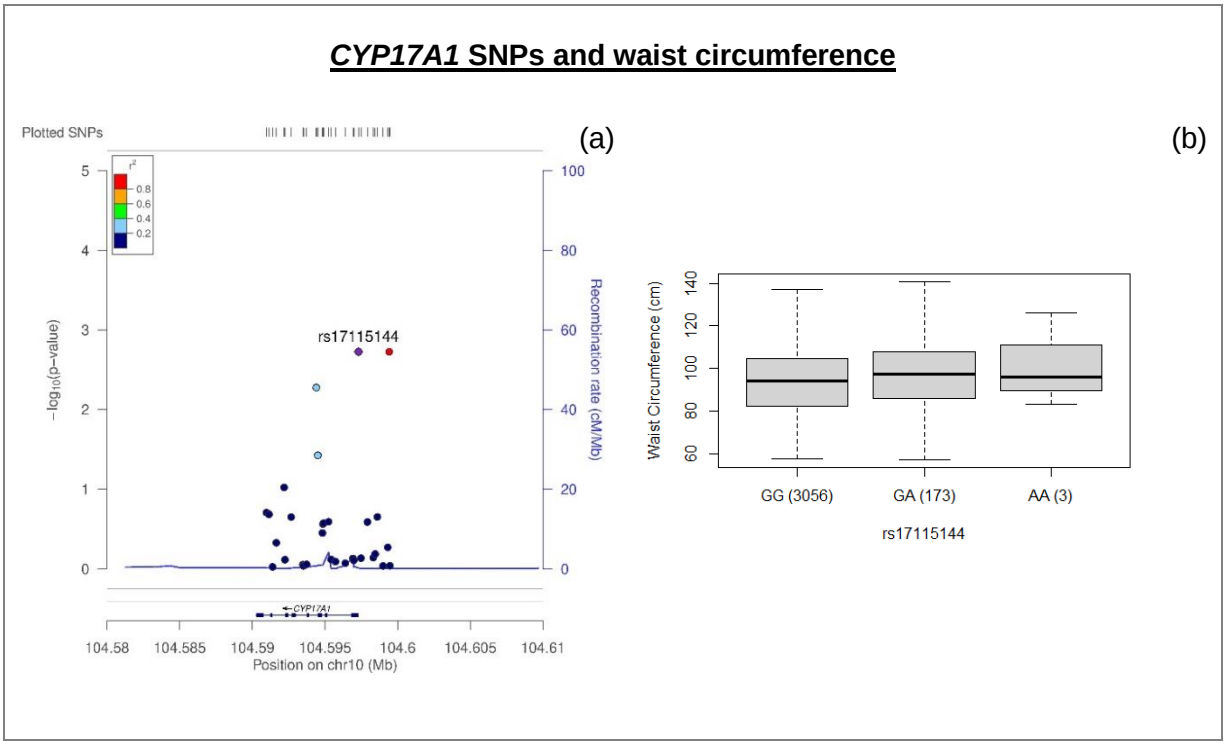


Figure 4.2: LocusZoom (a) and box and whisker (b) plots for the associations of rs17115144 at *CYP17A1* with waist circumference in men and women of African ancestry.

The LocusZoom plot association data was plotted against the AFR LD backgrounds from the 1000 genomes phase 1 dataset. The lead SNP (rs17115144) is represented by a purple diamond. Each SNP is coloured according to the r^2 value against rs17115144. The box and whisker plot shows medians and Interquartile ranges of waist circumference across the rs17115144 genotype groups.

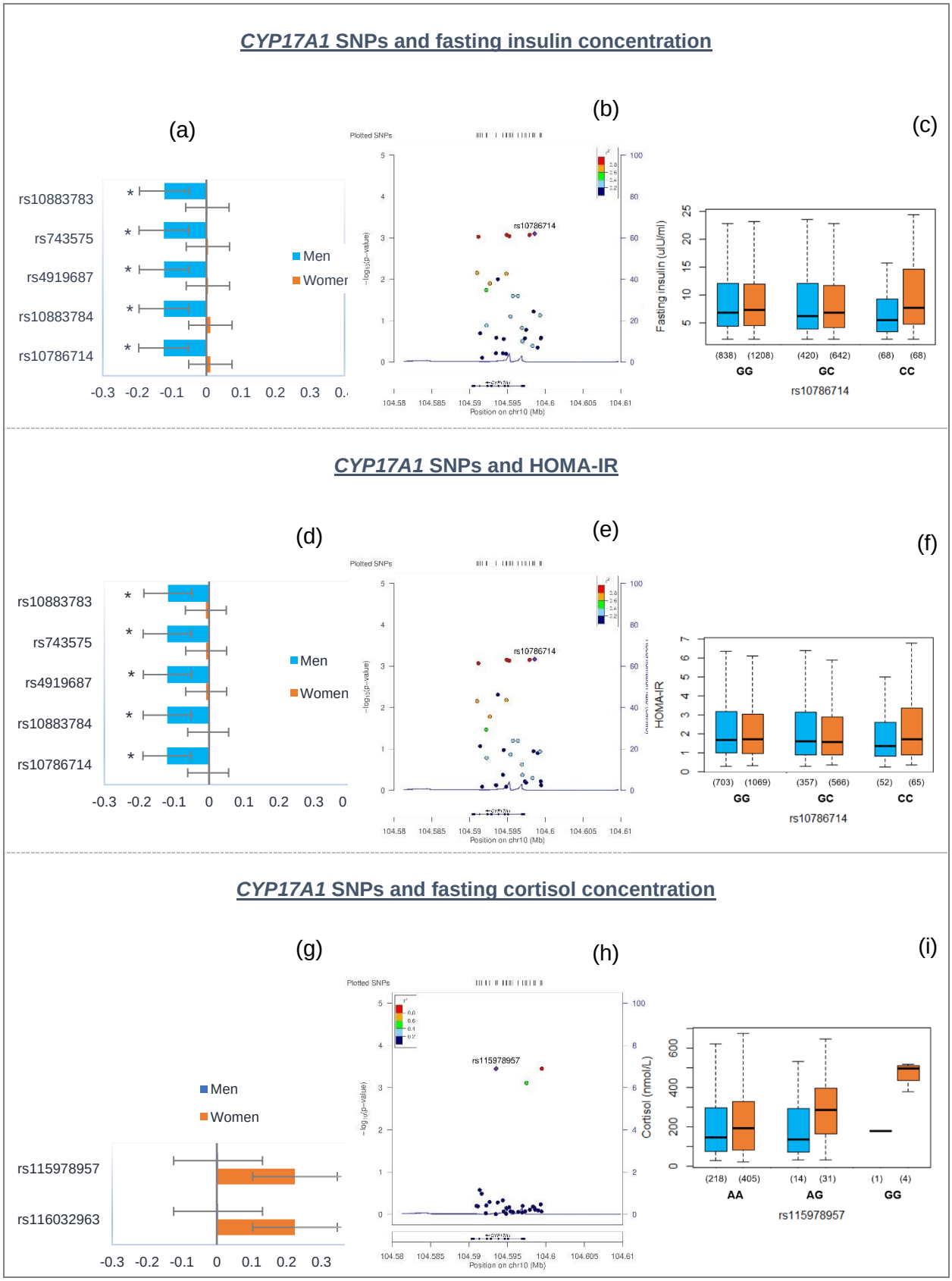


Figure 4.3: Sex-specific associations of *CYP17A1* SNPs with fasting insulin concentrations and HOMA-IR (a-f), and fasting cortisol concentrations in men and women of African ancestry (g-i). Plots (a), (d) and (g) show

standardized beta coefficients for the linear regression models after adjusted for age, smoking, alcohol, and BMI. Error bars represent 95% confidence intervals for the standardized beta coefficients. * p2 < 0.05, # p2 < 0.01. LocusZoom plots represent the sex-specific associations of *CYP17A1* SNPs with fasting insulin concentrations (b), HOMA-IR (e) in men only and fasting cortisol concentrations in women only (h). The LocusZoom plot association data was plotted against the AFR LD backgrounds from the 1000 genomes phase 1 dataset. The lead SNPs (rs10786714 and rs115978957) are represented by purple diamonds. Each SNP is coloured according to the r^2 value against the respective lead SNP. SNPs with missing LD information are coloured in grey. The box and whisker plots show medians and Interquartile ranges of fasting insulin concentrations (c), HOMA-IR (f) and fasting cortisol concentrations (i) across the respective SNP genotype groups in men and women. The number of observations in each group is shown in brackets.

There were no significant associations between any of the tested *CYP17A1* SNPs and measures of insulin sensitivity (fasting insulin and HOMA-IR) and fasting glucose (Table 4.2). However, rs10883783, rs743575, rs4919687, rs10883784 and rs10786714 at *CYP17A1*, showed significant sex interactions for both fasting serum insulin concentrations and HOMA-IR. Likewise, rs115978957 and rs116032963 *at CYP17A1*, showed significant sex interactions for fasting cortisol concentrations (Table 4.2). Hence, the study sample was sex-stratified, such that the regression models that showed significant sex-interactions in Table 4.2 were repeated for each sex group.

In accordance with the observed sex interactions, rs10883783, rs743575, rs4919687, rs10883784 and rs10786714 at *CYP17A1*, were associated with lower fasting serum insulin concentrations (p2 = 0.017, 0.016, 0.017, 0.016 and 0.015, respectively) and HOMA-IR (p2 = 0.017, 0.015, 0.016, 0.015 and 0.014, respectively) in men but not in women (Figure 4.3 (a) and (d)). The LocusZoom plots in Figure 4.3 (b) and (e) suggested that all five identified *CYP17A1* SNPs are in strong LD ($r^2 > 0.8$) in populations of African ancestry and may represent the same signal in the observed associations. Therefore, to summarize the associations for both fasting serum insulin concentrations and HOMA-IR, the medians and interquartile ranges were presented across the genotypes for rs10786714 (the lead SNP) in Figure 4.3 (c) and (f) in both men and women.

In contrast to the above, rs115978957 and rs116032963, at *CYP17A1*, were associated with higher fasting serum cortisol concentrations in women (p2 = 0.005 for both) but not in men (Figure 4.3g). Similar to the above observations, the LocusZoom plot in Figure 4.3 (h) suggests the identified two SNPs, are in strong LD ($r^2 > 0.8$) in populations of African ancestry. Hence, the medians and interquartile ranges for fasting cortisol concentrations for rs115978957 (lead SNP) genotypes in both men and women are presented in Figure 4.3 (i).

4.4.3 *SERPINA6/A1* SNPs and metabolic syndrome components

There were no significant associations between any of the tested *SERPINA6/A1* SNPs and the odds of having the metabolic syndrome in the present study. Table 4.3 shows that rs17090691 and rs877084 at *SERPINA6/A1* were associated with higher diastolic blood pressure in the study sample comprising both men and women, with no significant sex interaction. The LocusZoom plot in Figure 4.4 (a) suggested that the identified *SERPINA6/A1* SNPs are in strong LD in populations of African ancestry and may represent the same signal in the observed associations. Therefore, to summarize the associations for diastolic blood pressure, the medians and interquartile ranges were presented across the genotypes for rs17090691 (the lead SNP) in Figure 4.4 (b) in the combined sample of men and women.

Table 4.3*:* Associations of *SERPINA6/A1* SNPs in men and women of African ancestry.

Phenotype	SNP	Genotypes			Linear regression model adjusted for confounders			
		MM	Mm	mm	Beta (95% CI)	p	p2	Sex Int
Diastolic blood pressure	rs17090691	AA	AG	GG	0.065 (0.036, 0.094)	9.5×10^{-6}	0.003	0.813
	rs877084	TT	TA	AA	0.088 (0.047, 0.130)	3.3×10^{-5}	0.008	0.910
HDL cholesterol	rs60643124	AA	AG	GG	-0.021 (-0.036, -0.006)	0.008	0.704	**0.047**
	rs12101216	AA	AT	TT	-0.018 (-0.032, -0.003)	0.016	0.899	**0.026**

	rs74074941	CC	CT	TT	-0.016 (-0.032, -0.001)	0.040	0.992	**0.013**
	rs74074947	GG	GT	TT	-0.016 (-0.032, -0.001)	0.040	0.991	**0.010**
	rs58460454	CC	CT	TT	-0.017 (-0.032, -0.001)	0.033	0.985	**0.013**
	rs1051052	AA	AG	GG	0.016 (0.004, 0.028)	0.008	0.733	**0.006**

Linear regression model adjusted for age, sex, smoking, alcohol, and BMI; **M:** Major allele; **m:** Minor allele; **Beta:** Unstandardized beta coefficient for the linear regression model; **95% CI:** 95% confident intervals; **p:** P value for the unstandardized beta coefficient; **p2:** P value after adjusting for multiple testing using 10 000 permutations; **Sex Int:** P value for sex interaction.

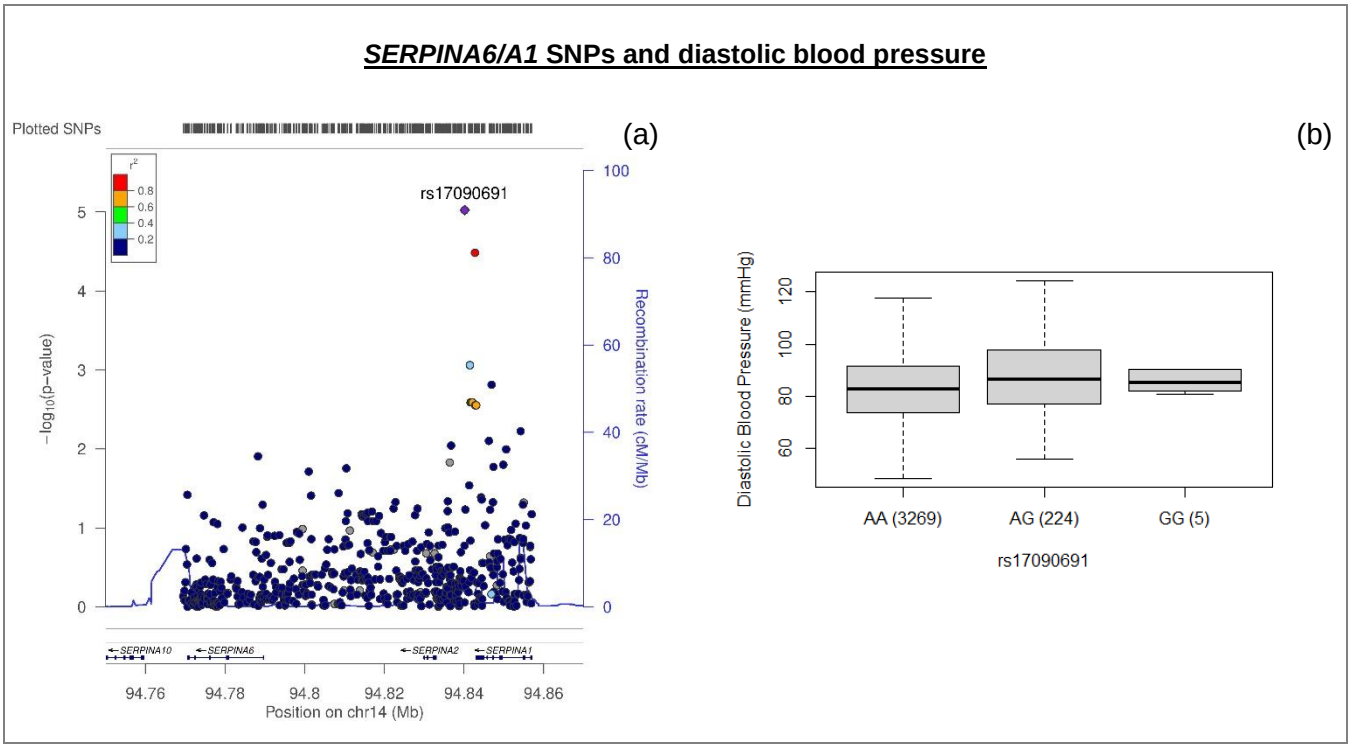


Figure 4.4: LocusZoom (a) and box and whisker (b) plots for the associations of rs17090691 at *SERPINA6/A1* with diastolic blood pressure in men and women of African ancestry.

