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Type 2 diabetes mellitus risk in middle-aged black South African women: dissecting the role of menopause, HIV infection and adipose tissue biology

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2022

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TYPE 2 DIABETES MELLITUS RISK IN MIDDLE-AGED BLACK SOUTH AFRICAN WOMEN: DISSECTING THE ROLE OF MENOPAUSE, HIV INFECTION AND ADIPOSE TISSUE BIOLOGY



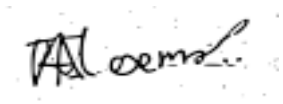
Maphoko Adelade Masemola

A thesis submitted to the Faculty of Health Sciences, University of Witwatersrand, School of Clinical Medicine, in fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg, 2022

DECLARATION

I, Maphoko Adelade Masemola, declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



.....

Maphoko Adelade Masemola

Signed on the.....19th.....day of.....October.....2022

DEDICATION

To

Ranape Rorišang Masemola

RESEARCH OUTPUTS

Conference presentations

Masemola M, Micklesfield LK, Kufe C, Chikowore T, Lichaba M, Crowther NJ, Norris S, Kengne AP, Olsson T, Karpe F, Goedecke JH.. 2021. **Increased insulin response in pre- and post-menopausal African women living with HIV,** Oral presentation at the 54th Society for Endocrinology, Metabolism and Diabetes of South Africa (SEMDSA) congress 26-28 March 2021, virtual conference.

Publications I contributed towards during my PhD

Ratshikombo T, Goedecke JH, Soboyisi M, Kufe C, Makura-Kankwende CBT, **Masemola M**, Micklesfield LK, and Chikowore T. **Sex Differences in the Associations of Nutrient Patterns with Total and Regional Adiposity: A Study of Middle-Aged Black South African Men and Women**. Nutrients 13, no. 12 (2021): 4558.

Goedecke JH, Nguyen K, Kufe C, **Masemola M**, Chikowore T, Mendham AE, Norris SA, Crowther NJ, Karpe F, Olsson T, Kengne AP. **Waist circumference thresholds predicting incident dysglycaemia and type 2 diabetes in Black African men and women**. Diabetes, Obesity and Metabolism. (2022)

Kufe C, Micklesfield LK, **Masemola M**, Chikowore T, Kengne AP, Karpe F, Norris S, Crowther NJ, Olsson T, and Goedecke JH. **Increased Risk for Type 2 Diabetes in Relation to Adiposity in Middle-Aged Black South African Men compared to Women.** medRxiv (2022).

Kufe C, Goedecke JH, **Masemola M**, Chikowore T, Soboyisi M, Smith A, Westgate K, Brage S, Micklesfield L. **Physical behaviours and their association with type 2 diabetes risk in urban South African middle-aged adults: An isotemporal substitution approach**. medRxiv. (2022)

ABSTRACT

Background:

The prevalence of type 2 diabetes (T2D) mellitus in sub-Saharan Africa has increased in recent decades and is projected to increase more than any region in the world. Black African women are at greater risk of T2D due to the higher rates of obesity and insulin resistance compared to white European women. The risk of T2D increases with menopause and is hypothesised to be due to changes in reproductive hormones, body fat distribution and adipose tissue biology. As life expectancy increases, more South African women living with human immunodeficiency virus (HIV) will be transitioning through menopause into post-menopause as they live longer on antiretroviral therapy (ART). Thus, aim of this thesis is twofold, firstly to examine the differences in body fat distribution and insulin sensitivity and response, measured using oral glucose tolerance tests (OGTT) and frequently sampled intravenous glucose tolerance tests (FSIGT), between pre- and post-menopausal women living with and without HIV. Secondly, to explore how abdominal and gluteal adipose tissue expression of adipokines, inflammatory, glucocorticoid and lipid metabolism genes differ by menopause and HIV and how these gene associate with insulin sensitivity. To address these aims, this thesis has 3 results chapters with the following objectives; 1) to compare conventional body composition and insulin sensitivity and response assessment methods to more precise measures, 2) to investigate the effect of menopause and HIV status on glycaemia, insulin sensitivity and response in black African women, 3) and to determine the difference in adipokines, inflammatory, glucocorticoid and lipid metabolism gene expression between abdominal and gluteal subcutaneous adipose tissue (SAT) depots and how they relate to insulin sensitivity, in pre- and post-menopausal women with and without HIV.

Methods:

This thesis was nested within a larger cohort study investigating the determinants of T2D risk in middle-aged black South African men and women: dissecting the role of sex hormones, inflammation and glucocorticoids. For the main study, all the men (n=502) and women (n=527) participants were recruited and provided informed consent, and completed a series of questionnaires including a demographic and food frequency questionnaire (FFQ). Body fat and its distribution were associated using anthropometry and dual energy x-ray absorptiometry (DXA); glycaemia and insulin sensitivity and response were measured from

fasting blood samples and an OGTT. For this thesis, a sub-sample of 92 of the women who met the following inclusion criteria were included and recruited to additionally undergo FSIGT and abdominal and gluteal adipose tissue biopsies. The inclusion criteria were: (1) age and menopause status: pre-menopausal women 40-45 years and post-menopausal women 55-65 years old; (2) HIV status: HIV negative women or women living with HIV (LWHIV); (3) BMI 20-40 kg/m^2; (4) not diabetic. The 92 women who were recruited were divided into four groups based on HIV and menopause status: (1) pre-menopausal HIV-negative (PRE-; n=21); (2) pre-menopausal women LWHIV (PRE+; n=11); (3) post-menopausal HIV-negative (POST-; n=42); (4) post-menopausal women LWHIV (POST+; n=18). From the gluteal and abdominal fat biopsies, adipose tissue expression of adipokines, inflammatory, glucocorticoid and lipid metabolism gene expression were measured. The frequently sampled intravenous glucose tolerance test was conducted in 82 of these participants to estimate insulin sensitivity (S_I), acute insulin response to glucose (AIRg) and beta cell function (disposition index, DI). The OGTT-derived outcomes included, insulin sensitivity measured by Matsuda Index, insulin response measured by Insulinogenic index (IGI) and beta cell function measured by oral disposition index (DI_O).

Results:

The results are reported in 3 separate results chapters. Firstly, I compared conventional body composition and insulin sensitivity and response methods to methods that are more precise and strongly associated with gold standards. In this study, I showed that waist circumference was positively correlated with VAT (r_s =0.665), SAT (r_s=0.743) and android fat (r_s=0.834), but the strongest correlation was between hip circumference and gynoid fat mass (r_s =0.929). There was homoscedasticity between each of these correlations. For insulin sensitivity, there was a significant correlation between the OGTT-derived Matsuda Index and FSIGT-derived S_I (r_s =0.518) and for insulin response, there was a significant positive correlation between OGTT-derived IGI (OGTT) and FSIGT-derived AIRg (r_s =0.517). There was a weak but significant positive correlation between DI_O (OGTT) and DI (FSIGT) (r_s = 0.336). Furthermore, there was no proportional bias and there was homoscedasticity between the OGTT- and FSIGT-derived measures of insulin sensitivity, response and beta cell function. I then used both OGTT and FSIGT- derived measures of insulin sensitivity and response and beta cell function to explore associations with menopause and HIV status in chapter 4. In chapter 5, the OGTT-derived insulin sensitivity measure were used to examine the association

between insulin sensitivity and adipose tissue function, since showed OGTT-derived Matsuda Index showed a strong correlation and agreement with the FSIGT-derived S_I and indicates a more physiological response.

In the second study I investigated the effect of menopause and HIV status on glycaemia, insulin sensitivity and response in black African women. Results from the second study (chapter 4) reported that a greater proportion of HIV negative women presented with obesity compared to the women LWHIV (62% vs. 43%), and body fat % (BF%), fat mass index (FMI) and SAT were lower in women LWHIV compared to HIV negative women. Postmenopausal women had greater VAT compared to the premenopausal women ($p=0.027$). Despite no differences in glycaemia or insulin sensitivity, insulin response to glucose, derived from both OGTT and FSIGT, which were higher in women LWHIV ($p=0.015$ and 0.005, respectively). This hyperinsulinaemia shown was associated with higher insulin secretion and not due to differences in insulin clearance. Postprandial glycaemia was higher in post-menopausal women compared to their premenopausal counterparts and this was independent of the higher VAT in postmenopausal women ($p=0.032$).

In chapter 5, my results show that although insulin sensitivity was not different between the HIV and menopausal groups, women LWHIV had greater expression of adiponectin in both abdominal and gluteal depots (abdominal: $p=0.057$; gluteal: $p=0.007$), a corresponding lower expression of leptin (abdominal: $p=0.005$; gluteal: $p=0.002$), and lower abdominal cell size ratio compared to HIV negative women ($p=0.001$). Postmenopausal women had greater expression of M1 adipose tissue macrophages (abdominal: $p=0.040$; gluteal: $p=0.018$). Markers of systemic inflammation (hsCRP and IL-6) and adiposity (android fat, circulating leptin, abdominal LEP and gluteal LEP) were associated with lower insulin sensitivity. Gluteal adipogenic transcription factor (PPARγ) and PPARγ-responsive genes (LPL and adiponectin) were associated with higher insulin sensitivity.