The LocusZoom plot association data was plotted against the AFR LD backgrounds from the 1000 genomes phase 1 dataset. The lead SNP (rs17090691) is represented by a purple diamond. Each SNP is coloured according to the r^2 value against rs17090691. The box and whisker plot shows medians and Interquartile ranges of waist circumference across the rs17090691 genotype groups.

However, although the rs60643124, rs12101216, rs74074941, rs74074947, rs58460454 and rs1051052 SNPs at *SERPINA6/A1* were not associated with HDL cholesterol in the combined sample of men and women there were significant sex interactions (Table 4.3). When stratified by sex, rs60643124 and rs12101216 at *SERPINA6/A1* were associated with lower HDL cholesterol in women (p2 = 0.041 and 0.047, respectively), but not in men (Figure 4.5a). In contrast, another *SERPINA6/A1* SNP (rs1051052) was associated with higher HDL cholesterol in women (p2 = 0.005) but not in men. In addition, the LocusZoom plot in Figure 4.4 (b) shows that the lead SNP (rs1051052) was not in strong LD ($r^2 < 0.2$) with the other two SNPs (rs60643124, rs12101216) at *SERPINA6/A1* in populations of African ancestry. Therefore, the medians and interquartile ranges for HDL cholesterol concentrations were presented across the genotypes for both rs60643124 and rs1051052 in men and women separately.

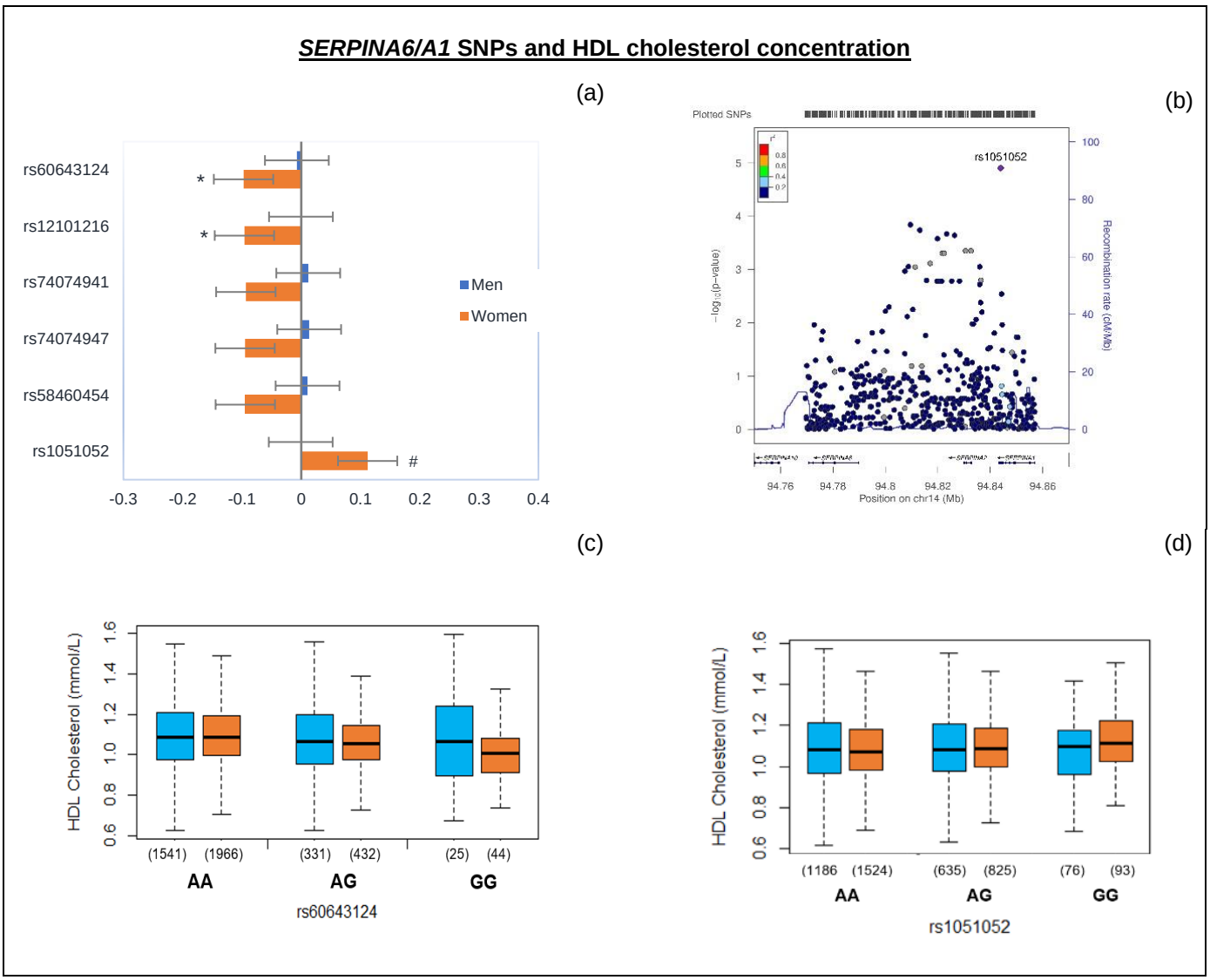


Figure 4.5: Sex-specific associations of *SERPINA6/A1* SNPs with HDL cholesterol concentrations and in women of African ancestry.

Plots in (a) show standardized beta coefficients for the linear regression models after adjusted for age, smoking, alcohol, and BMI. Error bars represent 95% confidence intervals for the standardized beta coefficients. * p2 < 0.05, # p2 < 0.01. LocusZoom plots represent the sex-specific associations of *SERPINA6* SNPs with HDL cholesterol concentrations in women only (b). The LocusZoom plot association data was plotted against the AFR LD backgrounds from the 1000 genomes phase 1 dataset. The lead SNP (rs1051052) is represented by purple a diamond. Each SNP is coloured according to the r^2 value against the respective lead SNP. SNPs with missing LD information are coloured in grey. The box and whisker plots show medians and Interquartile ranges of HDL cholesterol concentrations across the rs60643124 (c) and rs1051052 (d) genotype groups in men and women. Both rs60643124 and rs1051052 were shown due to lack of correlation between the two SNPs in Africans and observed differences in the direction of association. The number of observations in each group is shown in brackets.

4.4.4 Minor allele frequencies of the identified SNPs

The MAFs of the identified SNPs are compared to those of other populations of African ancestry and other major populations of different ancestries in Table 4.4. Most of the identified SNPs had similar MAFs when compared to other populations of African ancestry. However, the two *CYP17A1* SNPs (rs17115144 and rs114190228) that were associated with waist circumference in the present study are monomorphic in all populations of European and Asian ancestry, with low MAFs (< 1%) in populations of American ancestry. Similarly, the two *CYP17A1* SNPs (rs115978957 and rs116032963) that were associated with fasting cortisol concentrations in women in the present study are monomorphic in populations of Asian ancestry, with low MAFs (< 1%) in populations of European and American ancestry. In contrast, the MAFs for the *SERPINA6/A1* SNPs (rs60643124, rs12101216 and rs1051052) associated with HDL cholesterol in the present study vary widely across different ethnic groups. For example, rs60643124 at *SERPINA6/A1* was monomorphic in populations of Asian ancestry, but with low MAFs (< 1%) in populations of European and American ancestry. Conversely, the MAF for the lead SNP (rs1051052) was higher in all populations of non-African ancestry (40-66%) compared to populations of African ancestry (20-21%) (Table 4.4).

Table 4.4: Minor allele frequencies of the identified SNPs in the present study sample and other populations.

Locus	Associated outcome (Sex)	SNP	Minor allele	Minor allele frequencies by ancestry					
				Present study	Other Africans	Europeans	Americans	East Asians	South Asians
CYP17A1	Waist circumference (Both)	rs17115144	A	0.03	0.05	0.00	< 0.01	0.00	0.00
		rs114190228	A	0.03	0.05	0.00	< 0.01	0.00	0.00
SERPINA6/A1	Diastolic BP (Both)	rs17090691	G	0.03	0.05	0.13	0.10	0.32	0.32
		rs877084	A	0.02	0.04	0.13	0.10	0.31	0.33
CYP17A1	Fasting insulin and HOMA-IR (men only)	rs10883783	A	0.21	0.11	0.30	0.20	0.25	0.10
		rs743575	G	0.21	0.11	0.30	0.20	0.27	0.10
		rs4919687	A	0.21	0.11	0.30	0.20	0.25	0.10
		rs10883784	T	0.21	0.11	0.30	0.20	0.25	0.10
		rs10786714	C	0.21	0.11	0.30	0.20	0.25	0.10
CYP17A1	Fasting cortisol (Women only)	rs115978957	G	0.03	0.02	< 0.01	< 0.01	0.00	0.00
		rs116032963	A	0.03	0.02	< 0.01	0.01	0.00	0.00
SERPINA6/A1	HDL cholesterol (Women only)	rs60643124	G	0.02	0.10	< 0.01	< 0.01	0.00	0.00
		rs12101216	T	0.12	0.10	< 0.01	< 0.01	0.03	0.07
		rs1051052	G	0.21	0.20	0.61	0.54	0.66	0.42

BP: Blood pressure; Fasting insulin, cortisol and HDL cholesterol concentrations were measured in **fasting serum**. Minor allele frequencies from the other populations were obtained from the **1000 genomes phase 3 dataset**. **Other Africans** include Yoruba in Ibadan (Nigeria), Luhya in Webuye (Kenya), Gambian in Western Divisions (Gambia), Mende (Sierra Leone), Esan (Nigeria), Americans of African Ancestry in SW (USA) and African Caribbeans (Barbados). **Europeans** include Utah Residents (CEPH) with Northern and Western European Ancestry, Toscani (Italia), Finnish (Finland), British (England and Scotland) and Iberian Population (Spain). **Americans** include Mexican Ancestry from Los Angeles (USA), Puerto Ricans (Puerto Rico), Colombians from Medellin (Colombia) and Peruvians from Lima (Peru). **East Asians** include Han Chinese in Beijing (China), Japanese in Tokyo (Japan), Southern Han Chinese, Chinese Dai in Xishuangbanna (China) and Kinh in Ho Chi Minh City (Vietnam). **South Asians** include Gujarati Indian from Houston (Texas), Punjabi from Lahore (Pakistan), Bengali (Bangladesh), Sri Lankan Tamil (the United Kingdom) and Indian Telugu (the United Kingdom).

4.5 Discussion

The present study shows that common variants at *CYP17A1* and *SERPINA6/A1*, two loci previously linked to circulating glucocorticoid concentrations, were associated with key components of the metabolic syndrome in men and women of African ancestry, but not with the metabolic syndrome itself. There was evidence of association between a *CYP17A1* signal and elevated waist circumference in both men and women. Another *CYP17A1* signal was associated with lower fasting insulin concentrations and HOMA-IR in men only, and another with higher fasting cortisol concentrations in women only. Further, there was evidence of association between a *SERPINA6/A1* signal and higher diastolic blood pressure in both men and women. In women only, another *SERPINA6/A1* signal was associated with lower HDL cholesterol concentrations, whilst another with higher HDL cholesterol concentrations.

This is the first study to report the association between two *CYP17A1* SNPs, rs17115144 and rs114190228, and waist circumference in humans. Notably, these two SNPs are monomorphic in populations of European and Asian ancestry and are present in low frequencies in populations of American ancestry. The *CYP17A1* gene catalyzes both 17-alpha-hydroxylase and 17,20-lyase activities, which are crucial in the biosynthesis of steroid hormones, including mineralocorticoids, glucocorticoids and sex hormones (383,384) (Figure 4.6). However, the identified *CYP17A1* SNPs that were associated with elevated waist circumference in the present study do not form part of the transcribed gene but are instead located within the 2 kilobase upstream region of the *CYP17A1* gene (Table 4.5). As these associations are novel, no functional studies have been undertaken for these SNPs. Further studies are required to investigate whether the observed genetic association occurs via steroid hormone biology and body fat distribution profiles. Notably, glucocorticoids and sex hormones have been differentially associated with centralization of body fat, as shown in Figure 4.6.

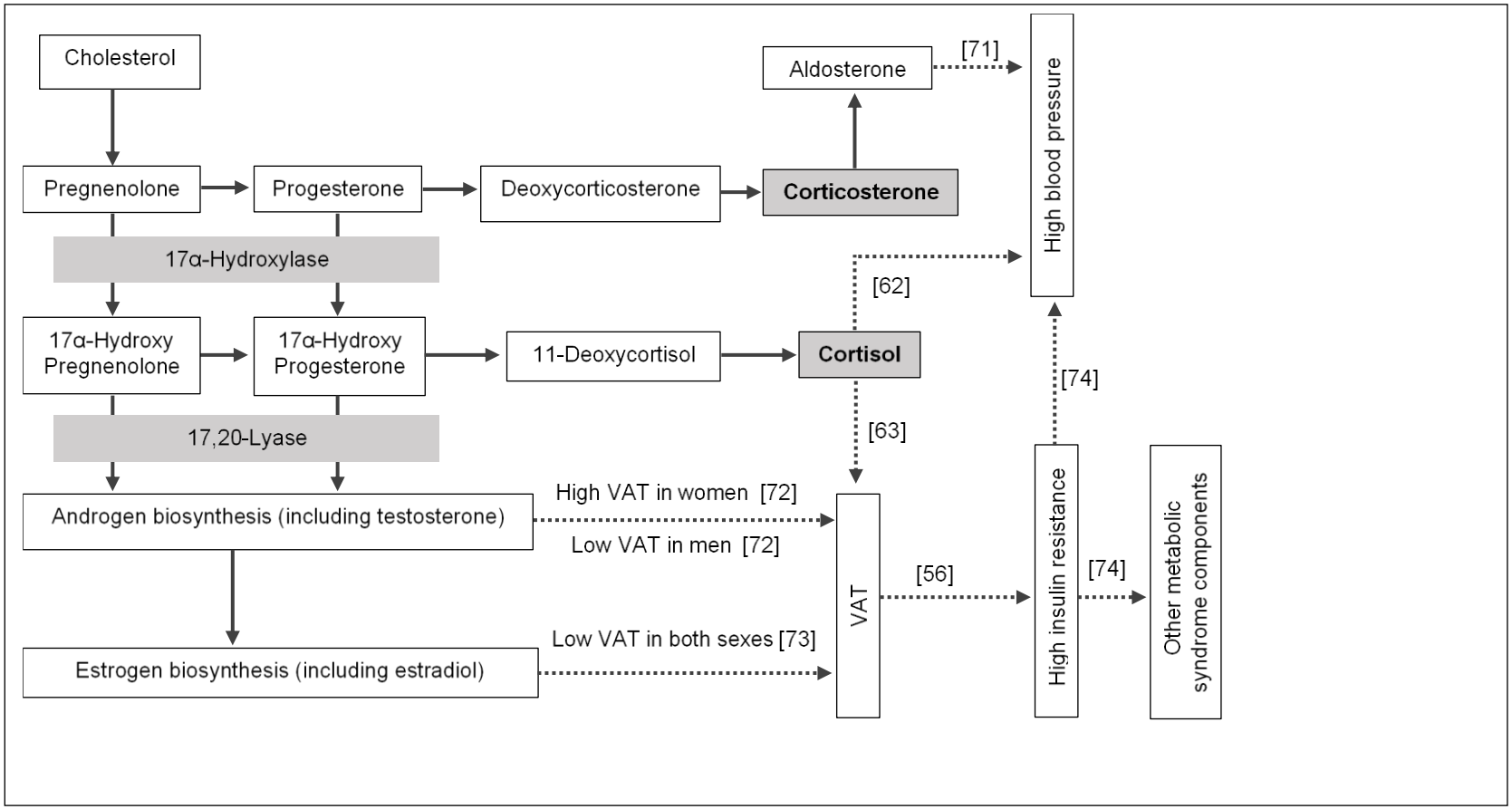


Figure 4.6: CYP17A1 enzyme functions in the synthesis of steroid hormones and relationships with components of the metabolic syndrome

The CYP17A1 enzyme catalyses both 17α-hydroxylase and 17,20-lyase which are key in the synthesis of glucocorticoids and sex hormones. VAT: Visceral adipose tissue.

Our finding that some of the genetic associations of *CYP17A1* SNPs were sex-specific, partly supports the involvement of sex hormones and body fat distribution profiles, as these significantly differ between sexes. In men, testosterone predominates and fat is preferentially stored as visceral adipose tissue, whilst in women, estrogen predominates and fat is preferentially stored as subcutaneous adipose tissue (365).

Table 4.5: A summary of previous genetic associations with the significant SNPs identified in this study

Present study		Single Nucleotide Polymorphism (SNP) info			Associations from previous studies	
Sample	Outcome	Locus	SNP ID	Genomic location	Outcome	Ancestry (Sex)
Both sexes	Waist	*CYP17A1*	rs17115144	< 1kb upstream of *CYP17A1*	None	NA
			rs114190228	2.1 kb upstream of *CYP17A1*	None	NA
	Diastolic BP	*SERPINA6*/A1	rs17090691	50.0 kb upstream of *SERPINA6*; 2.9 kb downstream of *SERPINA1*	None	NA
			rs877084	< 1kb downstream of *SERPINA1*	None	NA
Men only	Fasting insulin and HOMA-IR	*CYP17A1*	rs10883783	Intron	Prostate cancer-specific mortality (385)	European (Males)
					Not associated with BMI and fat mass (386)	European (Both)
			rs743575	Intron	Bone mineral density (387)	European (Males)
			rs4919687	Non-coding transcript exon	Coronary artery disease (388,389)	East Asian (Both)
					Elevated blood pressure (390)	East Asian (Males, not Females)
					Elevated BP and systolic BP (391)	East Asian (Both)
					Breast cancer (392)	Multi-ethnic (Females)
					Preeclampsia (393)	East Asian (Females)
					Colorectal cancer (394)	East Asian (Both)
			rs10883784	< 1kb upstream of *CYP17A1*	No *In Vitro* functional effects (340)	European (Both)
			rs10786714	1.3 kb upstream of *CYP17A1*	No *In Vitro* functional effects (340)	European (Both)
Women only	Fasting cortisol	*CYP17A1*	rs115978957	Non-coding transcript exon	None	NA
			rs116032963	2.2 kb upstream of *CYP17A1*	None	NA
	HDL cholesterol	*SERPINA6*/A1	rs60643124	19.8 kb upstream of *SERPINA6*; 33.6 kb downstream of *SERPINA1*	None	NA
			rs12101216	23.4 kb upstream of *SERPINA6*; 29.9 kb downstream of *SERPINA1*; Promoter flanking region	None	NA
			rs1051052	3 prime UTR for *SERPINA1*	Childhood asthma (395)	European (Both); not African (Both)
					Chronic obstructive pulmonary disease (396)	East Asian (Both)

All SNP bioinformatic data are reported using the Genome Reference Consortium human genome Build 37 (*GRCh37*). **Waist:** Waist circumference; **BP:** Blood pressure; **kb:** Kilobases; **UTR:** Untranslated region.

While this is the first study to report associations between *CYP17A1* SNPs and lower fasting insulin concentrations and HOMA-IR in men only, most of the identified SNPs have been previously associated with other human complex traits, such as elevated blood pressure, coronary artery disease, bone mineral density and certain cancers, in populations of non-African ancestry (Table 4.5). Indeed, the identified *CYP17A1* SNPs are in high LD ($r^2 > 0.8$) with each other in all ancestral groups and the MAFs do not differ much between populations of African and non-African ancestry. Hence, it is possible that the associations observed in the present study also occur in men of other populations. However, molecular consequences of these SNPs remain unclear, as *in vitro* studies based on European SNPs have shown no significant effects on *CYP17A1* transcription (Table 4.5). Moreover, the lack of evidence of association between SNPs at the identified haplotype block and measures of whole body adiposity in the present study, and in a previous European study (386), suggests that adiposity may not mediate the observed genetic associations. Rather, this association may be via sex hormones and body fat distribution, particularly VAT, which is strongly associated with both insulin and HOMA-IR (81). Indeed, for the same level of adiposity men generally accumulate more VAT compared to women (90,91). However, the lower levels of adiposity (including waist circumference) in the men of the present study are likely to have been accompanied by lower levels of VAT compared to women. Hence, it is possible that the identified SNPs reduce the CYP17A1 enzyme activities (both 17-alpha-hydroxylase and 17,20-lyase activities) and limit the biosynthesis of glucocorticoids and/or sex hormones (particularly testosterone), leading to low VAT, and ultimately, low fasting insulin and insulin resistance in men (Figure 4.6). These mechanisms may have also occurred in women, but the

associations may have been confounded by higher concentrations of circulating oestrogen (Figure 4.6).

The associations of the other two *CYP17A1* SNPs, rs115978957 and rs116032963, with higher fasting cortisol concentrations in women only, are also likely to have influenced the *CYP17A1* enzyme. However, these two *CYP17A1* SNPs are located at a different haplotype block from those that were associated with lower fasting insulin concentrations in men (Table 5). Moreover, the minor allele of these two *CYP17A1* SNPs are not found in any population of Asian ancestry but are present in low frequencies (< 1%) in other populations of non-African ancestry (European and American). Consequently, the two SNPs could not be flagged in the GWAS meta-analysis of circulating cortisol concentrations, which was conducted in populations of European ancestry (163). It is possible that these two *CYP17A1* SNPs may have particularly influenced the 17-alpha-hydroxylase activity of the CYP17A1 enzyme, thus favouring the production of both cortisol and sex hormones (Figure 4.6). Certainly, further studies that include assessment of sex hormones and body fat distribution profiles may shed some light on the mechanisms underlying the observed associations. Such studies should also include populations of European ancestry, as women of African ancestry have lower cortisol concentrations compared to their European ancestry counterparts (73,124,313).

In addition to the above novel associations of *CYP17A1* SNPs, the present study showed for the first time that the rs17090691 and rs877084 SNPs at *SERPINA6/A1* were associated with higher diastolic blood pressure in both men and women of African ancestry. Notably, the MAFs of these SNPs are lower in populations of African ancestry compared to other populations (~13% in European and 31-33% in Asian versus 2-5% in African). Although the SNPs at *SERPINA6/A1* that have been associated with circulating cortisol concentrations in populations of European ancestry (163) reside in a separate

haplotype block, the associations with diastolic blood pressure in this study may also be mediated by circulating cortisol concentrations. Notably, the lack of association between *SERPINA6/A1* SNPs and serum glucocorticoid concentrations in the present study does not completely negate mediation by glucocorticoids. Circulating glucocorticoid concentrations change dynamically and are influenced by several known and unknown confounders (213). For example, circulating glucocorticoid concentrations exhibit a diurnal nature, such that peaks are seen 30-45 minutes after awakening and the concentrations decline throughout the day (217). It is possible that the identified *SERPINA6/A1* SNPs were associated with the daily circulating glucocorticoid concentrations instead of the morning concentrations that were measured in the present study. In support of mediation by glucocorticoids, fasting cortisol concentrations have been consistently shown to be associated with measures of blood pressure in both men and women of African and non-African ancestry (221,242,251,257,258,261,272,276,277,281,296,322). Certainly, glucocorticoids increase blood pressure by several mechanisms, including through impairment of nitric oxide-mediated renal vasodilation in the kidneys (303).

Another novel finding in this study was that the rs60643124 and rs12101216 SNPs at *SERPINA6/A1* were associated with lower HDL cholesterol, while the rs1051052 SNP was associated with higher HDL concentrations, in women only. These observations suggest two separate mechanisms for the associations between *SERPINA6/A1* SNPs and HDL cholesterol concentrations in women. This notion is further supported by the observation that whilst rs1051052 is located at the 3'UTR region of *SERPINA1*, the other two SNPs that are associated with lower HDL concentrations are intergenic between *SERPINA6* and *SERPINA1* genes (Table 4.5). As functional studies for these SNPs are lacking, the exact mechanisms involved are unknown. However, based on the biology of the proteins encoded by these two genes, the associations may be mediated by

circulating glucocorticoids. In support of this hypothesis, some studies reported inverse associations between circulating cortisol concentrations and HDL cholesterol in populations of predominantly European ancestry (276,278). Certainly, further investigations that include other populations and functional studies are required to understand the mechanisms involved in these associations.

Investigating a population of African ancestry, comprising both men and women, was one of the major strengths of the present study. The novel genetic associations observed in the present study were likely missed by previous studies due to the fact that most genetic association studies have been conducted in populations of European ancestry. For example, the ancestral composition of previously published GWAS is about 78% European, 10% Asian and only 2% African (397). Moreover, the previous genetic association studies in populations of African ancestry were limited by the use of genotyping arrays that are not designed to capture the diversity observed in the African genome (398,399). Hence, by conducting the present study in a population of African ancestry, who are more genetically diverse with lower levels of linkage disequilibrium, and by using an African-specific genotyping array that captures this genetic diversity, the probability of finding these novel genetic associations was optimized. Moreover, the inclusion of both men and women in the present study provided an opportunity to observe genetic associations that were sex specific. Notably, several genetic models, including multifactorial (interactions between biological and environmental factors) (400), the sex-dependent liability threshold (401), and sex-disparities in the underlying genetic architecture (402), have been proposed to explain the often observed sex-specific associations in human traits. However, the two latter models, which rely on the differences in the frequency of the effect alleles (402), are unlikely to be involved in the present study, as the MAFs in the identified SNPs were similar in men and women. The present study also has some limitations. A power calculation was not conducted as the sample size was

dependent on the available AWI-Gen dataset and the criteria used to select the final participants. Hence, lack of genetic associations with some of the key components of the metabolic syndrome, such as circulating lipid concentrations, and the odds of having the metabolic syndrome, may be attributed to a lack of statistical power. Furthermore, the role of body distribution, menopausal status, and oral contraceptive use were not assessed in the present study. These factors may have influenced circulating glucocorticoid concentrations and influenced some of the reported results. Moreover, circulating glucocorticoid concentrations were only measured at one time point and the influence of the circadian rhythm could not be assessed. Nevertheless, this limitation was minimized by adjusting for blood sampling time in the statistical models. Future studies should also measure the diurnal curves to draw better conclusions regarding mediation by circulating glucocorticoids. Moreover, the data used in the present study were cross-sectional, making it impossible to draw conclusions about the causality.

In summary, the findings reported in the present study have shown that some *CYP17A1* and *SERPINA6/A1* SNPs are associated with components of the metabolic syndrome in South African men and women of African ancestry. The observed sex-specific associations highlight the importance of testing for sex interactions in future genetic studies of glucocorticoids and metabolic syndrome components in populations of African ancestry. Certainly, the genetic associations observed in the present study, as well as follow up functional studies, need to be explored in other African populations. Further investigations into the identified loci may help in improving our understanding of the mechanisms underlying the metabolic syndrome.

Chapter 5 General Discussion

This chapter summarizes the key findings from research papers presented in chapter 2 to 4 and gives a general discussion and conclusions after considering all research findings. Overall strengths and limitations are highlighted and protentional future studies are also discussed. The chapter finally draws conclusions of the new knowledge provided in this thesis.

5.1 General discussion

Previous studies have clearly demonstrated that populations of African ancestry exhibit a unique metabolic phenotype, particular with regard to the metabolic syndrome and its components. For example, comparing populations of African ancestry to those of European ancestry showed ethnic differences in components of the metabolic syndrome, presenting with high prevalence of central obesity and elevated blood pressure, but low fasting triglyceride and HDL-cholesterol concentrations and a low prevalence of elevated fasting glucose concentrations (68,74). Further, although populations of African ancestry have higher prevalence of obesity (84) and insulin resistance (85–88) than populations of European ancestry in similar environmental settings, the differences in insulin sensitivity are independent of body fat and its distribution (89).

The pathophysiology of the metabolic syndrome in populations of African ancestry remains poorly understood. However, evidence in women of African ancestry suggested that glucocorticoid signalling may be partly involved (318). Indeed, excess glucocorticoid exposure had been consistently shown to negatively influence several components of the metabolic syndrome including body fat distribution, insulin resistance, blood pressure, and serum lipid profile in populations of non-African ancestry (242,245,246,251,257,258,261,270,272,274–278,280,281,295,296). However, most of these associations remained to be investigated in men and women of African ancestry.

These findings from cross-sectional studies of non-Africans were further supported by genetic association studies which demonstrated that SNPs at *CYP17A1* and *SERPINA6/A1*, two loci involved in the biology of glucocorticoids, were associated with circulating glucocorticoid concentrations and/or components of the metabolic syndrome (163,223,334). However, genomes from individuals of African ancestry are known to be more complex and diverse with lower levels of linkage disequilibrium compared to those

from people of European ancestry. Hence, the primary aim of the study was to investigate the relationships between circulating glucocorticoids and key components of the metabolic syndrome, and to explore the genetic basis of these relationships in men and women of African ancestry.

To address this aim, three studies were conducted in individuals of African ancestry. The research questions and their main findings are summarised in Table 5.1 below.

Table 5.1: Summary of the findings from the thesis

Chapter	Research Question	Main Findings
2	Are circulating glucocorticoids associated with the metabolic syndrome and its components in men and women of African ancestry?	Fasting serum cortisol concentrations were associated with greater odds of presenting with the metabolic syndrome and higher systolic and diastolic blood pressure, but lower LDL and higher HDL cholesterol concentrations, in both men and women. In contrast, fasting corticosterone concentrations were only associated with higher insulin sensitivity in men and women, but not independently of BMI. Sex-specific associations were observed, such that both fasting serum cortisol and corticosterone concentrations were associated with higher fasting plasma glucose and HbA1c concentrations in men, but lower HbA1c concentrations in women.
3	Are SNPs at *CYP17A1* and *SERPINA6/A1* that have been previously associated with components of the metabolic syndrome and/or circulating glucocorticoids in Europeans, also associated with the metabolic syndrome and its components in women of African ancestry?	There was evidence of an association between the minor allele of rs2749527 at *SERPINA6/A1* and higher fasting plasma glucose concentrations, and paradoxically, the same allele was associated with lower odds of having the metabolic syndrome, when assuming an additive model. Similarly, in an additive model, the minor allele of rs7147098 at *SERPINA6/A1* was associated with higher diastolic blood pressure.
4	Are *CYP17A1* and *SERPINA6/A1* SNPs, including those not previously reported in Europeans, associated with the metabolic syndrome and its components and serum glucocorticoid concentrations in men and women of African ancestry?	Minor alleles of *CYP17A1* SNPs were associated with higher waist circumference in both men and women. There was also evidence of associations between minor alleles of other *CYP17A1* SNPs with lower fasting insulin concentrations and the homeostatic model assessment of insulin resistance in men only, whilst minor alleles of other *CYP17A1* SNPs were associated with higher fasting cortisol concentrations in women only. There was also evidence of an association between *SERPINA6/A1* SNPs and diastolic blood pressure in both men and women. In women only, minor alleles of other *SERPINA6/A1* SNPs were associated with lower high-density lipoprotein (HDL) cholesterol concentrations, and another minor allele was associated with higher HDL cholesterol concentrations.