Conclusion:

In conclusion, this study has provided evidence that postmenopausal women have greater postprandial glycaemia than premenopausal women, however this was independent of the higher VAT in postmenopausal women. This supports the hypothesis of a preferential increase in abdominal adiposity in postmenopausal women. This study demonstrates for the

first time that insulin response to glucose was higher in women LWHIV, irrespective of their menopausal status. However, the cause and significance of this higher insulin response in women LWHIV requires further investigation. Moreover, despite a tendency for lower insulin sensitivity, women LWHIV had greater expression of adiponectin in both abdominal and gluteal depots, a corresponding lower expression of leptin and lower abdominal cell size ratio compared to HIV negative women which requires further exploration. Lastly, irrespective of menopause and HIV status, gluteal adipogenic transcription factor and (PPARγ) and PPARγ-responsive genes (LPL and adiponectin) were associated with favourable insulin sensitivity, whereas markers of adiposity (android fat, circulating leptin, abdominal LEP and gluteal LEP) were associated with low insulin sensitivity. Thus, future research should explore biological pathways involved in SAT gene expression and insulin sensitivity in this population.

ACKNOWLEDGEMENTS

I would like to express my most sincere appreciation and gratitude to **Professors Julia Goedecke, Lisa Micklesfield and Fredrik Karpe** for their guidance, supervision and investment in my academic growth and development. Your pearls of wisdom have been invaluable to me. **Prof Julia Goedecke**, thank you for your understanding and patience throughout this journey and for always reminding me that it is attainable. **Prof. Lisa Micklesfield**, thank you for your consistent support and redirecting me when I lost focus. To **Prof. Fredrik Karpe**, thank you for your motivation, commitment, and dedication towards this journey. You have all deeply inspired me and it is a great privilege to work under your guidance.

This study was funded by **GlaxoSmithKline, South African. Medical Research Council and National Research Fund.** Without their support, all of this would have been but a dream.

I am grateful to the **MRC/Wits Developmental Pathways for Health Research Unit** for hosting me throughout my PhD. I extend my thanks to **Clement Nyuki Kufe, Tinashe Chikowore, Nomses Baloyi, Siphumele Sekwati, Bonisiwe Mlambo, Dr Mamosilo Lichaba, Melikhaya Soboyisi, Tshifhiwa Rachikombo** and **Onke Godongwna** for the valuable contributions made towards data collection for my PhD project and all the moments in-between. I cannot think of a better team to have worked with.

I am thankful to the **Oxford Centre for Diabetes, Endocrinology and Metabolism** for hosting me during data collection and to all the staff (**Prof. Fredrik Karpe, Amy Barret, Dr. Marijana Todorcevic, Prof. Anne Clarke, Dr Senthil Vasan, Dr Katherine Pinnick and Dr Matt Neville**) for welcoming me to their lab and creating wonderful memories with. I will cherish these memories forever and I hope to one day go punting again ☺.

I would also like to thank the **Biomedical Research and Innovation Platform** for allowing me to use their facilities to process samples. I thank **Dr Carmen Pfeiffer** for her time and patience.

Finally, I would like to thank my family (**Mama, Papa, Tumišang le Tsedile**) for their love, kindness and support. Without whom, I wouldn't have achieved anywhere near as much as I have and who deserve this success as much as I do. Thank you to my close friends who have shared this journey with me. Ke leboga go menagane.

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LIST OF ABBREVIATIONS

ADIPOQ:	Adiponectin
AIRg:	Acute insulin response to glucose
AUC:	area under curve
BF:	Body Fat
BF%:	Body Fat %
BMI:	Body mass index
CD11c:	Integrin alpha X
CD206:	Mannose receptor 1
CRP:	C-reactive protein;
DI:	Disposition Index (FSIGT)= AIRg x SI;
DI_O:	Oral Disposition Index=IGI x Matsuda Index
E_2:	Oestrogen
ERß:	Oestrogen receptor beta
Erα:	Oestrogen receptor alpha
FMI:	Fat Mass Index
FSH:	Follicle Stimulating Hormone
GRα:	Glucocorticoid receptor alpha
HbA1c:	Glycated haemoglobin; Hb, haemoglobin
HC:	Hip circumference
HDL:	High density lipoprotein cholesterol
HOMA-IR:	Homeostatic Model Assessment of Insulin Resistance
iAUC:	incremental area under curve
IGI:	Insulinogenic Index
IL-6:	Interleukin-6
ISI:	Insulin secretion index
LDL:	low density lipoprotein cholesterol
LEP:	Leptin
LH:	Luteinizing Hormone
LPL:	Lipoprotein Lipase
OGTT:	Oral glucose tolerance test
OPLS:	Orthogonal Partial Least Squares
PCA:	Principal Component Analysis

PPARγ:	Peroxisome proliferator-activated receptor gamma
SAT:	Subcutaneous Adipose Tissue
Sg:	glucose effectiveness
SHBG:	Sex Hormone Binding Globulin
S_I:	Insulin sensitivity index
T2D:	Type 2 Diabetes
tAUC:	total area under curve
TG:	triglycerides
VAT:	Visceral Adipose Tissue;
WC:	Waist circumference
WHR:	Waist-to-Hip ratio;

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PREVIEW OF THESIS STRUCTURE

This thesis is made up of six chapters, followed by references and appendices.

Chapter one provides an introduction and an overview of the literature pertinent to the study. This chapter provides a background on the risk of T2D in pre- and post-menopausal women with and without HIV, highlighting the results and knowledge gaps arising from related studies focused on African women. This chapter also provides rationale for the study and concludes with an outline of the aim and objectives.

Chapter two details the material and methods used in this study, and the training and experience I gained during my PhD.

The results of the study that I have completed for my PhD are presented in chapters 3, 4 and 5 and include an introduction, results and discussion for each of the chapters. **Chapter three** compares different methodologies of body fat distribution as well as different methodologies of insulin sensitivity and response. **Chapter four** then examines how body fat distribution and OGTT and FSIGT- derived measures of glycaemia, insulin sensitivity and secretion and beta-cell function differ by menopause and HIV status. **Chapter five**, investigates differences in subcutaneous adipose tissue gene expression between menopause and HIV groups and how they associated with insulin sensitivity in pre- and post-menopausal women with and without HIV.

Chapter six gives a summary of the major findings of the study and highlights the potential applications of the study findings and key conclusions of the study. The study limitations are presented, and future work is proposed.

Chapeter 1: Introduction and review of current literature

1.1 Introduction

The prevalence of type 2 diabetes mellitus has increased in recent decades in developed and developing countries, and is projected to increase further, particularly in sub-Saharan Africa [1]. The factors involved in the development of diabetes mellitus are complex and multifactorial. There is evidence that the development of diabetes is the result of an interaction between environmental and genetic factors, with obesity featuring prominently in the development of type 2 diabetes (T2D) [2]. The prevalence of T2D varies among different ancestry groups, with people from Black African, African Caribbean and South Asian backgrounds at a higher risk of developing T2D compared to White Europeans [3].

T2D is characterized by elevated fasting or postprandial glucose, resulting from either reduced insulin action (insulin resistance) and/or reduced insulin secretion as a result of pancreatic β-cell dysfunction. Insulin resistance refers to a decrease in the response (insulin sensitivity) of target tissues (muscles, liver and adipose tissue) to insulin, and is a risk factor for T2D [4]. Although the exact mechanism(s) underlying the development of T2D are complex and not fully elucidated, the widely accepted paradigm is that with insulin resistance, the pancreatic β-cells secrete more insulin to compensate for a reduced action in metabolic tissues [5]. Finally, an ensuing failure of the pancreatic β-cells to compensate for the reduced level of insulin sensitivity (ability of insulin to promote peripheral glucose disposal), leads to hyperglycaemia, leading to the development of T2D [5,6].

A study in premenopausal SA women have shown that the determinants of insulin resistance differ between ethnicities [7]. However, there is a dearth of studies on post-menopausal women who are at greater risk of developing T2D than their pre-menopausal counterparts [8]. There is evidence to suggest that increased T2D risk in postmenopausal women may be due to changes in body fat distribution which includes the accumulation of central fat, which is a risk factor for T2D [9–11]. One of the mechanisms by which obesity contributes to the development of T2D includes adipose tissue dysfunction [12]. Adipose tissue plays a central role in lipid and glucose metabolism and produces a large number of hormones and cytokines [13] and is intricately linked to T2D risk.

In addition to the burden of non-communicable diseases (NCD), South Africa (SA) also has an additional challenge of a high burden of infectious diseases, particularly HIV. The

distribution and availability of antiretroviral (ARV) treatment means more people will live longer with the virus. It is therefore of great importance that we understand the association between HIV infection, ARV treatment and T2D risk. Considering this, Dillion et al., (2013) suggested that specific assessment and management guidelines for sub-Saharan African countries are required for managing HIV positive individuals presenting with cardio-metabolic risk factors [14]. However, information on the interaction between HIV and T2D risk in women, considering how this may be influenced by the menopausal transition, is required to inform these guidelines. Thus, understanding the key components of the pathogenesis of T2D in black pre- and postmenopausal South African women living with HIV (LWHIV) and without HIV is of particular interest, as it may provide effective prevention and management solutions.