Findings from the epidemiological study of this thesis demonstrated that there was evidence of associations between circulating glucocorticoid concentrations and the metabolic syndrome and its individual components (Chapter 2). Circulating cortisol concentrations were associated with higher systolic and diastolic blood pressure, as well as greater odds of having the metabolic syndrome, but were also associated with a favourable lipid profile. In contrast, circulating corticosterone concentrations were associated with higher insulin sensitivity. These glucocorticoid associations were similar to a recent European study that investigated both cortisol and corticosterone in populations of European ancestry (154). The relationship between circulating cortisol concentrations and greater odds of having the metabolic syndrome is generally thought to reflect underlying associations with the components used in the clinical definition of the metabolic syndrome (44). In this thesis, the relationship between serum cortisol concentrations and the metabolic syndrome is likely to have been driven by blood pressure. Indeed, circulating cortisol concentrations were not associated with elevated waist circumference or serum triglyceride concentrations, and the association with HDL cholesterol concentration was positive.

Notably, the associations between circulating cortisol concentrations and blood pressure, were in accordance with the genetic associations between *SERPINA6/A1* SNPs and diastolic blood pressure (chapter 3 and chapter 4). The *SERPINA6/A1* locus was previously associated with circulating cortisol concentrations in men and women of European ancestry (163). Therefore, the genetic associations with diastolic blood pressure were likely related to inter-individual differences in circulating glucocorticoid concentrations. Glucocorticoids bind the GR or MR in the kidneys to impair nitric oxide-mediated renal vasodilation (303), by suppressing nitric oxide synthase and influencing salt and water retention (304), ultimately increasing blood pressure. The lack of evidence

of mediation of this effect by glucocorticoids in the genetic association study of both men and women in this thesis (Chapter 4) is likely due to the fact that circulating glucocorticoid concentrations change dynamically and are influenced by several known and unknown confounders (213). Notably, blood samples for glucocorticoid measurements were not collected at the same time of the day in the present thesis. Hence, the lack of evidence of cortisol mediation could alternatively reflect variation in the daily circulating cortisol concentrations which were not assessed in this thesis.

In addition to directly binding to the GR and MR to influence blood pressure, glucocorticoids can increase blood pressure via adiposity and insulin resistance. For example, glucocorticoids can promote adiposity by increasing adipocyte differentiation and proliferation (291). The involvement of adiposity was partly supported by the associations between the *CYP17A1* SNPs and higher waist circumference in both men and women (chapter 4). However, there was limited evidence to support the hypothesis that the associations between circulating glucocorticoids and blood pressure were mediated via adiposity in the present study, as the associations were observed even after adjusting for BMI. Moreover, there was no evidence to suggest associations between circulating glucocorticoid concentrations and measures of adiposity (BMI and waist circumference) in the epidemiology study (chapter 2). Based on reports from populations of European ancestry, the lack of evidence of association with adiposity is possibly due to increased metabolic clearance of cortisol within the adipose tissue and liver in obesity (259). Hence, the lower glucocorticoid concentrations in individuals with obesity compared to their normal-weight counterparts, usually reflects increased glucocorticoid activity within the adipose tissue (282).

While the main findings from the epidemiology study of this thesis confirmed that glucocorticoids are associated with the metabolic syndrome and its components, the

findings also suggested that the associations are glucocorticoid-specific (Chapter 4). The majority of previous studies investigating the role of glucocorticoids in the metabolic syndrome had exclusively focused on cortisol, with the assumption that corticosterone has similar functions (187). Although further studies are required to determine the mechanisms underlying the glucocorticoid-specific association observed in the present thesis, previous findings suggested the involvement of ABC transporters. Cortisol and corticosterone differ in their susceptibility to transmembrane transport by ABC transporters, which transport glucocorticoids across cellular membranes (363). For example, ABCB1, an ABC transporter expressed in the CNS, selectively exports cortisol but not corticosterone across the blood-brain barrier (205). On the other hand, another ABC transporter called ABCC1 exports corticosterone over cortisol in the adipose tissue (140). Thus, while corticosterone may contribute disproportionately to the negative feedback control of the HPA axis within the CNS, cortisol may contribute disproportionally to the detrimental effects of glucocorticoids within the adipose tissue. Certainly, the adipose tissue is thought to play a key role in the development of the metabolic syndrome. For example, the mechanisms by which the individual components are interrelated to form the metabolic syndrome are thought to occur via adiposity and the associated insulin resistance (54). However, glucocorticoid activity within the adipose tissue is also influenced by the region of adipose tissue involved. Cortisol activity and clearance are higher in VAT due to a higher number of glucocorticoid receptors compared to SAT (368). Although further studies that assess adipose tissue are still required, the associations observed in the present study were likely to be influenced by body fat distribution profiles.

The hypothesis that body fat distribution profiles mediated the associations between glucocorticoids and the metabolic syndrome and its components was in accordance with sex-specific associations observed in the thesis (chapter 4). Fat is preferentially stored

as VAT in men, but preferentially stored as SAT in women (365). Considering the differences in the number of GRs between the VAT and SAT, the observed sex-specific associations were likely to be related to metabolism of glucocorticoids within the adipose tissue.

Indeed, the findings from the epidemiology study (chapter 2) suggested that although women had higher concentrations of both cortisol and corticosterone, even after adjusting for adiposity, sex-specific associations of both glucocorticoids with measures of glycaemia were similar. As shown in Table 5.1, both fasting serum cortisol and corticosterone concentrations were associated with higher fasting plasma glucose and HbA1c concentrations in men, but lower HbA1c concentrations in women. It is unlikely that the observed sex-specific associations were due to chance, as the two genetic association studies in this thesis (Chapter 3 and 4) also demonstrated similar sex-specific associations. For example, the observation that circulating glucocorticoid concentrations were inversely associated with measures of glycaemia were partly supported by the pilot study that was conducted in women only (Chapter 3). In the pilot study, the minor allele of rs2749527, which had been previously associated with lower cortisol concentrations in populations of European ancestry, was associated with higher fasting glucose concentrations in African women. As circulating glucocorticoid concentrations were not measured in the pilot study, mediation by circulating glucocorticoids in this genetic association could not be assessed. Notably, in the genetic association study comprising both men and women (Chapter 4), circulating glucocorticoids were measured in an attempt to assess glucocorticoid mediation in the observed genetic associations. However, in that study, the associations between rs2749527 and measures of glycaemia were not observed in either men or women. These contradicting findings in the two genetic association studies may possibly be attributed to differences in menopausal

status and sex hormone concentrations in the study populations, which were not assessed. The associations between circulating glucocorticoid concentrations and measures of glycaemia are likely influenced by both sex hormones and body fat distribution profiles, which are intricately interlinked (403).

5.2 Study strengths and limitations

This thesis has some limitations that should be mentioned. As the present study was observational and cross-sectional, causality could not be inferred. Future experimental and Mendelian randomization studies may provide better evidence of association and causality, respectively. Moreover, women were overrepresented in the present study. Indeed, this is in accordance with many epidemiological studies in populations of African ancestry (9,369), but in contrast to European studies where men are often overrepresented (404). Missing data in some of the variables may have also introduced some potential bias. For example, missingness in research paper 1 study was largely due to the use of two study samples (sample 1 and 2), as some variables were not measured in study sample 1 (e.g. OGTT glucose and insulin) (Figure 2.1). As I could not find any evidence to suggest that missingness was systematic when assessing the source of missing values during data cleaning, all missingness was assumed to be random. Furthermore, the role of time of awakening, menopausal status, HIV medication, and oral contraceptive use were not assessed in the present study. These factors may have influenced circulating glucocorticoid concentrations and influenced some of the reported results. Notably, in South Africa, the prevalence of HIV in this age range was estimated at 21.3% (348). Hence, it is possible that HIV status and associated medication may have influenced the results. Another limitation was the lack of measurements of VAT and SAT which are likely to explain the observed sex-specific associations. Although waist circumference is often used as a proxy for VAT, for the same level of waist circumference,

women of African ancestry were shown to accumulate less VAT compared to their European ancestry counterparts (89,91–95). As suggested by several observational studies, waist circumference cut-offs that are based on European data may have not been an appropriate estimate of body fat distribution in this South African population (75). Furthermore, circulating glucocorticoids are often measured at a set time (e.g., 8:00 or 9:00 am) due to their diurnal nature. Circulating glucocorticoid concentration peaks are observed after awakening and the concentrations decrease throughout the day (216,217). Although blood sampling times in the present study varied widely (07:00-11:59 am), all samples were taken in the morning and blood sampling time was adjusted for in all models. Due to lack of studies assessing associations of the two glucocorticoids with the metabolic syndrome in populations of African ancestry, power calculation was conducted using effect sizes from studies in Europeans (154). The power analyses results suggested that an additional 542 samples are required in future to be able to assess the full spectrum of the associations between circulating glucocorticoid concentrations and the components of the metabolic syndrome (Appendix H). As the sample size was dependent on the available dataset and the criteria used to select the final participants, failure to show associations with some of the key components of the metabolic syndrome may have been attributed to the lack of statistical power. Moreover, as suggested by several cross-sectional studies, waist circumference cut-offs that are based on European data may have not been an appropriate estimate of body fat distribution in this South African population (75). Hence, it is possible that the prevalence of both elevated waist circumference and the metabolic syndrome in the present study were overestimated. Furthermore, the datasets used in the present research were subsets of much larger cohorts for which data was not made available. Hence, whether bias was introduced during sample selection could not be assessed. Moreover, whether the findings observed

in the present study of only black South Africans can be generalized for all Africans, remains to be investigated.

This is one of the first and largest studies to date in individuals of African ancestry that has investigated the role of glucocorticoids in the metabolic syndrome. The existence of associations of glucocorticoids with the metabolic syndrome and its components were observed in both epidemiological and genetic analyses. Certainly, this thesis has produced interesting leads and thought-provoking hypotheses that can be explored in future studies. Another strength was the use of a UPLC-MS method for measuring serum glucocorticoid concentrations which is preferred due to its high specificity and sensitivity.

5.3 Future studies and recommendations

Although the exact cause of the metabolic syndrome is unknown, the prevailing hypothesis suggests that centralization of body fat is the key driver. Accordingly, studies conducted predominantly in populations of European ancestry demonstrated strong associations between increased centralized body fat, particularly VAT, and insulin resistance, the proposed primary links in the metabolic syndrome (81). Hence, positive associations between VAT, insulin resistance and the other components of the metabolic syndrome are traditionally acknowledged (82). On the other hand, increased accumulation of SAT in the peripheral (gluteal-femoral) body regions has been traditionally associated with higher insulin sensitivity, and consequently lower risk of developing the metabolic syndrome components (83). Likewise, glucocorticoid activity is much higher in the VAT than SAT (368). Hence, the associations observed in this thesis, particularly, the sex-specific relations, are likely to have occurred via body fat distribution which significantly differs between sexes. The sex differences in body fat distribution profiles are thought to be largely driven by differences in predominating sex hormones (403). Indeed, the enzyme encoded by the *CYP17A1* gene also influences sex hormones

(377). Therefore, future studies should assess whether the associations observed in this thesis were mediated by sex hormones and body fat distribution profiles. The first step in these future analyses could be assessing the relationship between the identified predictor variables (circulating glucocorticoid concentrations and the identified *CYP17A1* and *SERPINA6/A1* SNPs) and sex hormone concentrations, as well as measures of adipose tissue distribution (VAT and SAT). Furthermore, the relationship between key confounders such as awakening time, HIV medication and oral concentratives, and circulating glucocorticoid concentrations should be further explored in an African population.

Several of the identified SNPs are located between *SERPINA6* and *SERPINA1* genes, which encode CBG and AAT. Hence, much of our hypotheses assumed that these SNPs influenced either one or both of these proteins. Functional studies that include measurement of CBG and AAT, and cortisol-binding capacity, are required in a population of African ancestry.

Many of the associations reported in the present study were novel. As populations of African ancestry have different metabolic and genetic profiles compared to other populations, these associations need to be confirmed in other populations.

5.4 Conclusions

The study demonstrated evidence of association between circulating glucocorticoid concentrations and the metabolic syndrome and its components in a population of African ancestry. These findings support the hypothesis that glucocorticoids play a role in the development of the metabolic syndrome. The study demonstrated for the first time in individuals of African ancestry that the associations of corticosterone with the components of the metabolic syndrome were not similar to those observed with cortisol.

Hence, the findings also support the hypothesis that corticosterone has a distinct role in human health and disease. The main and novel finding in the overall thesis was that the associations between circulating glucocorticoid concentrations, *SERPINA6/A1* and *CYP17A1* SNPs, and key components of the metabolic syndrome, exhibited sex-specificity in individuals of African ancestry. Therefore, future studies investigating the metabolic syndrome and its components in populations of African ancestry should include assessment of sex interactions.

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Appendices

Appendix A: Ethics clearance certificates

R14/49 Mr Siphiwe Dlamini

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M160225

NAME: (Principal Investigator)	Mr Siphiwe Dlamini
DEPARTMENT:	Developmental Pathways for Health Research Unit National Health Laboratory Services Division of Human Genetics BHF Centre for Cardiovascular Science, UK
PROJECT TITLE:	Non-Communicable Disease Risk in Black South Africans: Dissecting the Role of Glucoorticoids
DATE CONSIDERED:	26/02/2016
DECISION:	Approved unconditionally
CONDITIONS:	(Is this for BEC?)
SUPERVISOR:	Prof Julia Goedecke
APPROVED BY:	Professor P. Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	06/06/2016

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 10004,10th floor, Senate House/2nd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/We fully understand the the conditions under which I am/we are authorised to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated,from the research protocol as approved, I/we undertake to resubmit to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. in this case, the study was initially review in February and will therefore be due in the month of February each year.

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Mrs Nicole Jaff

CLEARANCE CERTIFICATE	**M090620**
PROJECT	An Investigation of the Metabolic, Hormonal and anthropometric Characteristics of the Menopausal Transition in Black Urban South African Women
INVESTIGATORS	Mrs Nicole Jaff.
DEPARTMENT	Department of Chemical Pathology
DATE CONSIDERED	09.06.26
DECISION OF THE COMMITTEE*	Approved unconditionally

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

DATE 2009/12/14 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Prof N Crowther

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

R14/49 Prof Lisa Micklesfield

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M160604

NAME: (Principal Investigator)	Prof Lisa Micklesfield
DEPARTMENT:	Paediatrics MRC/Wits Developmental Pathways for Health Research Unit Chris Hani Baragwanath Academic Hospital
PROJECT TITLE:	Determinants of Type 2 Diabetes Mellitus (T2D) Risk in Middle-Aged Black South African (SA) Men and Woman: Dissecting the Role of Sex Hormones, Inflammation and Glucocorticoids
DATE CONSIDERED:	24/06/2016
DECISION:	Approved with conditions
CONDITIONS:	Providing the name and status of the Doctor once appointed. The MTA must be submitted and approved by the HREC (Medical) before shipment of the specimen.
SUPERVISOR:	
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	26/09/2016

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 10004, 10th floor, Senate House/3rd Floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in June and will therefore be due in the month of June each year.