This review will focus on black African adult women including black South African women who have the highest prevalence of obesity in SA, and previous work has suggested that the pathogenesis of type 2 diabetes is different in black Africans who present with a different phenotype. As menopause has been shown to be associated with significant T2D risk in other ethnicities, this section will also include a review of this literature together with HIV due to the ~20% prevalence of HIV in South African women and its intersection with NCDs.

1.2 Global burden of Diabetes

Diabetes has become a global public health concern in recent decades and is among the top 10 causes of death in adults, estimated to have caused 6.7 million deaths globally in 2021[15]. Increases in diabetes prevalence have been shown in almost all regions of the world, with 536.6 million adults now living with diabetes worldwide [15] .These numbers are estimated to reach 578 million by the year 2030 and 700 million by 2045, with over two-thirds of all diabetes cases occurring in low- to middle-income countries (figure 1.1) [1]. The estimated global economic burden of diabetes and its complications in 2030 are estimated to be more than $2.1 trillion, with significant morbidity [16].

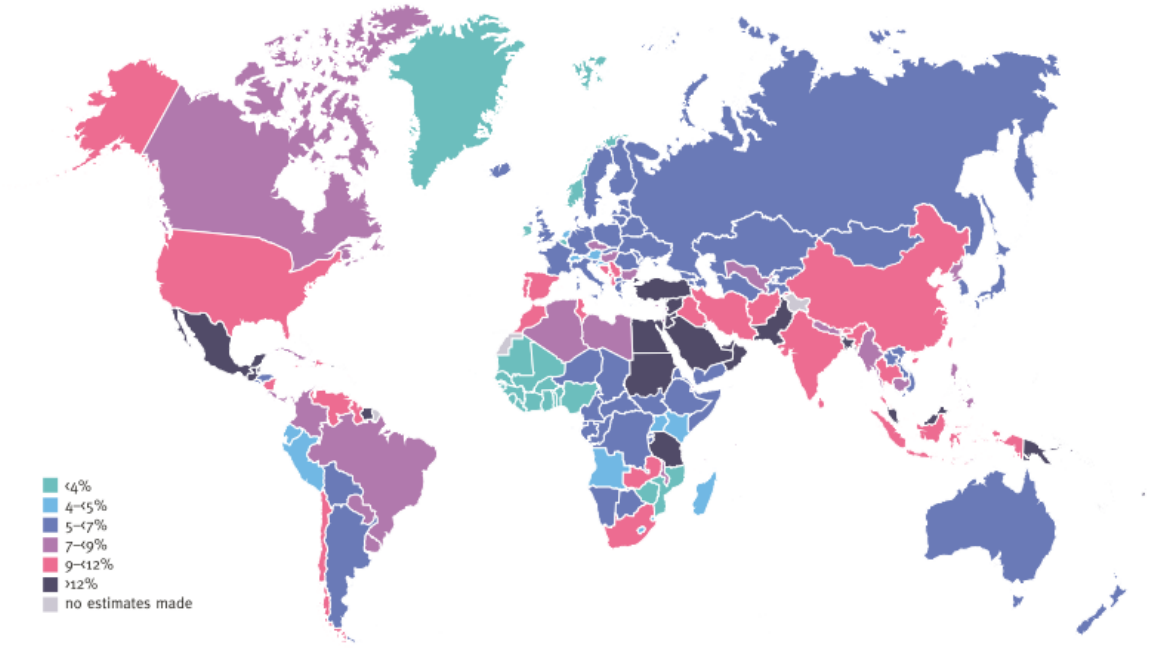


Figure 0.1: A schematic representation of the total number of adults (20-79 years old) with diabetes in 2021 [15]. Adapted from International Diabetes Federation. IDF diabetes atlas 10th edition (2021).

Sub-Saharan Africa has an estimated prevalence of diabetes of 4.5% (figure 1.2). Although it has the lowest prevalence among all IDF regions, it is also estimated that approximately 54% of adults living with diabetes in Africa remain undiagnosed [1].T2D accounts for approximately 90% of the total diabetes prevalence, with the rising trend attributed to ageing, a rapid increase in urbanisation, and increased prevalence of obesity [17,18]. An increase in diabetes prevalence will increase the number of chronic (cardiovascular diseases, kidney failure, retinopathy and neuropathy) and acute (hyperglycaemia and hypoglycaemia) complications in the general population, with profound effects on quality of life, demand on health services and economic costs [19]. The prevalence of T2D varies across Africa as different countries have different disease profiles and are at different stages of the epidemiological transition (increased longevity, changes in lifestyle and diet, and economic development) induced by urbanization [20].

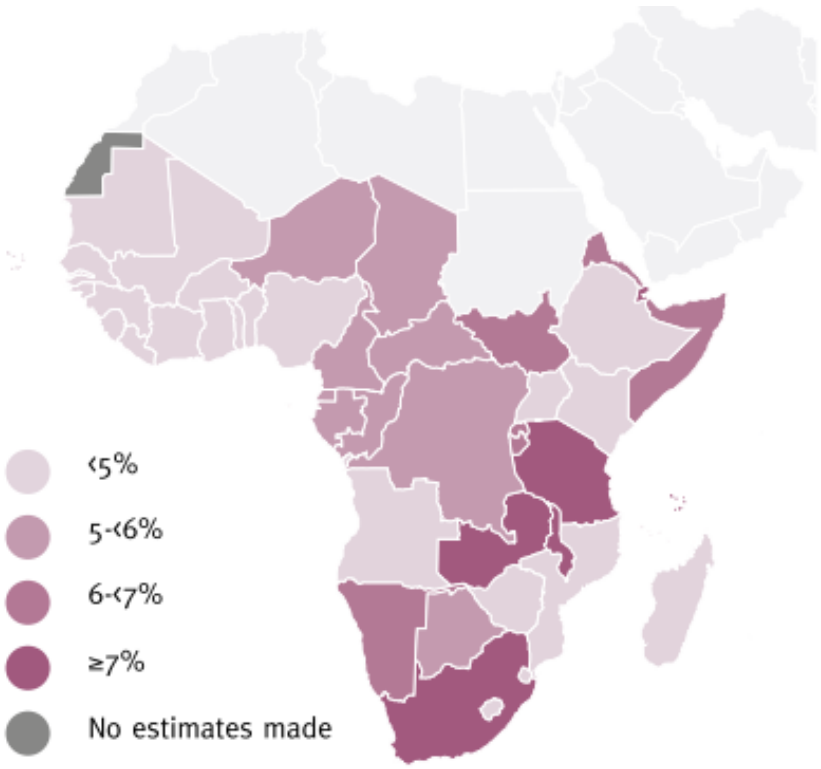


Figure 0.2: A schematic representation of age-adjusted comparative prevalence (%) of diabetes, IDF Africa region 2021 [15]. Adapted from International Diabetes Federation. IDF diabetes atlas 10th edition (2021).

1.3 The burden of Type 2 Diabetes in South Africa

The prevalence of T2D in SA is one of the highest in Sub-Saharan Africa [15,21] (figure 1.2). Within SA, T2D is the second leading cause of death in people of all ages and the leading cause of death in women of all ages [22]. The increasing prevalence of obesity is a major contributor to this growing pandemic, with national data reporting approximately 38% of men and 69% of women classified with overweight or obesity [23]. In 2000, 87% of all T2D in SA was attributed to high body mass index (BMI) [24]. Over the past 20 years the prevalence of T2D has increased in urban black African residents and it is projected to increase further in view of the high prevalence of impaired glucose tolerance and high levels of obesity in urban areas [8], as well as increasing urbanisation. Indeed, 79% of people living with T2D live in urban areas [25]. The migration from rural environments to urban areas is associated with lifestyle shifts from a relatively healthy traditional lifestyle pattern to an urban lifestyle which includes frequent consumption of fatty and high sugar foods, smoking and high alcohol availability, and low physical activity combined with high sedentary time [26].

A recent systematic review found that the prevalence of T2D was higher in South African women (16.8%) compared to men (12.4%), and this could be attributed to higher rates of obesity and insulin resistance [21]. Additionally, the prevalence of obesity in women also appears to differ by ethnicity with black SA women having a higher prevalence of obesity, (40.9%) compared to white (30.6%) SA women [23].

There are studies to suggest that the pathogenesis of T2D may differ by ethnicity [27–29] and an understanding of the pathogenesis of T2D in black Africans is essential for effective prevention and management in this under-studied but high risk population. Although this thesis focuses on black African women, the literature review uses White European women as the main comparator to highlight ethnic differences in the pathogenesis of T2D. There is limited information on black African women, therefore research relating to other populations of African ancestry, including African Americans, west Africans and Caribbean women living in Europe, will be included in this review. Although populations of African ancestry globally and living in Africa share ancestral lineage [30], it is important to acknowledge that their disease risk is also influenced by different environmental and cultural factors [31].

1.4 Pathophysiology of T2D

1.4.1 Overview of the pathophysiology of T2D

The development of T2D results from an interaction between genetic, lifestyle and environmental factors that result in insulin resistance with subsequent reduction in glucose uptake and insulin clearance, and compensatory insulin secretion and lipolysis resulting in hyperglycaemia and β-cell failure and ultimately T2D (figure 1.3). Lipolysis is a metabolic process through which triglycerides are hydrolysed into free-fatty acids and glycerol.