Micklesfield
Principal Investigator Signature

26-09-2016
Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Professor Michele Ramsay

CLEARANCE CERTIFICATE	**M121029**
PROJECT	WITS-INDEPTH Partnership: Genomic and Environment Risk Factors for Cardiometabolic Disease in Africans
INVESTIGATORS	Professor Michele Ramsay.
DEPARTMENT	School of Pathology/Division of Human Genetics
DATE CONSIDERED	26/10/2012
DECISION OF THE COMMITTEE*	Approved unconditionally

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

DATE 21/11/2012 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor :

DECLARATION OF INVESTIGATOR(S)
To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/~~We~~ fully understand the conditions under which I am/~~we~~ are authorized to carry out the abovementioned research and I/~~we~~ guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/~~we~~ undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**
PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

M. Ramsay 11 April 2013

R14/49 Asanda Mtintsilana

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M150530

NAME: (Principal Investigator)	Asanda Mtintsilana
DEPARTMENT:	MRC/Wits Developmental Pathways for Health Research Unit
PROJECT TITLE:	Identification of Metabolites and Metabolic Pathways that Predict Impaired Glucose Metabolism in Black South African Women
DATE CONSIDERED:	29 May 2015
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Associate Prof Julia Goedecke
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2015/06/29

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature _____ Date _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix B: Supplementary figures and tables

Table S2.1: Summary of the categorical variables of the epidemiology study sample.

	n	All (n = 1104)	Men (n = 316)	Women (n = 788)	p
Adiposity					
BMI categories	1094				**< 0.001**
Underweight (< 18.5 kg/m^2)		42 (3.8)	31 (9.8)	11 (1.4)	
Normal (18.5 – 24.9 kg/m^2)		222 (20.3)	125 (39.5)	97 (12.5)	
Overweight (25–29.9 kg/m^2)		243 (22.2)	80 (25.3)	163 (21.0)	
Obese (≥ 30 kg/m^2)		587 (53.7)	80 (25.3)	507 (65.2)	
Elevated waist circumference (≥ 80 cm in women; ≥ 94 cm in men)	1066	849 (79.6)	153 (48.4)	696 (88.3)	**< 0.001**
Blood pressure (BP)					
Elevated systolic BP (≥ 130 mmHg)	1076	519 (48.2)	167 (53.0)	352 (46.3)	**0.017**
Elevated diastolic BP (≥ 85 mmHg)	1076	595 (55.3)	193 (61.3)	402 (52.8)	**0.003**
Elevated BP (Elevated systolic and/or diastolic BP and/or using of BP medication)	1104	756 (68.5)	232 (73.4)	524 (66.5)	**0.030**
Glycaemia					
Fasting glucose (FG) categories	1038				**0.023**
Normal FG (< 5.6 mmol/L)		791 (76.4)	251 (79.4)	540 (74.7)	
Impaired FG (5.6-6.9 mmol/L)		161 (15.5)	50 (15.8)	111 (15.4)	
Elevated FG (≥ 7.0 mmol/L)		86 (8.2)	15 (4.7)	71 (9.8)	
Elevated blood glucose (Elevated FG and/or using diabetes medication)	1104	269 (24.4)	74 (23.4)	195 (24.7)	**0.699**
Serum lipids					
Elevated fasting triglycerides (≥ 1.7 mmol/L)	1012	105 (10.4)	48 (15.2)	57 (8.2)	**< 0.001**
Reduced fasting HDL-cholesterol (< 1.0 mmol/L in men, < 1.3 mmol/L in women)	1013	455 (44.9)	57 (18.0)	398 (57.0)	**< 0.001**

Data presented as n (%). All categorical variables were statistically compared between sexes using a chi-square test. **N:** Number of observations; **BMI:** Body Mass Index; blood pressure; **BP:** Blood pressure; **FG:** Fasting glucose; **HDL:** High Density Lipoprotein; **P**: P value of the chi-square test conducted.

Table S2.2: All regression models tested in both men and women of the epidemiology study.

		Unadjusted Model				Model 1 (Adjusted for confounders)				Model 2 (Adjusted for confounders and BMI)			
	Dependent variable	**Beta/OR**	**95% CI**	**p**	**Sex Int**	**Beta/OR**	**95% CI**	**p**	**Sex Int**	**Beta/OR**	**95% CI**	**p**	**Sex Int**
Corticosterone	BMI	0.005	(-0.033, 0.043)	0.794	0.173	-0.046	(-0.096, 0.004)	0.071	0.063	N/A	N/A	N/A	N/A
	Waist circumference	-0.011	(-0.034, 0.013)	0.375	0.274	-0.030	(-0.065, 0.005)	0.089	0.123	N/A	N/A	N/A	N/A
	Systolic BP	0.010	(-0.015, 0.035)	0.437	0.633	0.007	(-0.029, 0.043)	0.714	0.656	0.014	(-0.021, 0.049)	0.430	0.956
	Diastolic BP	-0.003	(-0.025, 0.020)	0.809	0.786	0.009	(-0.023, 0.041)	0.572	0.897	0.016	(-0.016, 0.047)	0.324	0.775
	Fasting glucose*	-0.032	(-0.071, 0.007)	0.113	**0.031**	-0.008	(-0.055, 0.039)	0.739	**0.009**	-0.001	(-0.048, 0.046)	0.953	**0.008**
	Fasting insulin	0.092	(-0.040, 0.223)	0.172	0.064	-0.119	(-0.301, 0.063)	0.200	0.219	-0.031	(-0.191, 0.129)	0.705	0.446
	Two-hour glucose	-0.008	(-0.093, 0.077)	0.854	0.791	-0.017	(-0.099, 0.065)	0.683	0.971	-0.001	(-0.082, 0.079)	0.972	0.824
	HbA1c*	-0.016	(-0.047, 0.014)	0.301	**0.007**	-0.013	(-0.045, 0.019)	0.435	**0.003**	-0.010	(-0.042, 0.022)	0.543	**0.002**
	HOMA2-IR	0.077	(-0.057, 0.211)	0.261	0.083	-0.129	(-0.312, 0.055)	0.170	0.300	-0.045	(-0.205, 0.116)	0.586	0.551
	Matsuda index	0.228	(0.042, 0.415)	**0.017**	0.560	0.220	(0.034, 0.407)	**0.021**	0.444	0.134	(-0.036, 0.303)	0.122	0.921
	Total cholesterol	-0.009	(-0.047, 0.029)	0.653	0.077	-0.053	(-0.108, 0.001)	0.056	0.060	-0.053	(-0.108, 0.002)	0.060	0.077
	LDL cholesterol*	-0.007	(-0.075, 0.062)	0.850	**0.012**	-0.095	(-0.193, 0.003)	0.057	**0.019**	-0.086	(-0.184, 0.012)	0.085	**0.029**
	HDL cholesterol	-0.024	(-0.084, 0.036)	0.431	**0.042**	0.018	(-0.066, 0.102)	0.678	0.263	-0.009	(-0.088, 0.071)	0.830	0.377
	Triglycerides	0.060	(-0.014, 0.135)	0.110	**0.013**	-0.066	(-0.177, 0.046)	0.247	0.063	-0.044	(-0.153, 0.066)	0.431	0.116
	Metabolic syndrome	0.983	(0.730, 1.321)	0.908	0.392	1.104	(0.792, 1.540)	0.558	0.142	NA	NA	NA	NA
Cortisol	BMI*	0.017	(-0.024, 0.059)	0.414	0.086	-0.011	(-0.064, 0.041)	0.679	**0.040**	N/A	N/A	N/A	N/A
	Waist circumference	0.008	(-0.018, 0.034)	0.540	0.085	0.000	(-0.016, 0.016)	0.984	0.055	N/A	N/A	N/A	N/A
	Systolic BP	0.036	(0.009, 0.063)	**0.009**	0.942	0.042	(0.005, 0.079)	**0.028**	0.955	0.043	(0.007, 0.080)	**0.018**	0.634
	Diastolic BP	0.046	(0.022, 0.070)	**1.790×10^{-4}**	0.954	0.051	(0.017, 0.085)	**0.004**	0.918	0.052	(0.019, 0.085)	**0.002**	0.546
	Fasting glucose*	0.027	(-0.017, 0.072)	0.229	**0.010**	0.042	(-0.010, 0.094)	0.110	**<0.001**	0.044	(-0.008, 0.095)	0.097	**<0.001**
	Fasting insulin	0.040	(-0.106, 0.186)	0.589	**0.027**	-0.065	(-0.261, 0.131)	0.516	0.073	-0.055	(-0.226, 0.117)	0.534	0.352
	Two-hour glucose*	0.032	(-0.054, 0.118)	0.467	0.255	-0.013	(-0.099, 0.074)	0.775	0.090	-0.010	(-0.095, 0.075)	0.810	**0.047**

	HbA1c*	-0.009	(-0.044, 0.026)	0.619	**0.022**	-0.001	(-0.036, 0.035)	0.965	**0.003**	-0.001	(-0.036, 0.035)	0.974	**0.001**
	HOMA2-IR	0.025	(-0.124, 0.175)	0.739	0.072	-0.057	(-0.254, 0.141)	0.573	0.241	-0.036	(-0.208, 0.137)	0.685	0.658
	Matsuda index	-0.054	(-0.248, 0.141)	0.589	0.128	0.083	(-0.117, 0.284)	0.415	0.572	0.048	(-0.133, 0.229)	0.603	0.848
	Total cholesterol	0.007	(-0.037, 0.050)	0.764	0.955	-0.048	(-0.106, 0.010)	0.102	0.690	-0.048	(-0.106, 0.010)	0.104	0.573
	LDL cholesterol	-0.007	(-0.082, 0.067)	0.846	**0.038**	-0.138	(-0.241, -0.034)	**0.009**	0.565	-0.136	(-0.239, -0.034)	**0.009**	0.768
	HDL cholesterol	0.037	(-0.027, 0.102)	0.254	**0.014**	0.105	(0.017, 0.193)	**0.020**	**0.038**	0.102	(0.019, 0.186)	**0.017**	0.083
	Triglycerides	0.073	(0.071, -0.006)	0.152	0.651	-0.032	(-0.146, 0.081)	0.577	0.708	-0.030	(-0.141, 0.081)	0.598	0.394
	Metabolic syndrome	1.397	(1.005, 1.946)	0.047	0.251	1.502	(1.044, 2.170)	0.029	0.245	NA	NA	NA	NA

Unadjusted Model: Unadjusted linear/logistic regression model; **Model 1 (Adjusted for confounders):** Linear/logistic regression model adjusted for age, sex, time of blood sample (calculated as minutes from the earliest sample (07h00)), smoking, alcohol and relevant chronic medication; **Model 2 (Adjusted for confounders and BMI):** Linear/logistic regression model adjusted the above confounders and BMI. **Beta/OR:** regression co-efficient/odds ratio value of the linear/logistic model; **95% CI:** 95% confidence intervals; **p:** P value of the regression model**; Sex Int:** P value for the sex interaction.

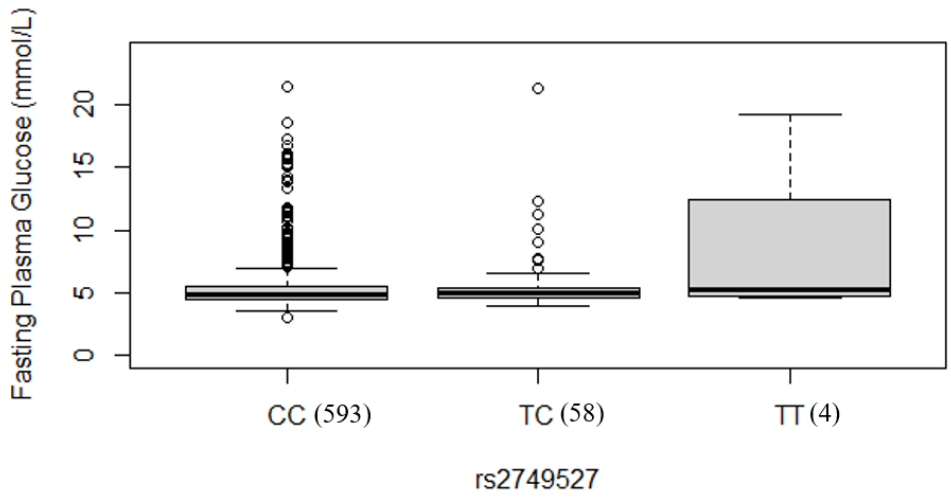


Figure S3.1: Fasting plasma glucose concentrations across the rs2749527 genotypes.