Obesity is associated with excessive lipid accumulation, where adipocytes expand to accumulate excess FFAs, which are then stored as triglycerides and is strongly associated with the development of T2D. According to the adipose tissue expandability and spillover hypothesis; when the storage capacity of SAT is exceeded, excess circulating lipids are then deposited into the VAT depot. In particular, central obesity, characterised by increased

visceral fat is associated with an increase in portal free fatty acid delivery to the liver resulting in lipotoxicity (lipid accumulation in ectopic tissues such as liver and pancreas), which induce hepatic insulin resistance and impair insulin secretion [32].

Insulin mediates its physiological actions through its binding to the insulin receptor located on membranes of target cells on many tissues including muscle, liver and adipose tissue. Increased plasma glucose levels stimulate the secretion of insulin from the vesicles in the β-cell in what is termed the "first phase" of glucose-mediated insulin secretion. This is followed by a "second phase" insulin secretion where newly synthesized insulin is secreted. The combination of insulin resistance, reduced glucose uptake in muscles, and increased lipolysis leads to hyperglycaemia as well as the hypersecretion of insulin by the β-cells, which ultimately results in failure to compensate for the required insulin action to maintain normoglycaemia, and the development of T2D.

However, some studies have suggested that hyperinsulinaemia may precede insulin resistance in the development of T2D [33], and therefore there is ongoing debate as to which is the initial driver to T2D: insulin sensitivity, insulin secretion or insulin clearance. Accordingly, maintaining the relationship between insulin sensitivity and insulin secretion is critical for normal glucose homeostasis. Furthermore, a hyperbolic relationship exists between insulin sensitivity (S_I) and insulin response (AIRg), such that as insulin sensitivity decreases, normal β-cells will increase their insulin response to maintain normoglycaemia (figure 1.3). The product of S_I and AIRg is known as the Disposition Index (DI) and is an estimate of β-cell function.

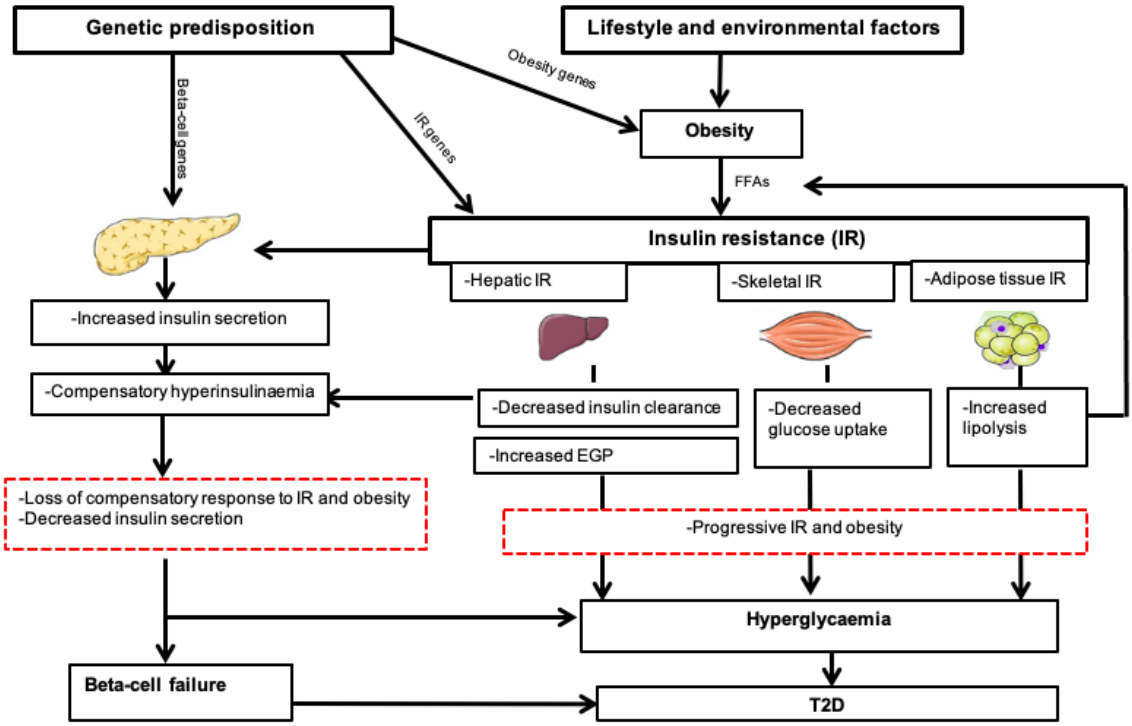


Figure 0.3: A schematic diagram describing the pathogenesis of T2D. The pathogenesis of T2D involves the interaction between genetic predisposition, lifestyle and environmental factors that result in insulin resistance with subsequent reduction in glucose uptake and insulin clearance, and compensatory insulin secretion and lipolysis resulting in hyperglycaemia and β-cell failure and ultimately T2D. Abbreviations: EGP, Endogenous glucose production; FFA, Free fatty acids; IR, Insulin resistance; T2D, Type 2 diabetes. Adapted from Mtintsilana (2021).

1.4.2 Pathophysiology of T2D in the African context

Studies from SA have shown that both normal weight and obese premenopausal black women had lower insulin sensitivity compared to their European counterparts [34,35]. Similar findings have been reported in African American women and black SA women, where insulin sensitivity was lower and acute insulin response to an intravenous glucose load was greater (AIRg), compared to their age and BMI matched White European counterparts [34–37]. Moreover, a meta-analysis concluded that women of African ancestry had higher acute insulin response to glucose compared to White European women [28]. When calculating the hyperbolic relationship between insulin sensitivity and insulin response, Kodoma et al., found that White European sub-populations clustered around the middle of the hyperbola, while African and East Asian sub-populations were located in unstable extreme points of insulin sensitivity and insulin response to maintain normal glucose concentrations [28] (Figure 1.4). This indicates that for the same level of insulin sensitivity, the insulin response is higher in black Africans.

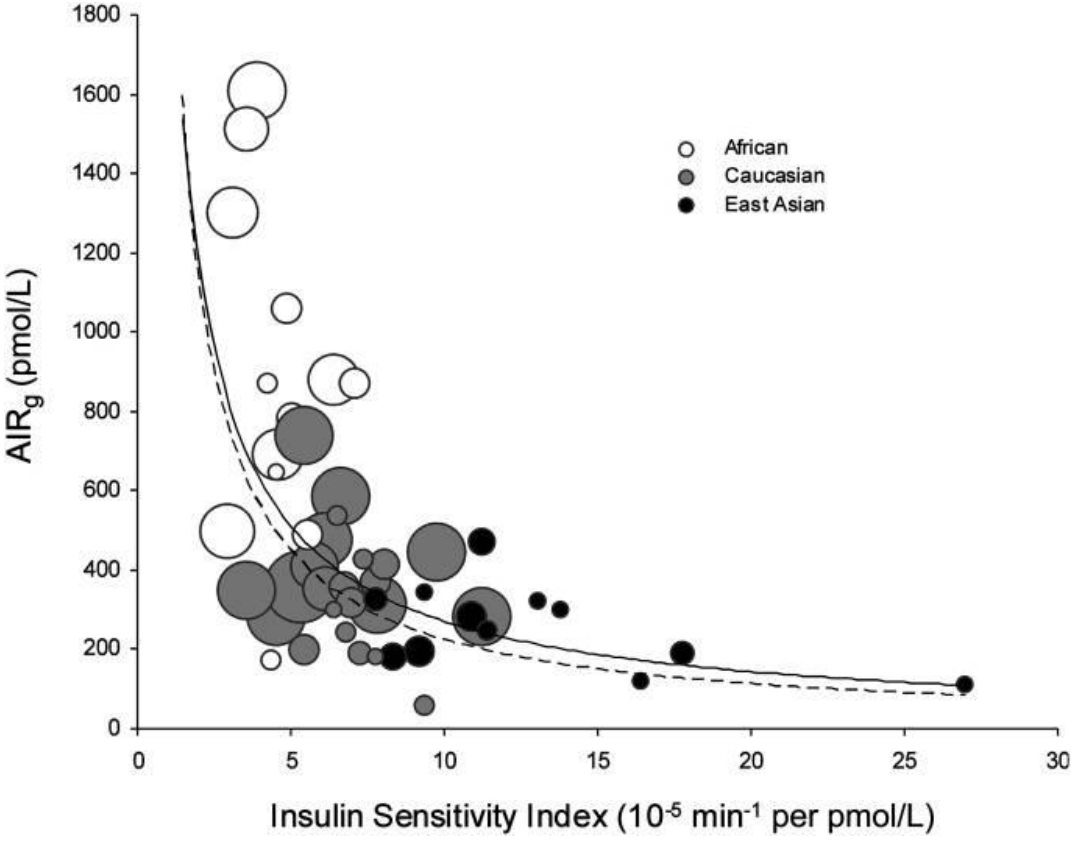


Figure 0.4: Ethnic differences in the hyperbolic relationship between insulin sensitivity and insulin response in NGT cohorts. Scatter plot of SI vs. AIRg measured in NGT (healthy) African, Caucasian, and East Asian cohorts. Each circle represents one study cohorts. Adapted from Kodama et al., 2013 [28].