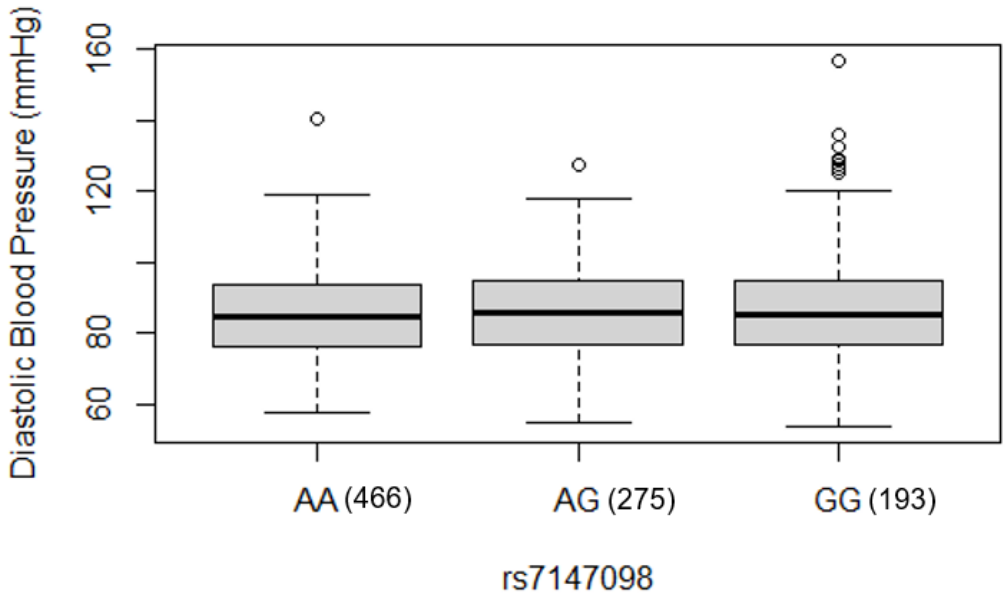
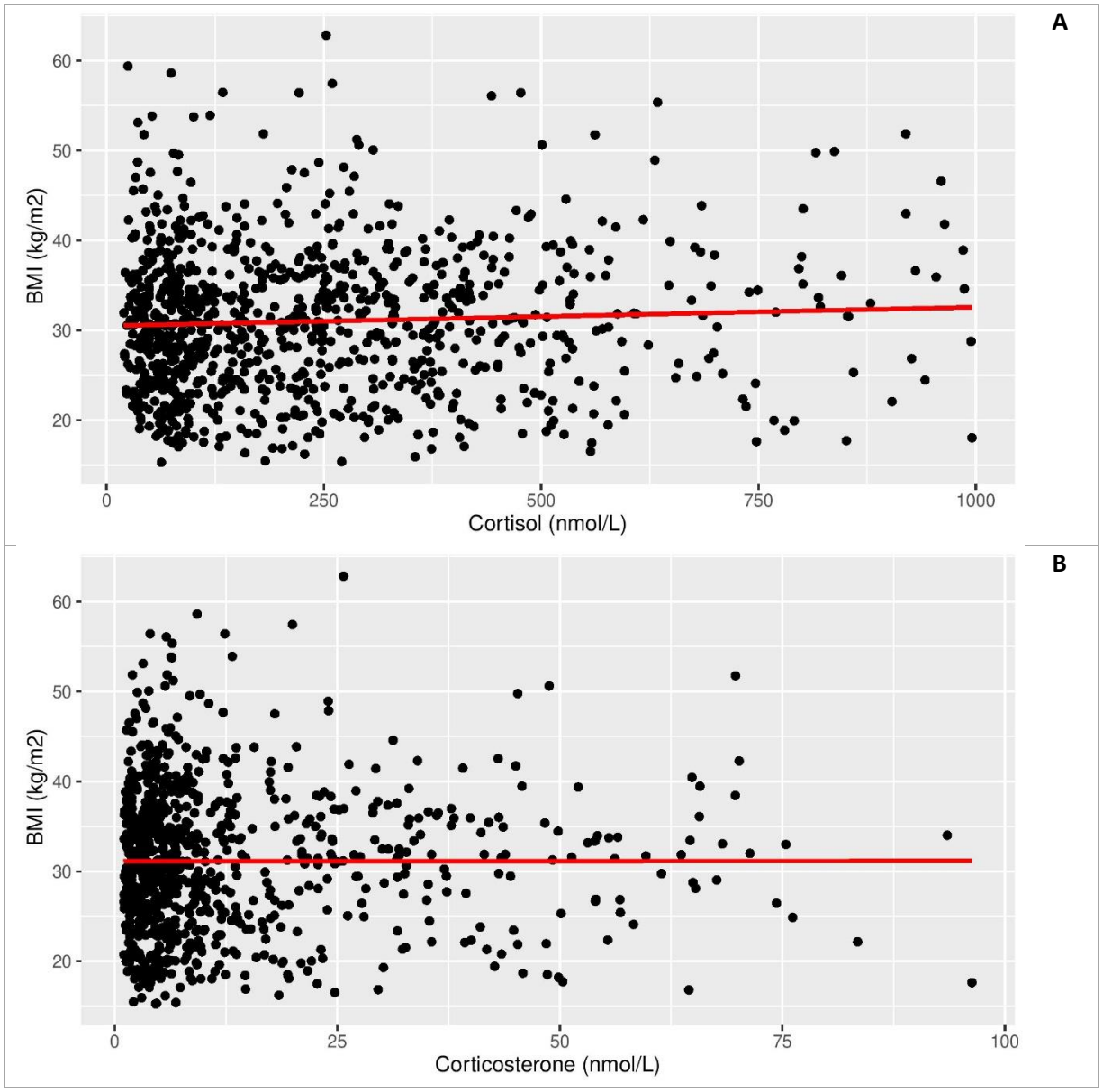
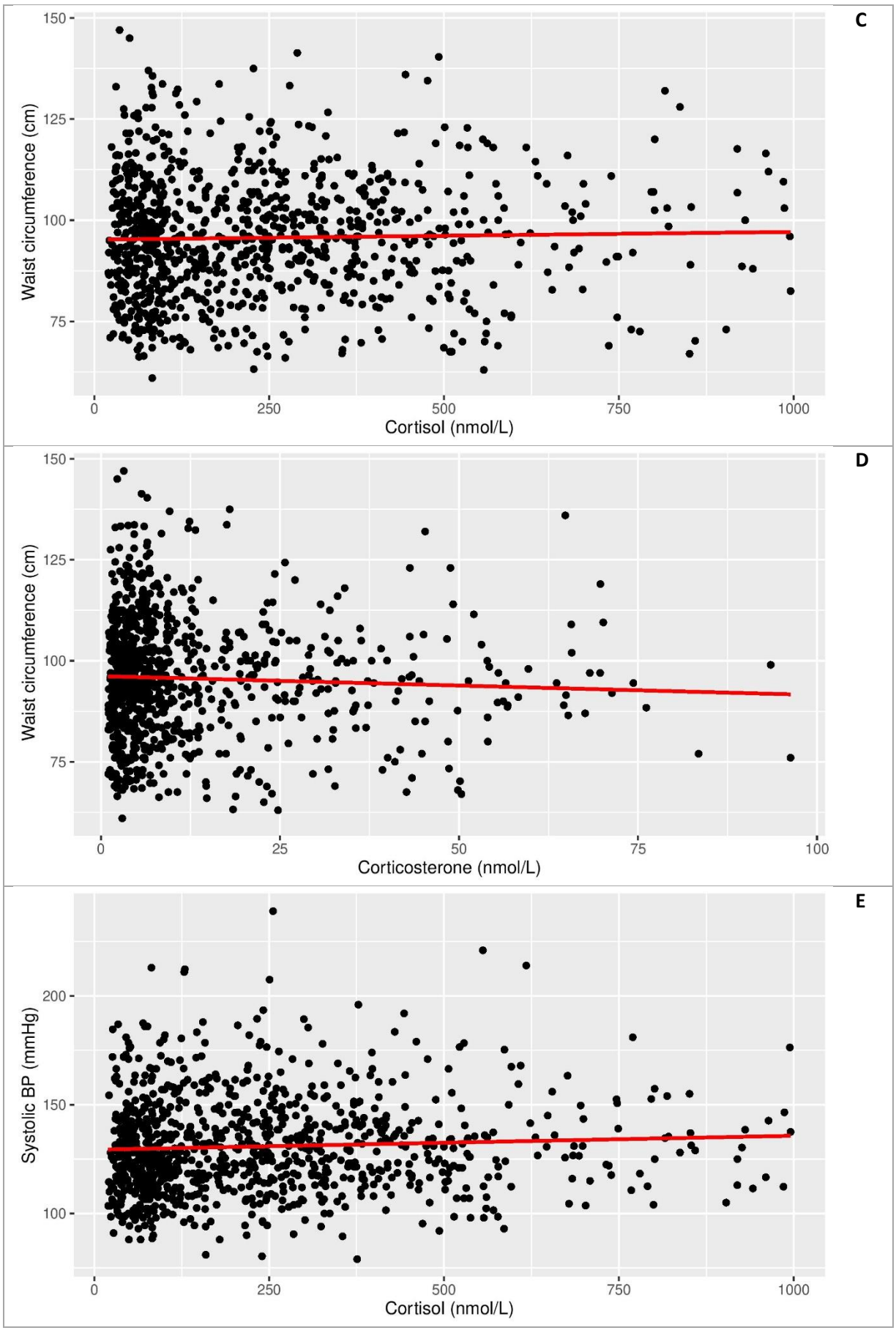


Figure S3.2: Diastolic blood pressure across the rs7147098 genotype.

Appendix C: Scatter plots (men and women combined).




C
Waist circumference (cm)
150
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250
500
750
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Cortisol (nmol/L)
D
Waist circumference (cm)
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25
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100
Corticosterone (nmol/L)
E
Systolic BP (mmHg)
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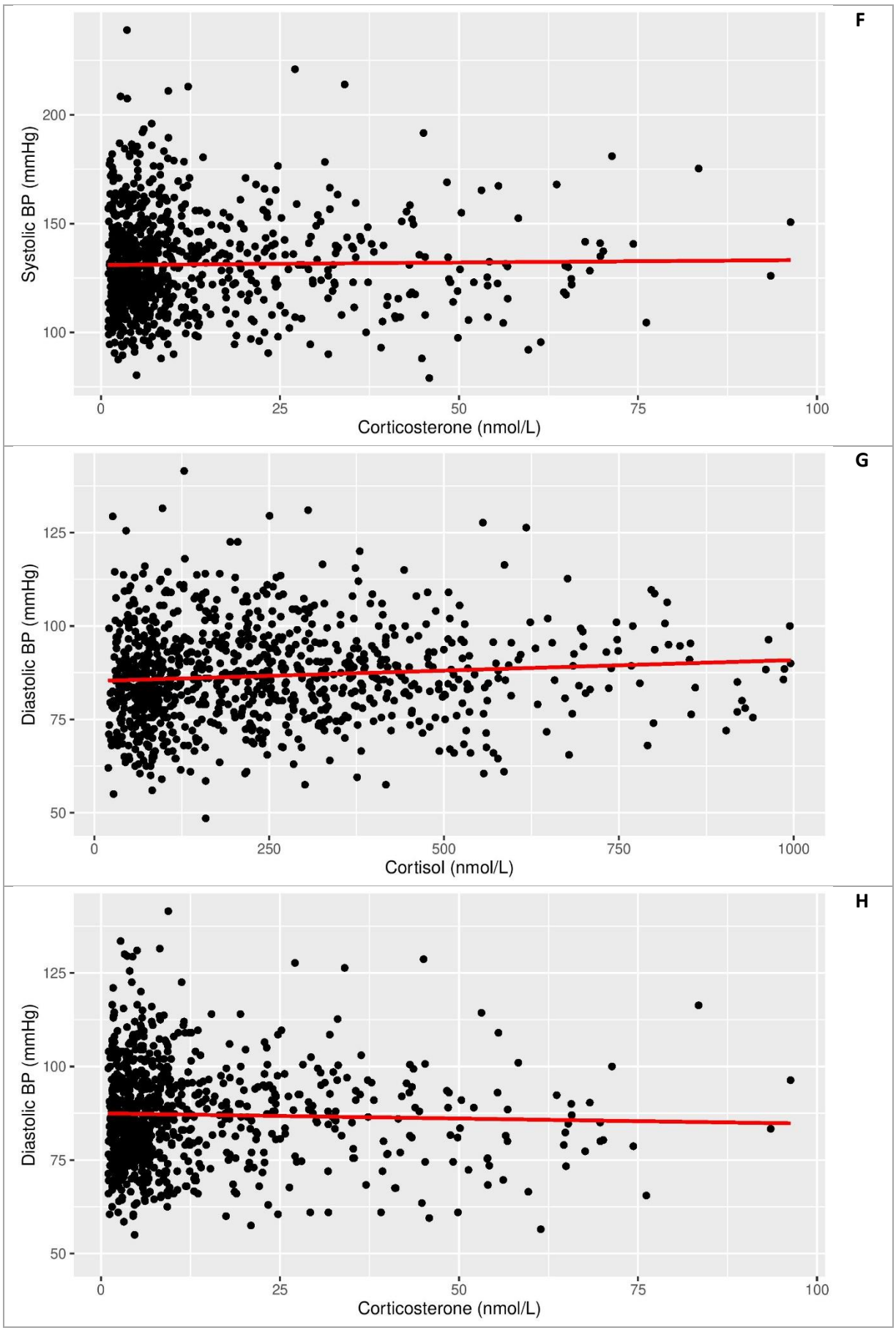
Cortisol (nmol/L)


F
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Cortisol (nmol/L)
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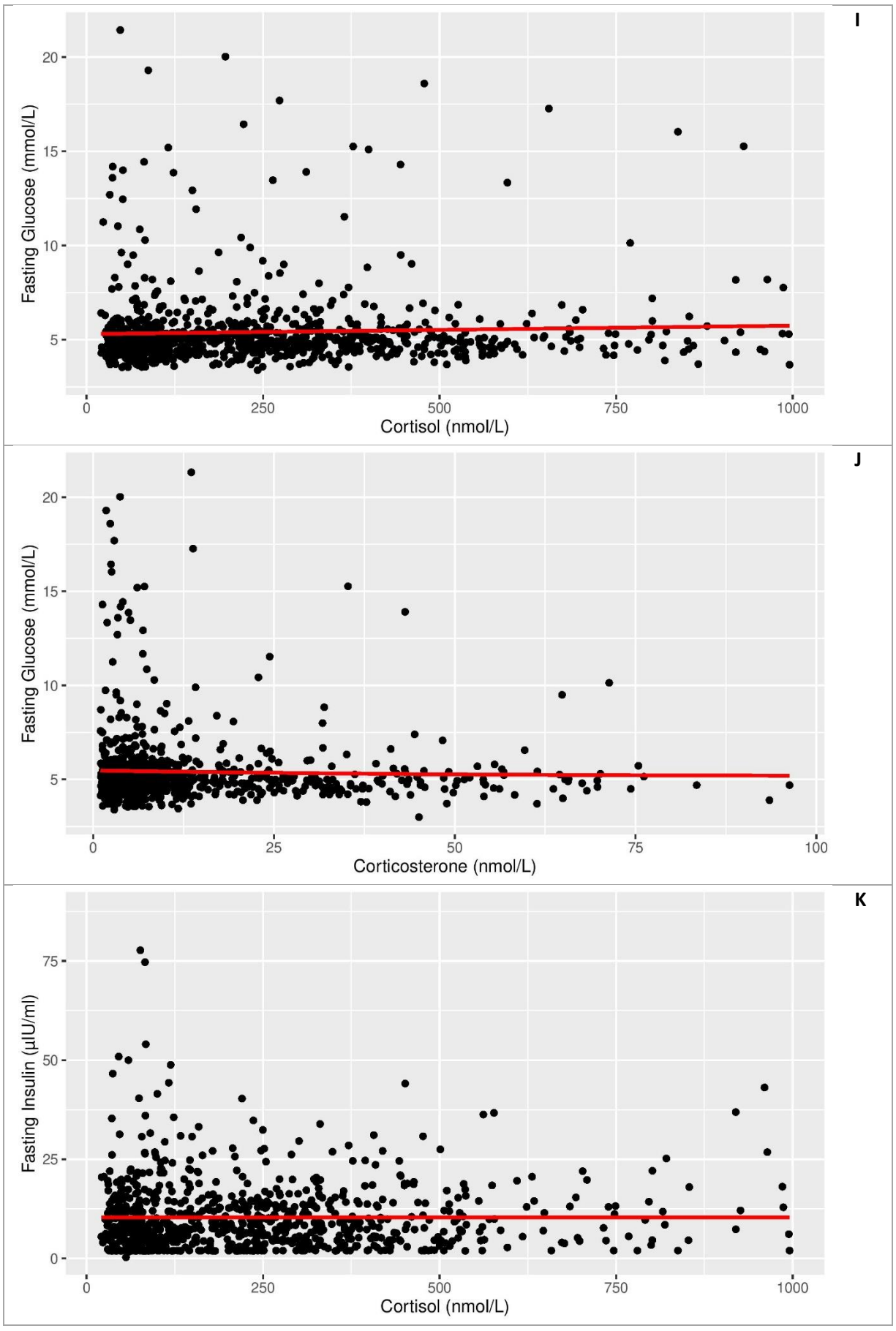
Corticosterone (nmol/L)