Black Africans are often characterised by hypersinulinaemia [35,38]. Hyperinsulinemia is characterized by abnormally high insulin levels in the blood and results from increased insulin secretion (enhanced β-cell responsiveness) or reduced insulin clearance, or both [38]. While some studies show greater β-cell function, as estimated using the DI, in black African and African American compared to White European women without diabetes [38], other studies report no differences in DI between black African and White women [34,39]. However, it is important to note that AIRg reflects pancreatic insulin secretion as well as insulin clearance. Older studies deduced that a higher insulin response in African Americans compared to White Europeans is due to higher insulin secretion, but a subsequent study that have measured C-peptide (which is secreted in equimolar concentrations with insulin) have shown that African

American women have lower hepatic insulin clearance, which may also contribute to hyperinsulinaemia [37,40]. Notably, it is hepatic and not extrahepatic insulin clearance that is different between African American and white European Women [40].
Thus, the observed hyperinsulinaemia in black women may be attributed to a combination of both increased insulin secretion and reduced hepatic clearance [40,41]. It is however not known if hyperinsulinaemia may be a compensatory response to insulin resistance or may drive insulin resistance and may be an independent predictor of T2D in Black Africans, as it has been shown in non-African populations [42,43]. Further, it is not known in this population who present with hyperinsulinaemia if the hyperinsulinaemia is altered by menopause or HIV infection.

Ethnic differences in oral and intravenous glucose tolerance test

Hyperinsulinaemia in black Africans has been confirmed consistently with frequently sampled intravenous glucose tolerance test (FSIGT) [35,37,44,45], whereas studies using oral glucose tolerance test (OGTT) provide inconsistent evidence with another reporting no ethnic difference in insulin response between black women and White women [35], another reporting a greater insulin response in black women compared to White women [38], and one study reporting lower response in men of West African descent living in UK to White European men [46]. This has been noted in a systematic review by Ladwa et al., (2019) where they reported that the majority of studies assessing insulin response to intravenous glucose provide consistent evidence that the insulin response is greater in black women compared to White women, whereas the studies using oral glucose or meal ingestion have much more variable findings [45]. Notably, these methodological differences in quantifying insulin secretion that may result in under reporting of hyperinsulinaemia with OGTT, or over-reporting hyperinsulinaemia when using intravenous infusion, must be carefully considered, noting too that insulin concentrations during an OGTT simultaneously reflect two interdependent physiological processes (insulin secretion and insulin sensitivity) [47].

1.4.3 Techniques to quantify insulin sensitivity, response and clearance

There are several methods to quantify insulin sensitivity, response and clearance, each with their own strengths and limitations. The gold standard for measuring insulin sensitivity is the euglycaemic hyperinsulinaemic clamp (EHC) technique [48]. One of the main advantages of this method is that it directly measures whole body glucose disposal at a given level of

insulinaemia under steady-state conditions [48,49]. The EHC is widely used to measure changes in insulin sensitivity after therapeutic intervention in individual patients. Although an EHC can estimate glucose disposal at maximum insulin stimulation, the determination of hepatic glucose production requires half maximal suppression of which is achieved by using a low insulin dose clamp situation. Therefore, in order to capture both maximum glucose disposal and the hepatic glucose production a two-step clamp procedure is needed. However, such clamp procedures are often considered technically difficult, invasive, costly, and time-consuming and not feasible for large studies, especially in resource poor settings.

Bergman et al [50] demonstrated that the minimal model analysis of the frequently sampled intravenous glucose tolerance test (FSIGT) yields a measure of insulin sensitivity equivalent to the parameter derived by the EHC [50], after showing a strong correlation (r= 0.89) between the insulin sensitivity indices measured by the two methods in White men and women. The FSIGT minimal model analysis is now widely used to evaluate insulin sensitivity index (S_I), acute insulin response to glucose (AIR_g), glucose effectiveness index (S_G; the capacity of glucose to enhance its own cellular uptake) and second phase insulin secretion in one sequence. A hyperbolic relationship has been demonstrated between insulin sensitivity and the acute insulin response to glucose (AIR_g) measured during FSIGT, with the product of the two variables providing an estimate of beta cell function, DI. A low DI has been reported as an early marker of inadequate β-cell compensation and a predictor of future T2D [51].

Research has indicated FSIGT is an accurate and valid technique for the measurement of insulin sensitivity in White populations [52]. It is also a useful tool for identification of subtle, non-symptomatic metabolic changes prior to the onset of type 2 diabetes [53]. The FSIGT is less labour intensive, requires less specialised equipment than the EHC, measures first and second phase insulin response, yields information about β-cell function, and can use c-peptide measures to model both hepatic and extrahepatic insulin clearance [40]. However, unlike the EHC, the FSIGT does not measure hepatic glucose production, and it also not feasible in a large-scale epidemiological setting due to the costly and time-consuming nature of the test (32 blood samples) and as such may not be feasible in a resource poor setting.

In large epidemiological studies, the OGTT is often used to estimate insulin sensitivity and response, as well as for the categorisation of glycaemic status [54]. In a systematic review,

Otten et al, (2014) showed that the OGTT-based surrogate measures of insulin sensitivity have a moderate correlation with the EHC, and this is likely due to differences in the actual test outcome as the EHC primarily quantifies peripheral insulin sensitivity, whereas the OGTT-based indices are likely to measure both hepatic and peripheral insulin sensitivity [55]. There are several OGTT-derived estimates of insulin sensitivity (Stumvoll MCR, OGIS, Stumvoll ISI and Matsuda-index), however the strength of correlation of the different surrogate measures with the EHC is moderate at best (r values ranges between 0.65 and 0.72). Furthermore, the OGTT-derived indices of insulin sensitivity have been shown to be less likely to detect differences between different ethnic groups, including Africans [47]. Another study indicated that OGTT-derived measures are less accurate in measuring insulin sensitivity in subjects with subtle β-cell defects, after they found that a reduction in β-cell function resulted in pronounced overestimation of insulin sensitivity [47]. This could be because exogenous insulin is needed to determine a direct measure of insulin sensitivity [56] and the OGTT relies on endogenous insulin secretion. While the FSIGT and OGTT have been compared in an African population [35], they have not been compared between pre-and postmenopausal women and between those LWHIV and those without.

While a separate test, the hyperglyceamic clamp, is required in addition to the EHC to measure insulin secretion [48], it can be calculated from the OGTT (Insulinogenic and c-peptide Index) [55]. The insulinogenic index$_{t30\ min}$= insulin/glucose ratio$_{t30\ min}$ and ratio of the area under the curve for insulin to glucose$_{t0\text{-}30\ min}$ were closely correlated with AIR$_g$ (FSIGT-derivative) [57]. The OGTT has been argued to provide a more physiological measure of insulin sensitivity, and is influenced by gastric emptying, glucose absorption, insulin secretion and incretin hormones [47].

Directly measuring insulin clearance in humans is not possible because accessing the portal and hepatic veins is difficult. However, surrogate indices have been developed to noninvasively estimate total insulin clearance. The conversion of proinsulin in the pancreatic β-cells leads to equimolar concentrations of insulin and C-peptide. Thus, given the equimolar secretion of C-peptide and insulin, and the negligible extraction of c-peptide by the liver, it is possible to quantify the hepatic insulin clearance using the C-peptide:insulin molar ratio [58]. During EHC, insulin clearance can be determined from the molar ratio of C-peptide to insulin concentration in basal plasma specimens and at steady state [59]. During FSIGT, measures of plasma insulin and c-peptide can be used to estimate both hepatic and extrahepatic insulin

clearance and similarly, the ratio between the incremental areas under the curves (AUC) of the same peptides can be used to estimate insulin clearance during an OGTT [58,60].

1.4.4 Obesity, body fat distribution and insulin sensitivity

Traditionally, total adiposity and more importantly body fat distribution are important determinants of T2D risk. With increasing obesity, the storage capacity of SAT is exceeded and this may lead to the ectopic fat deposition in other tissues that regulates glucose homeostasis, such as the liver, skeletal muscle and pancreas [61,62]. This ectopic fat deposition has been proposed to be closely associated with reduced insulin sensitivity and integral to the development of T2D.

Insulin promotes glucose uptake in the skeletal muscle by stimulating the translocation of GLUT4 glucose transporter to the plasma membrane, thus impaired insulin signalling results in reduced glucose disposal [63]. While in the liver, insulin inhibits the expression of key gluconeogenic enzymes and thus insulin resistance in the liver leads to elevated hepatic glucose production [64]. Insulin signalling in adipose tissue results in decreased hormone sensitive lipase activity which inhibits free fatty acid efflux out of adipocytes. Thus, impaired insulin signalling can result in increased circulating free fatty acids (FFA) which can in turn result in decreased insulin sensitivity in skeletal muscle due to an increase in intracellular lipid products [65]. Moreover, the Randle cycle describes the reduction in the uptake and utilisation of glucose that occurs in muscle when fatty acid oxidation is increased[66]. According to this model, an increased oxidation of muscle fatty acids yields increased levels of intracellular acetyl-CoA and citrate, which then inhibit the activities of enzymes involved in glucose utilization, pyruvate dehydrogenase and phosphofructokinase. The lowering of pyruvate oxidation and glycolysis would then result in glucose-6-phosphate accumulation, increased intracellular glucose content and reduction in glucose uptake[66–68].