I
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Cortisol (nmol/L)
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Corticosterone (nmol/L)
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Fasting Insulin (µIU/ml)
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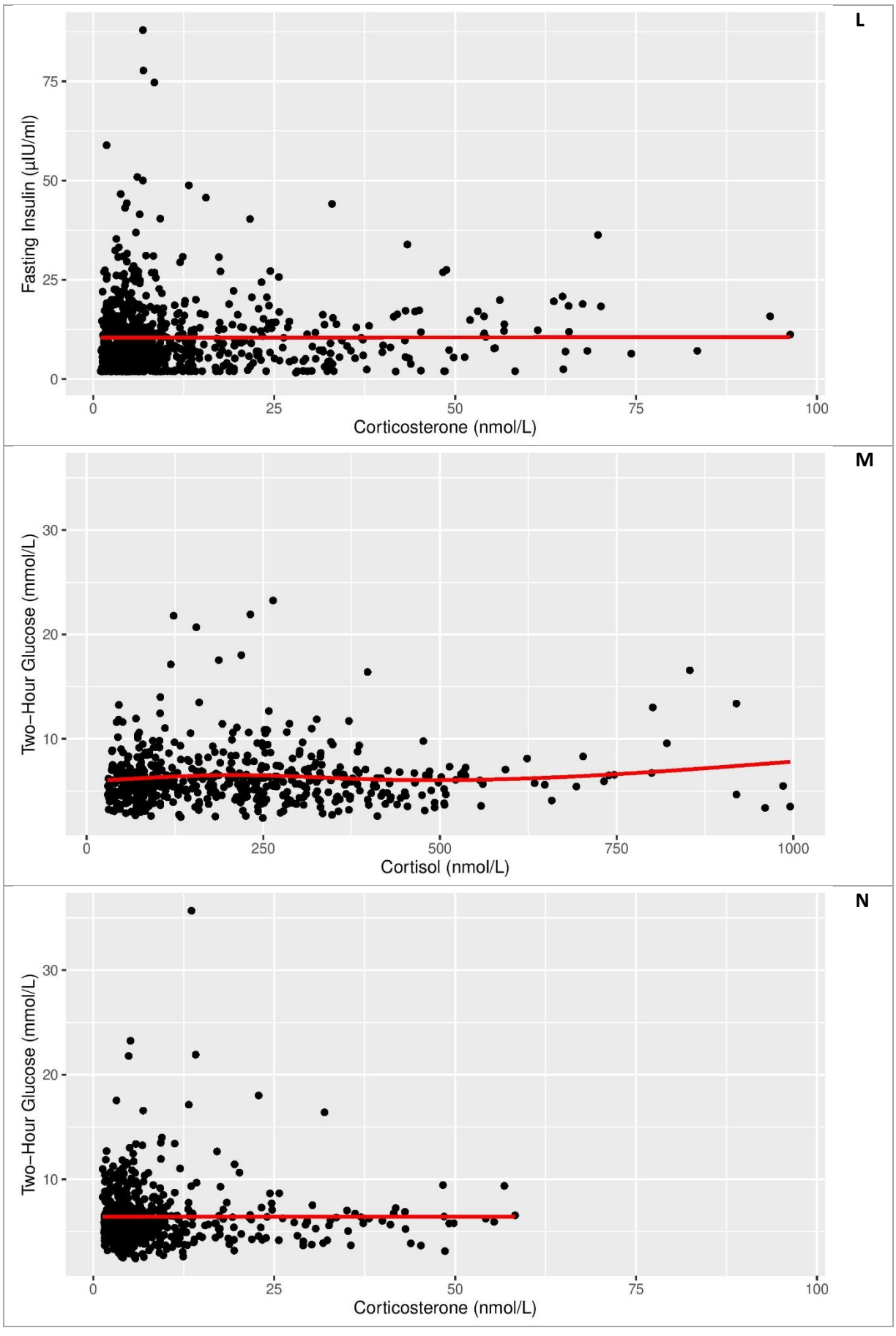
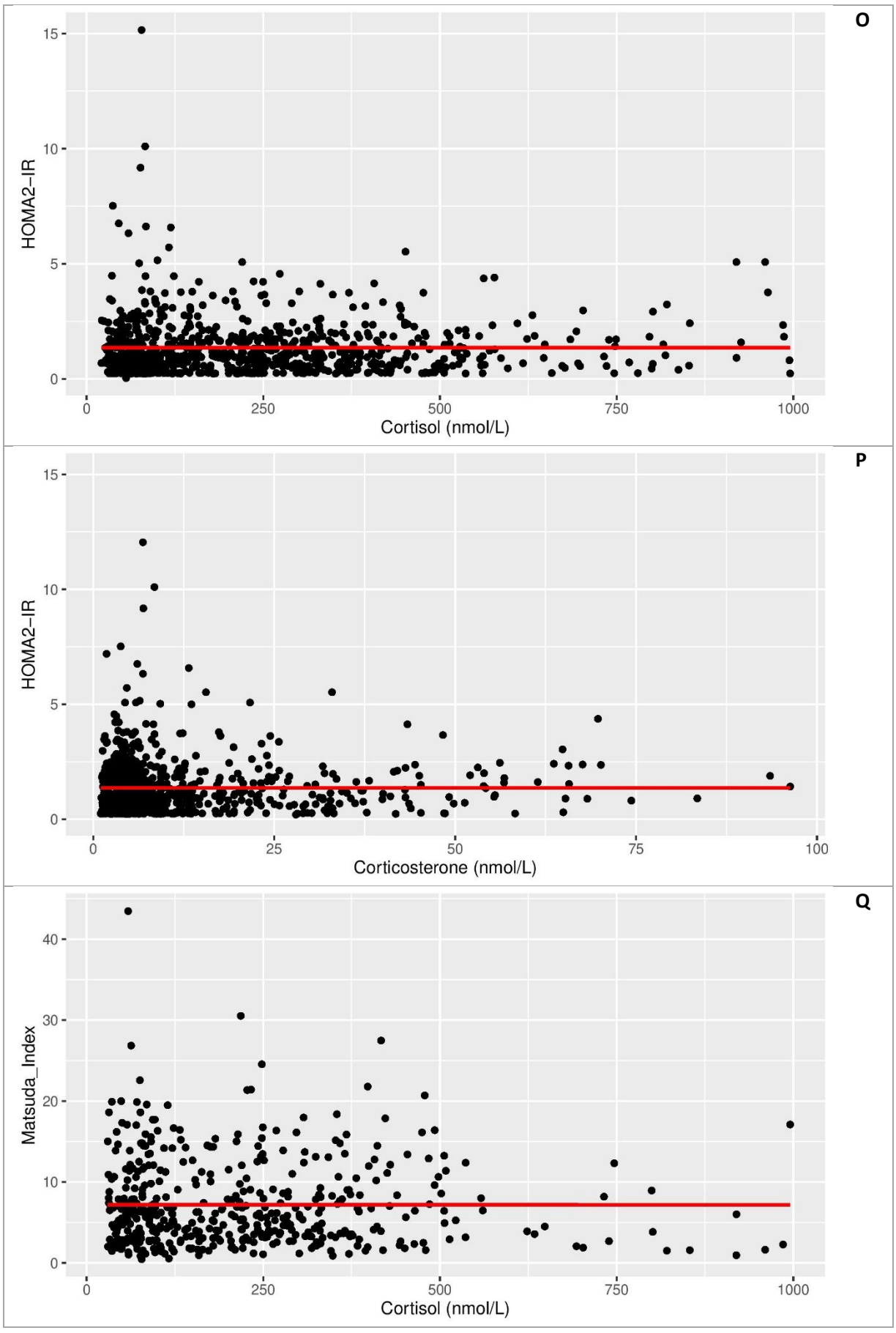
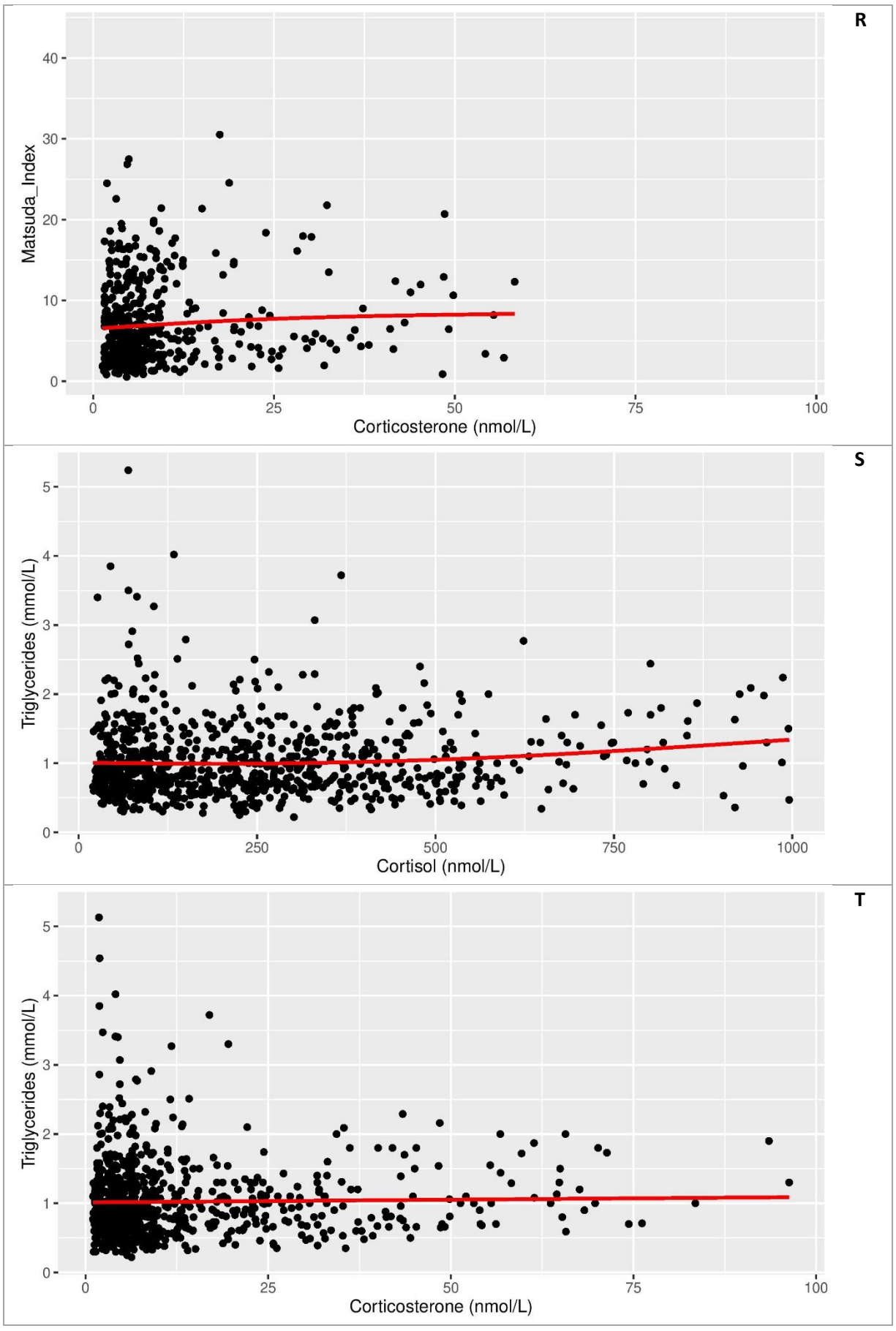
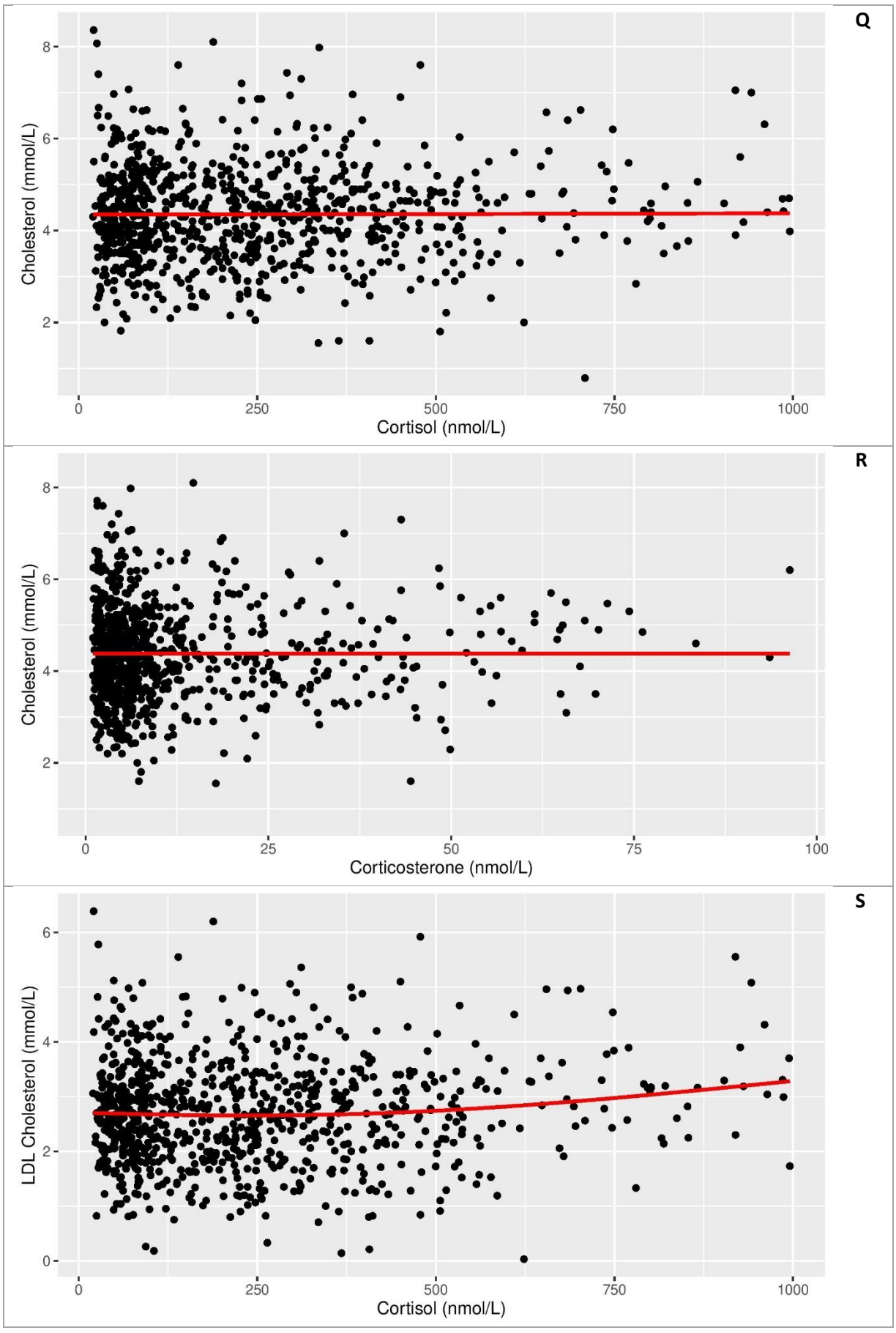
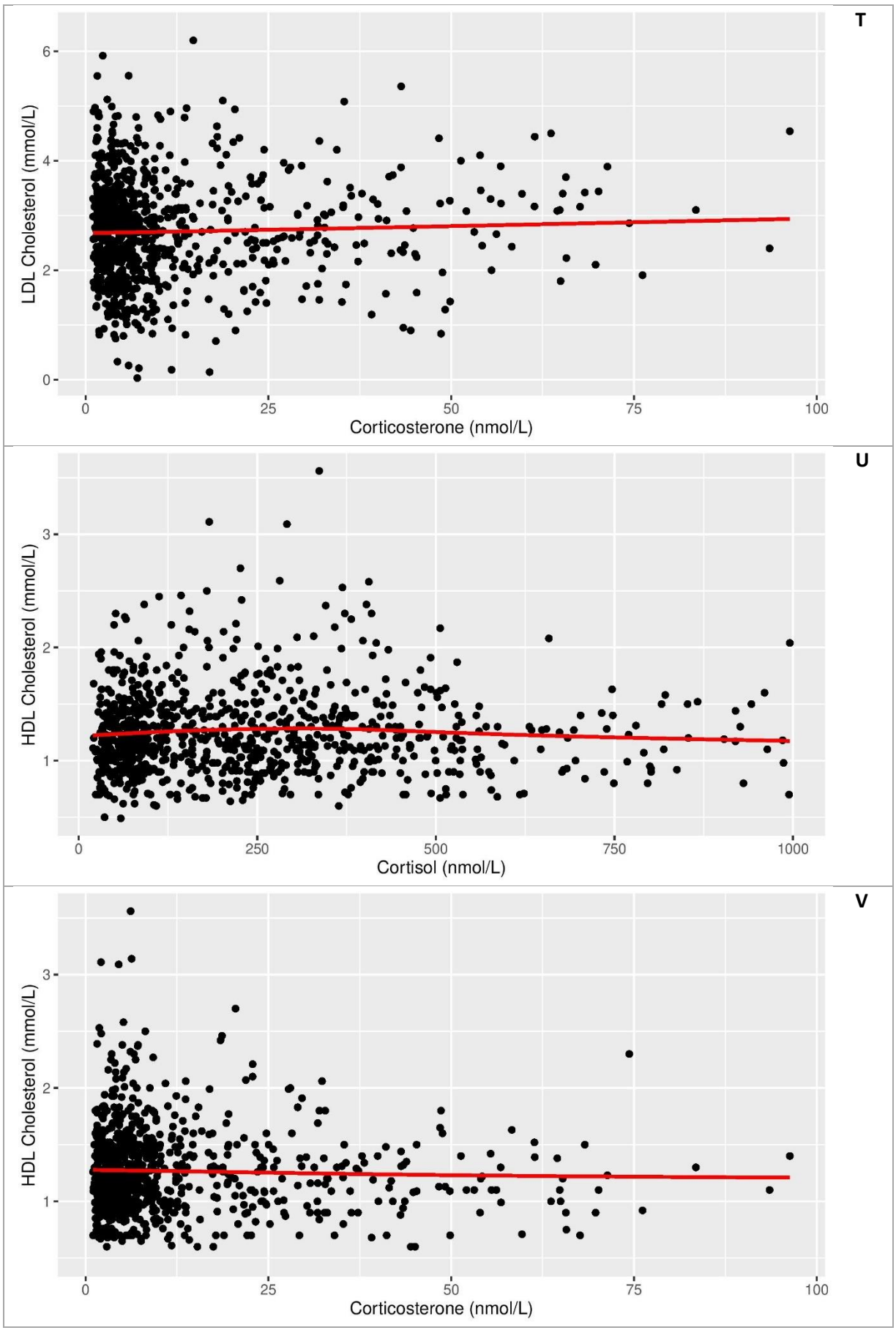
750
1000
Cortisol (nmol/L)


L
Fasting Insulin (µIU/ml)
0
25
50
75
0
25
50
75
100
Corticosterone (nmol/L)
M
Two−Hour Glucose (mmol/L)
10
20
30
0
250
500
750
1000
Cortisol (nmol/L)
N
Two−Hour Glucose (mmol/L)
10
20
30
0
25
50
75
100
Corticosterone (nmol/L)

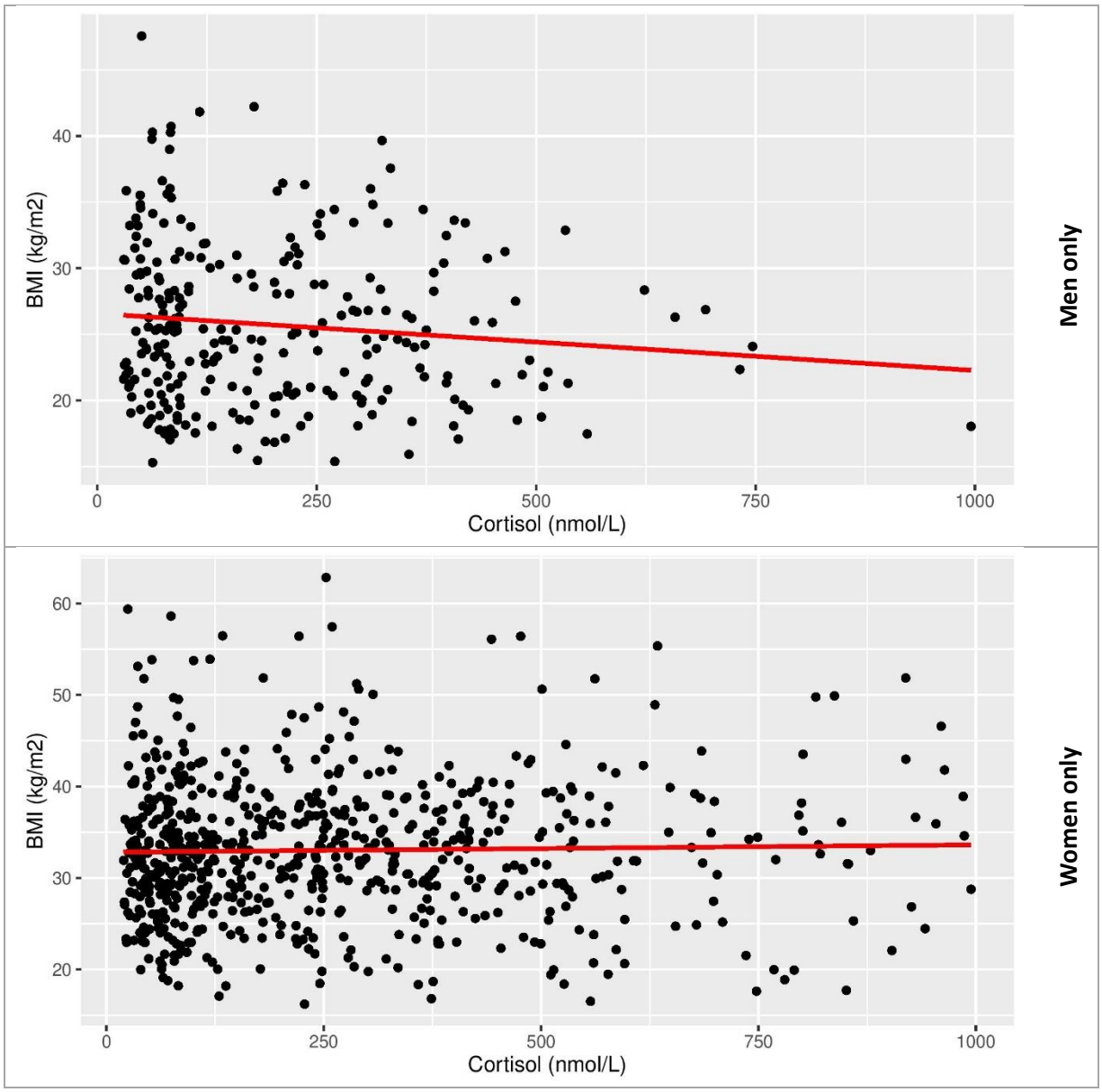

O
HOMA2–IR
Cortisol (nmol/L)
P
HOMA2–IR
Corticosterone (nmol/L)
Q
Matsuda_Index
Cortisol (nmol/L)


R
Matsuda_Index
Corticosterone (nmol/L)
S
Triglycerides (mmol/L)
Cortisol (nmol/L)
T
Triglycerides (mmol/L)
Corticosterone (nmol/L)


Q
Cholesterol (mmol/L)
8
6
4
2
0
250
500
750
1000
Cortisol (nmol/L)
R
Cholesterol (mmol/L)
8
6
4
2
0
25
50
75
100
Corticosterone (nmol/L)
S
LDL Cholesterol (mmol/L)
6
4
2
0
0
250
500
750
1000
Cortisol (nmol/L)


T
LDL Cholesterol (mmol/L)
0
2
4
6
0
25
50
75
100
Corticosterone (nmol/L)
U
HDL Cholesterol (mmol/L)
1
2
3
0
250
500
750
1000
Cortisol (nmol/L)
V
HDL Cholesterol (mmol/L)
1
2
3
0
25
50
75
100
Corticosterone (nmol/L)

Appendix D: Scatter plots (BMI: men and women separated).

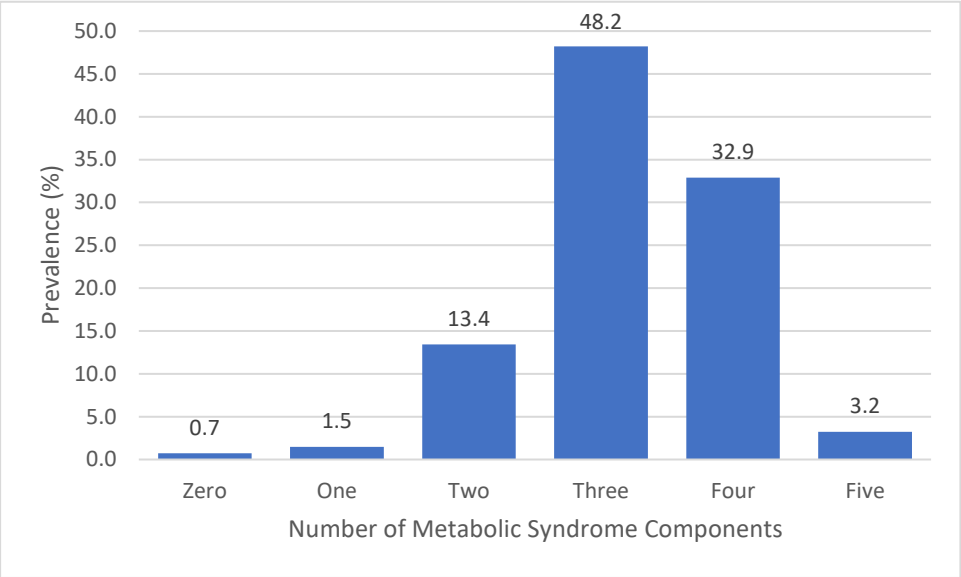


Appendix E: Sex comparison of circulating glucocorticoids.

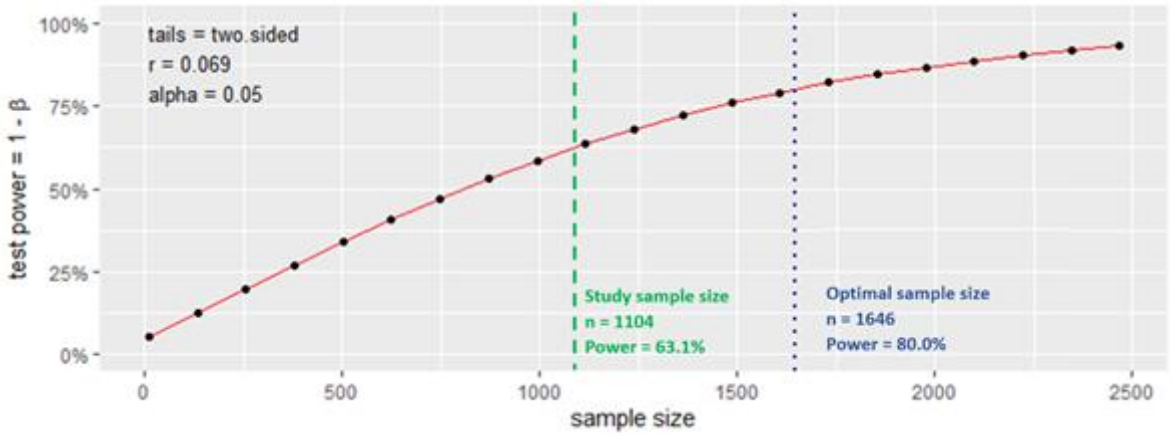
Comparing circulating glucocorticoids by sex			ANCOVA P values after adjusting for the indicated confounder/s						
	Men	Women	Unadjusted	BMI	Waist	Sampling time	Smoking	Alcohol	All confounders except waist
Cortisol (nmol/L)	153.8 (76.3-295.8)	201.2 (82.5-371.9)	<0.001	<0.001	<0.001	<0.001	<0.001	1.000	0.873
Corticosterone (nmol/L)	5.0 (3.3-9.0)	6.8 (3.6-17.9)	<0.001	<0.001	<0.001	<0.001	<0.001	0.147	0.149

Glucocorticoid values presented as median (25th-75th percentiles). ANCOVA P values are statistical p values of the ANCOVA test when comparing average glucocorticoid concentrations between men and women.

Appendix F: Proportions of the study population above each cut off point.



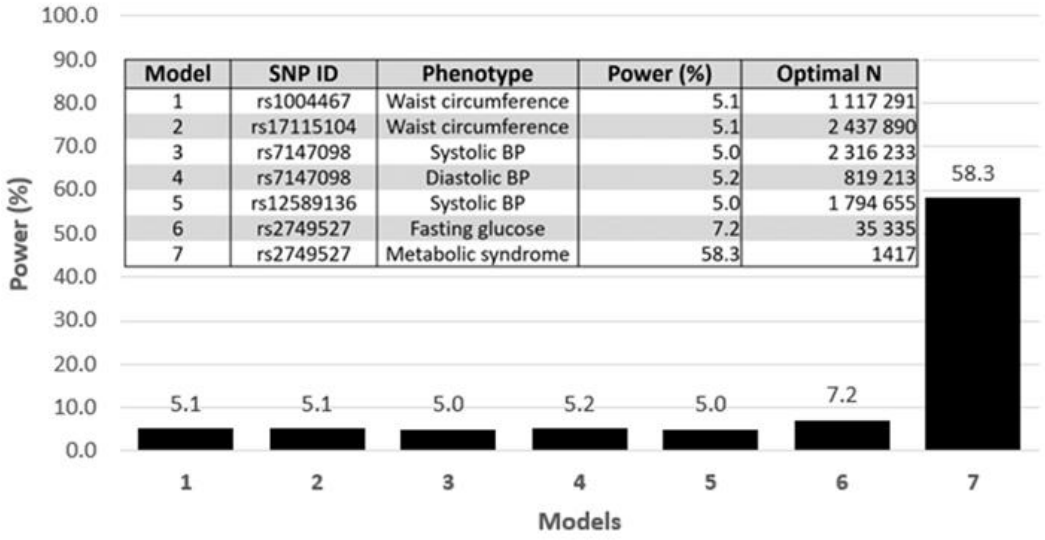
Appendix G: Research paper 1 power calculation results.



The power was estimated using correlation (arctangh transformation) using 'pwr package' in R, version 3.6.2 (RStudio, Boston, USA). The plot shows that, for a sample size of 1,104, the study was approximately 63.1% powered to detect associations between circulating cortisol and BMI.

Appendix H: Research paper 2 power calculation results.

Model	SNP ID	Phenotype	Power (%)	Optimal N
1	rs1004467	Waist circumference	5.1	1 117 291
2	rs17115104	Waist circumference	5.1	2 437 890
3	rs7147098	Systolic BP	5.0	2 316 233
4	rs7147098	Diastolic BP	5.2	819 213
5	rs12589136	Systolic BP	5.0	1 794 655
6	rs2749527	Fasting glucose	7.2	35 335
7	rs2749527	Metabolic syndrome	58.3	1417



Post hoc power estimates and required sample sizes for testing associations between the selected SNPs and the metabolic syndrome, and its components. BP: Blood pressure; REF: Optimal N: Required sample size for 80% power.

Appendix I: LCMS method used to measure glucocorticoids.

Extraction of steroids from serum: All calibrators, internal standard (labeled with stable isotope deuterium) mix, and controls were purchased from Chromsystems (Grafelfing, Germany). A liquid-liquid extraction technique (Agilent technologies, California, United States) was used to extract all steroids. Briefly, 50ul of the internal standard mix was added to 500ul of serum. 3ml of methyl tert-butyl ether (MTBE) was then added to each sample and vortexed at maximum speed for 3 x 30 seconds, with 30 seconds rests in between. The MTBE top layer was then transferred into a clean glass tube and dried down using nitrogen at room temperature. The extracted steroids were dried down under nitrogen and then reconstituted in 100ul of 50% Methanol-water which was transferred into limited volume analytical vials (Kinesis inc., Vernom Hills, United States) for analyses on the Ultra-higher Pressure Liquid Chromatography (UPLC) Mass Spectrometry (MS).

Steroid quantitation in the UPLC-MS: Ten microliters of each extracted sample was injected onto a Kinetex 2.6u 50 x 2.1 mm Column (Phenomenex, California, United States) utilising an Acquity UPLC that was connected to a Xevo-TQS MS (Waters Corporation, Milford, United States). The UPLC-MS method was optimized and validated to quantitate the following serum steroid analytes (and their respective deuterated internal standards): cortisol, cortisone, corticosterone, 11-deoxycortisol, testosterone, 17-hydroxyprogesterone, and androstenedione. A flow rate of 0.6 ul/min was used to was set on a gradient profile with mobile phase A was (5mM Ammonium formate and 0.1% formic acid in deionized water), and mobile phase B (1mM Ammonium formate and 0.1% formic acid in acetonitrile) to elute off each of the steroids over a total run time of 5.50min. The binary gradient programme was set as follow: 5% B for the first 0.95min; 30% B for 0.05min; 70% B for 0.50min; 90% B for 3.50min; 95% B for 0.50min.

The following mass spectrometry parameters were used:

Electrospray ionisation in positive mode (ESI+) was used for analysis. Capillary voltage was 3kV with cone and collision energies optimised to each compound analysed. Source temperature and desolvation temperature were set at 150°C and 350°C respectively. Cone gas was set at 550 L/hr and Desolvation gas was set at 150 L/hr. The analytes were identified with multiple reaction monitoring identified for each individual compound.