Additionally, the increase in FFA efflux stimulates resident macrophages and adipocytes, causing increase in pro-inflammatory cytokines, culminating in adipose tissue inflammation and insulin resistance [64]. The resulting efflux of FFA and increased secretion of cytokines can lead to secondary insulin resistance in other tissues. This process is illustrated in figure 1.5.

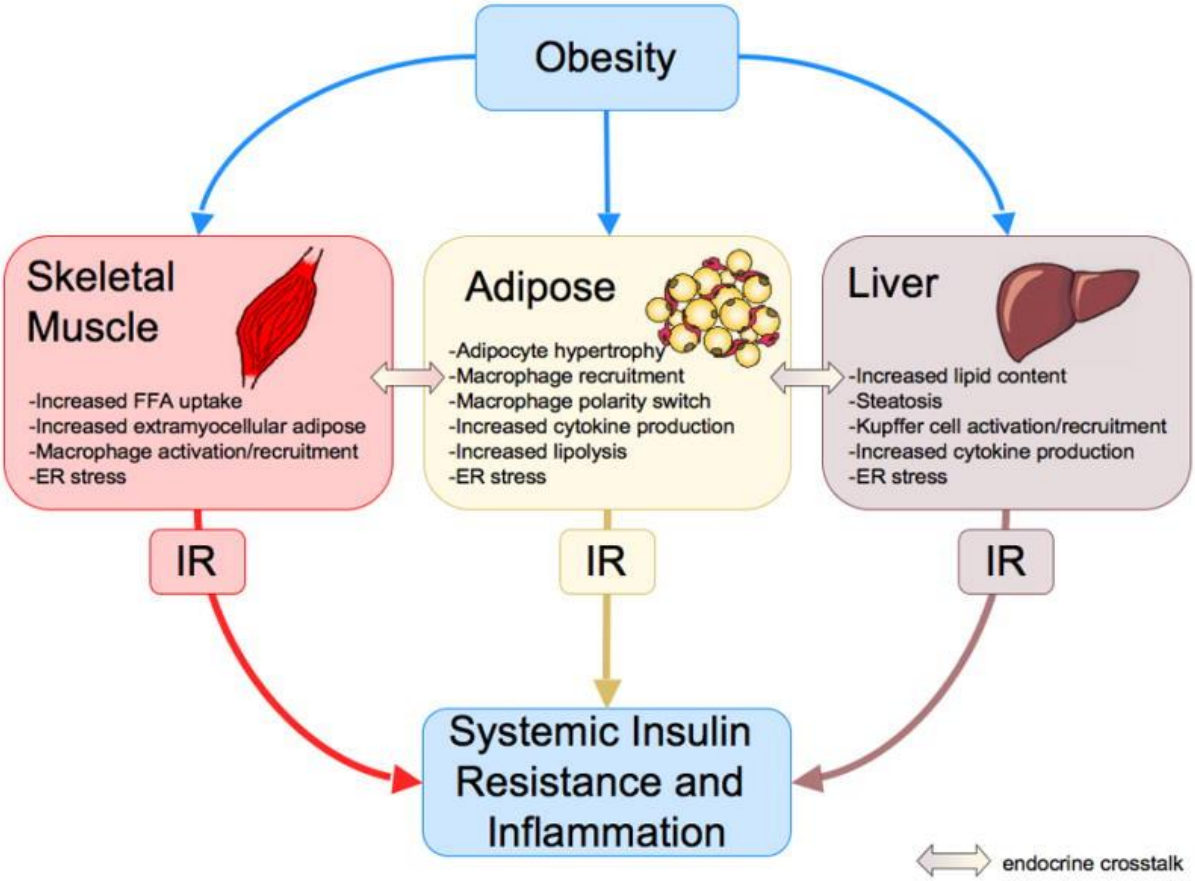


Figure 0.5: Obesity and the development of inflammation and insulin resistance. Obesity-induced changes in skeletal muscle, adipose tissue and the liver result in localized inflammation and insulin resistance (IR) through autocrine and paracrine signalling. Endocrine-mediated cross-talk between insulin target tissues contributes to insulin resistance in distant tissues. Systemic inflammation and insulin resistance are the overall effect of these changes. Adapted from de Luca and Olefsky (2008).

Furthermore, ethnicity appears to be an important factor in determining body fat distribution, as African American and black SA women have more gluteo-femoral fat, and relatively less VAT compared to White women despite similar BMI and waist circumference measurements [69–71]. However, despite having less VAT, black SA women and African American women have been shown to be more insulin resistant than their White counterparts, even when matched for age and BMI [34,35,72]. A study in SA has shown that VAT is more closely associated with insulin sensitivity in White women compared to black women [7], while abdominal SAT is more closely associated with insulin sensitivity in black women from SA and the US compared to white women [7,72], which may explain, in part, why black women are more insulin resistant that their BMI-matched White counterparts despite having less VAT [73]. A small study in SA has shown that gluteal SAT was negatively correlated with insulin sensitivity in black women, but not in White women [74]. This study found that obese black

SA women had reduced gluteal SAT expression of adipogenic and lipogenic genes compared with White women, which were associated with reduced insulin sensitivity. Nonetheless, a longitudinal study has shown that higher central fat mass, particularly VAT, and lower leg fat mass predicted T2D risk in middle-aged black SA women 13 years later [75] and that age was associated with increase in central fat and relative decrease in gynoid fat, which were associated with reduced insulin sensitivity [76].

1.4.5 Techniques to quantify body fat distribution

Body fat distribution is an important determinant of T2D risk [77]. Previous studies have shown that anthropometric measures such as waist circumference (WC) and the waist-to-hip ratio (WHR) are stronger predictors for T2D than overall adiposity [78]. Anthropometric indices remain the most used measurements of adiposity in epidemiologic studies because of their simplicity. However, these indices do not directly measure the amount of adipose tissue and cannot distinguish between fat mass and lean mass [79]. Moreover, the WC and WHR measures reflect abdominal adiposity, and cannot distinguish visceral fat from abdominal subcutaneous adipose tissue (SAT).

Studies that have directly measured adipose tissue depots by magnetic resonance imaging (MRI) or computed tomography (CT) have consistently reported that visceral adipose tissue (VAT) was associated with insulin resistance and T2D [80,81]. These gold standard techniques are not always available, especially in resource poor settings such as in SA. In comparison with the CT and MRI, the dual-energy x-ray absorptiometry (DXA) provides a more practical approach to directly measuring adiposity [82]. DXA has often been used to measure whole body and regional distribution of fat and lean tissue accurately and precisely, and is readily available, relatively inexpensive, and has minimal radiation exposure (0.04 to 0.86 mrem). Studies have shown strong correlations between DXA and CT measures of adiposity [83,84], indicating that DXA can serve as a reference method for adiposity measurement in epidemiologic studies. Moreover, studies have shown strong correlations between body composition parameters obtained by DXA and those obtained by CT or MRI in adults of normal weight [85]. However, with DXA, the scanning bed or stretcher has an upper weight limit and the whole-body field-of-view cannot accommodate very large persons. DXA estimates of fat mass are influenced by ‘trunk thickness’ with the error increasing as the individual’s trunk thickness increases [86].

1.5 Menopause

Peer et al., reported that the prevalence of T2D doubled in urban black South African women from 10.1 to 21.5% between the age range of 24-44 years and 45-54 years [8]. This period coincides with the menopausal transition in women [87]. Menopause is the permanent cessation of menstrual cycles and reproductive function, and the transition from pre-menopause to post-menopause is characterised by significant endocrine changes, including the reduction in oestrogen (E_2) and progesterone, and an associated increase in follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Figure 1.6). The menopause transition can have long term effects on overall health, including effects on bone health, lipids, loss of lean body mass and imbalance in energy metabolism, and is associated with increased fat mass [88–90]. The reduction in E_2, in combination with changes in lifestyle factors such as an increase in sedentary behaviour, are considered to be the main drivers of these health effects [91]. In addition to increasing body fat, the changes in sex hormones, particularly the decrease in E_2 has both direct (endocrine) and indirect (body composition) effects which are associated with a significant reduction in insulin sensitivity, a major risk factor for T2D [92].

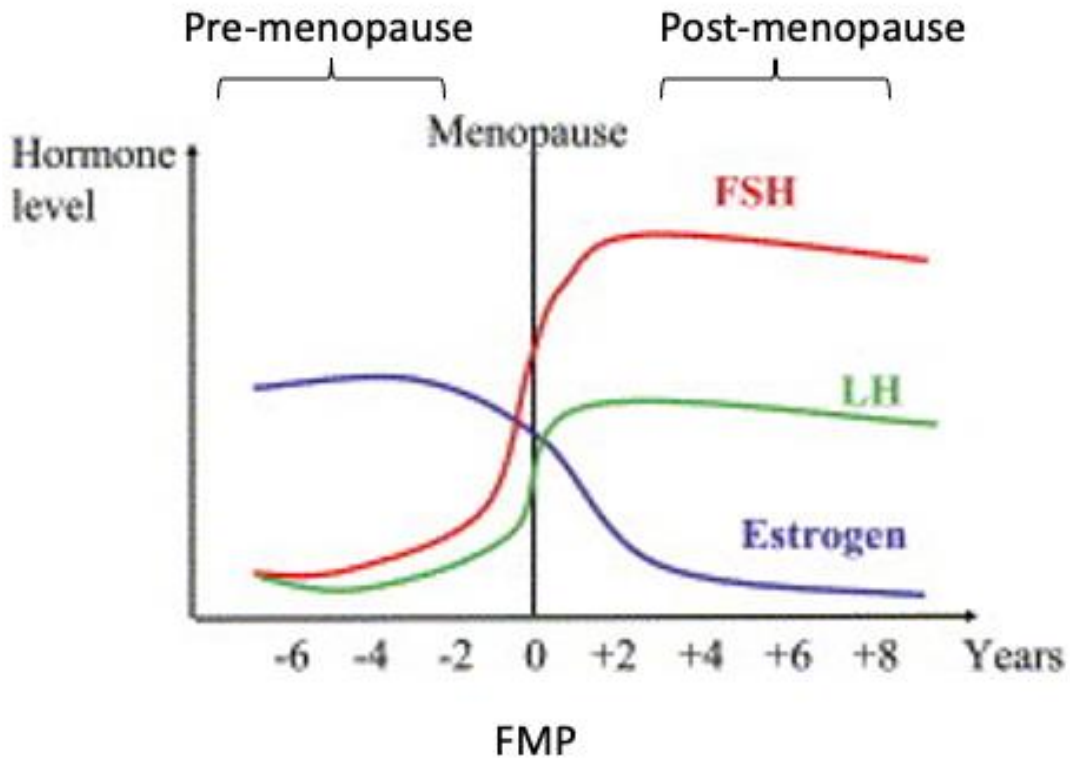


Figure 0.6: Schematic of follicle-stimulating hormone (FSH), luteinizing hormone (LH) and oestrogen (E2) changes around the final menstrual period (FMP). Adapted from Baron 2006 [93]. Only the hormones that were measured and analysed in this thesis were included in the figure.

1.5.1 Menopause and Body composition

Evidence from a recent meta-analysis reported that the increase in fat mass in postmenopausal women was predominantly attributable to increasing age, whereas changes in the body fat distribution were attributable to menopause-associated hormonal changes [94]. This supports the notion of a redistribution of fat from the gluteo-femoral (peripheral) region to the abdominal (central) region, with a consequent increase in abdominal fat accumulation in post-menopausal women [95–97]. The causal association with oestrogen deficiency is supported by studies that demonstrate that HRT in postmenopausal women reversed the accumulation of central fat, as indicated by reduced waist circumference [98], lower waist-to-hip ratio [99], reduced trunk fat mass (FM) [100], and reduced android fat [101]. Indeed, Papadakis et al., also showed a reduction in VAT, BMI, and android FM among White European postmenopausal women on HRT [102]. Thus, oestrogen is thought to regulate fat distribution and this may contribute to improved metabolic profile in women receiving HRT [98],

although the precise mechanism by which oestrogen acts on human adipose tissue function is not well understood. However, it is known that oestrogen signalling is predominantly mediated via the two nuclear oestrogen receptors α and β (ERα and ERβ), both of which are present in human adipose tissue. McInnes et al.(2013) showed that ERβ, but not ERα was higher in the abdominal SAT of post- compared to pre-menopausal women, and that this was associated with up-regulation of 11HSD1 activity [103], an enzyme that converts cortisone to cortisol, and is associated with increased VAT accumulation and insulin resistance in transgenic mice and in humans [104].

The majority of studies characterising body fat and fat distribution following menopause have been conducted in White European women, with few data in Africans. Similar to studies done in White Europeans, a Ghanaian cross-sectional study showed that postmenopausal women had greater waist-to-hip ratio (WHR) compared to premenopausal women, and this was accompanied by a greater prevalence for metabolic syndrome in postmenopausal women [105]. However, this study used conventional anthropometric measures and did not provide accurate measures of the differences in regional body fat distribution between pre- and postmenopausal women. A cross-sectional study in SA, showed that in women of mixed-ancestry, post-menopausal women had greater %FM, waist and visceral adipose tissue (VAT), and less gynoid %FM than pre-menopausal women [106]. To our knowledge, only one cross-sectional study has examined total body fat and body fat distribution in black African women at different stages of the menopause transition [107]. This study showed premenopausal women had lower abdominal SAT. measured using ultrasound, than postmenopausal women, but no other differences in body fat distribution. Although these few studies in Africa have shown differences in body fat distribution between menopausal groups, none have explored differences in T2D risk.

1.5.2 Menopause and T2D risk

Indeed, a study in black SA population showed that with increasing age, there is a decrease in insulin response, but no changes in insulin sensitivity [108]. However, it is not known if these changes correspond with the menopausal transition. Studies are required to determine if menopause is associated with insulin sensitivity and response. Although some studies show that the risk of developing T2D is significantly greater in postmenopausal women than premenopausal women [109–112], others have found no association between postmenopausal

status and diabetes risk [113,114]. Notably, it is difficult to separate menopause from aging, and this may explain the discrepancies between studies.

Post-menopausal women have been shown to have lower insulin sensitivity than their BMI-matched premenopausal counterparts [112]. Experimental studies suggest that lower E_2 concentrations, as well as lower oestrogen receptor-α (ERα) activity, can cause insulin resistance in peripheral tissues in postmenopausal women [115]. The Study of Women's Health Across the Nation (SWAN) showed that lower E_2 concentrations resulted in a 47% higher risk of T2DM during the menopausal transition [116] . In further support of the role of E_2 as a hormone with anti-diabetic properties, two large randomized trials have shown that E_2 replacement therapy in post-menopausal women reduced the incidence of T2D compared to the controls [117,118]. There is also evidence to suggest that E_2 prevents β-cell failure, and studies in post-menopausal women have shown an improvement in glucose-stimulated insulin secretion with E_2 replacement therapy [119].

The majority of studies on T2D risk in black African women from SA and USA are cross-sectional and are mostly conducted in pre-menopausal women, and yet those in post-menopausal stage, whom are at highest risk for T2D, are understudied. Moreover, a large proportion of the population is LWHIV and will be transitioning into post-menopause as they live longer on ART.

1.6 HIV in South Africa

SA has the highest number of people living with HIV in the world, with 7.7 million people living with HIV [120]. HIV/AIDS was the fifth leading cause of death in South Africa in 2017, with tuberculosis being the leading cause of death followed by diabetes mellitus, cerebrovascular disease and other forms of heart diseases [22]. Women are disproportionately more affected than men, with an estimated 4.8 million women compared to 2.5 million men LWHIV in South Africa in 2019 [121]. This trend contrasts global HIV infection trends which indicate that the prevalence of HIV is highest among men who have sex with men, suggesting that the demographics of people most affected by HIV in SA are different [120]. The factors that predispose Black SA women to increased risk of HIV infection include gender and racial inequalities, as perpetuated by structural inequalities, such as the level of education, socio-economic status, and contextual factors, such as socio-cultural norms, stereotypes, and beliefs [122].

SA has the world's largest ART programme, which has undergone further expansion with the implementation of 'test and treat' guidelines. The Universal Test and Treat is a strategy which allows all HIV infected individuals to receive ART irrespective of their CD4+ T-cell count, and was further expanded to advocate for ART initiation on the day of the patients' HIV diagnosis [123]. SA began the use of a fixed drug/dose combination (FDC) of tenofovir disoproxil fumarate (TDF), emtricitabine (FTC), and efavirenz (EFV) in 2013 and further advanced to the use of Tenofovir (TDF), lamivudine (3TC) and Dolutegravir (DTG) (an integrase inhibitor) as a first line regimen for non-pregnant (or planning to conceive) patients older than 10-years and weighing more than 35-kg [124] in 2019, and were in use as a first-line treatment of HIV at the time of this study. These were later (April 2018) replaced with a recent class of antiretroviral medications termed integrase strand transfer inhibitors (INSTI); e.g., raltegravir, dolutegravir and elvitegravir).

The implementation of effective combination ART (cART) has transformed HIV infection from a life-threatening condition into a chronic disease, with about 68% of people LWHIV on treatment as of December 2018 [120]. The success of SA's ART programme is evident in the increase in national life expectancy from 56 years in 2010 to 63 years in 2018 [125]. This significant increase in life expectancy means that people LWHIV are potentially at a risk of developing non-communicable diseases associated with ageing, HIV itself, and HIV treatments that are associated with metabolic complications [126] .

1.6.1 HIV and body fat distribution

Historically, HIV/AIDS has been associated with weight loss and "wasting", however this has changed in recent decades as the prevalence of overweight and obesity are as high amongst people LWHIV as they are in the general population globally [127]. However, some studies suggest that wasting in people LWHIV is associated with a progressed disease state. Wrottesley et al., 2013, showed that black, urban SA women LWHIV had lower body weight and BMI, as well as lower fat and muscle mass than their HIV-negative counterparts, but this was associated with low CD4 counts pre-ART initiation [128]. This study further concluded that weight loss may be a symptom of severe disease in women LWHIV who are not on ART. Other studies have shown that weight gain in people LWHIV is associated with pre-ART weight, such that

low baseline CD4 cell count is correlated with excess weight gain upon initiating ART, surpassing the return-to-health phenomenon [129]. Furthermore, using data from 44 sub-Saharan countries, Coetzee et al., showed that initiation of ART was associated with a 14% increase in the prevalence of obesity [130]. Furthermore, in pooled analyses of 8 phase 3 trials, Sax et al, (2020) showed that black women LWHIV gained approximately twice as much weight as women of other races (Asian and White) following initiation of ART[131]. Although the mechanisms are not known, the results of this study mirror the disproportionately high prevalence of obesity in black women LWHIV in the United States and in Africa. Indeed, ART was associated with increased BMI, waist circumference, overweight/obesity and abdominal obesity in Nigerian adults LWHIV[132]. Certainly, older generation NNRTI and PI-based ART were associated with increased central fat and reduced peripheral fat in black SA women LWHIV [133]. It was previously thought that PIs and thymidine-based NRTIs are the cause of lipodystrophy (the redistribution of body fat from the periphery to the central adipose tissue depots) . In a cross-sectional study, Goedecke et al, showed lipodystrophy, as indicated by greater centralization of body fat and more peripheral wasting, in black South African women receiving older generation NNRTI-based ART (d4T or AZT, 3TC, and efavirenz or nevirapine) [134]. This study went on to suggests that the increase in centralization of body fat on ART may be due to an increase in visceral, rather than subcutaneous adipose tissue deposition.

Although INSTI-based regimens avoid the use of medications implicated in lipodystrophy [135], and are highly efficacious for viral suppression, they appear to cause excessive weight gain and treatment emergent obesity than non-INSTI-based regimens and may increase the risk of weight-related co-morbidities [136]. Indeed, in a randomised, phase III trial, Venter et. el., (2020) showed that there was higher weight gain in the groups given the dolutegravir-containing regimens than the group given the efavirenz-containing regimen, among South Africans LWHIV [137]. Moreover, recent evidence suggests that NNRTIs (rilpivirine),

INSTIs (raltegravir, elvitegarvir and dolutegravir) and PI (darunavir) are associated with fat gain to the same extent, which may partly be a “return to health” fat gain [138].

1.6.2 HIV and diabetes risk.

Data from a NCD Risk Factor Collaboration (NCD RisC) of sub-Saharan African countries show that HIV and ART use are associated with increased prevalence of diabetes and obesity, and conclude that this may be due to the HIV infection itself, the side effects of receiving ART, and/or the development of age-related diabetes in an ageing HIV-infected population [130]. HIV infection itself may contribute to metabolic changes both directly through HIV immune activation and inflammation, and indirectly through immunodeficiency [139–141]. Accordingly, abnormalities in glucose metabolism are common in people LWHIV [142]. Traditional risk factors such as obesity may also play a role in the development of T2D in those LWHIV.

Additionally, cART associated weight gain has also been demonstrated as a risk factor for incident diabetes in people LWHIV, and this may be due to direct effects of medications by promoting hyperglycaemia via insulin resistance or decreased insulin secretion, an effect primarily observed for protease inhibitors (PIs) [143]. Certain PIs, such as indinavir (IDV), lopinavir, and ritonavir, have been shown to reversibly induce insulin resistance [144] and this is postulated to be due to the inhibition of glucose translocation through GLUT4 [145]. Furthermore, the NNRTIs, zidovudine and stavudine, have been shown to have direct and indirect effects on glucose metabolism through reduced insulin sensitivity which has been shown in male patients in the absence of changes in fat distribution [145]. Furthermore, in a cohort of 56,298 South African men and women LWHIV, NNRTI-based efavirenz was found to be significantly associated with a higher incidence of diabetes [146]. There are several probable mechanisms by which efavirenz mediates insulin resistance; including mitochondrial toxicity which may causes impaired adipogenesis, increased lipolysis, and release of free fatty acids and inflammatory cytokines resulting in insulin resistance and T2D [147]. Although, little is known about the effect of the newer INSTI-based regimes on diabetes risk, as these regimens have shown to increase weight more significantly than NNRTIs we hypothesize that they will also be associated with greater diabetes risk. However, this remains to be established.

1.7 Adipose tissue biology

Adipose tissue accounts for between 20 and 30% of the total body weight in people of normal body weight and up to 50% in people with obesity [148]. Most adipose tissue depots are comprised of white fat which is responsible for energy storage. It is well established that adipose tissue is involved in the regulation of glucose and lipid metabolism, energy homeostasis and inflammation [149–151]. Adipose tissue also functions as an endocrine organ, that can secrete adipokines (e.g leptin and adiponectin) that signal other organs and adipocytokines, which are inflammatory cytokines (e.g Il6 and TNF) [13] . In metabolically active tissue, adipokines mediate important metabolic processes such as fatty acid oxidation, de-novo lipogenesis, gluconeogenesis, glucose uptake, insulin signalling, and energy expenditure.

The expansion of adipose tissue is marked by hypertrophy, which is an increase in adipocyte size and/or hyperplasia, which is an increase in adipocyte number due to the recruitment of new adipocytes [152]. With excess caloric intake and obesity, the adipocytes initially become hypertrophic and secrete adipokines which then results in the recruitment of additional pre-adipocytes, which later differentiate into mature adipocytes. However, fat begins to accumulate in ectopic sites such as visceral depots, the liver, skeletal muscle, and pancreatic beta cells when the capacity for adipocyte recruitment and hypertrophy is overwhelmed [149].

Furthermore, the expansion of adipose tissue during obesity is coupled with changes in inflammatory profile within the adipose tissue, which may contribute to chronic low-grade systemic inflammation that is characterized as moderately elevated levels of circulating cytokines and chemokines [149]. Additionally, the expansion of adipose tissue depots is accompanied by the infiltration of new inflammatory cells, including macrophages, which suggests that obesity can directly cause low-grade systemic inflammation, and thus contributes to insulin resistance [64,153], though the mechanisms are complex and involve multiple pathways.

1.7.1 Ethnic differences in adipose tissue biology

As described in an earlier section studies have shown ethnic differences in phenotypic body fat distribution between black African women and white European women, characterised by greater peripheral and less central fat accumulation in black compared to white South African (SA) women [34,35,154]. Black SA women have been shown to have greater ERA and reduced ERB mRNA levels in the gluteal depot of black compared to white SA women, which accounted for the ethnic differences in regional gene expression [155]. Furthermore in a study of premenopausal South African women, it has been shown that the higher SAT inflammatory gene expression in the black women correlated with the lower expression of peroxisome proliferator activated receptor γ (PPARγ) and PPARγ-responsive genes in the gluteal depot, and was associated with reduced insulin sensitivity [74]. Down-regulation of these adipogenic and lipogenic genes in the peripheral adipose tissue of obese black women may limit further accumulation of peripheral adipose tissue and favour central deposition of fat [74]. Indeed, it has been shown that the higher inflammatory SAT profile of obese black South African women was associated with down-regulation of GRα expression [156]. Lower GRα mRNA levels in black women may attenuate the immunomodulatory effects of glucocorticoids, resulting in a vicious cycle, which could protect black SA women from VAT accumulation, but increase SAT inflammation with consequent negative effects on adipogenesis and insulin sensitivity [157].
However, all of these studies were conducted in premenopausal women, and there is a lack of information on the adipose tissue biology of post-menopausal black African women, in whom diabetes risk may be greater due to differences in their adipose tissue biology.

1.7.2 Adipose tissue biology and menopause

Menopause is associated with changes in AT phenotype related to metabolic dysfunction in both SAT and VAT [158]. Indeed, Gomez-Santos et. al., showed that changes in gene expression between premenopausal and postmenopausal Spanish women with obesity may be associated with the alteration of several key biological processes in postmenopausal women, including the immune system, metabolic processes, translation, cadmium ion binding, or the regulation of gene expression [159].

Furthermore, the adipose tissues of postmenopausal and premenopausal women exhibit differential lipid metabolism [160]. Basal lipolysis rate in the gluteal adipose tissue of postmenopausal women has been reported to be 77% lower than that in perimenopausal women, who were matched for race, BMI and percentage body fat [161]. Moreover, this study showed that adipose tissue lipoprotein lipase (LPL), which catalyzes the conversion of TG into FFAs for uptake and storage by the adipocytes, and plays a major role in the accumulation and distribution of fat stores, was significantly higher in the gluteal and abdominal adipose tissues of postmenopausal women than that of perimenopausal women, suggesting that postmenopausal women may be predisposed to gain body fat [161].

However, there is currently no information on the adipose tissue biology of pre- and post-menopausal African women, and it would be interesting to study due to known ethnic differences in body fat distribution between black African women and White European women (described in section 1.4.4). There are currently no studies that have examined adipose tissue function and how it differs by menopause status in black African populations.

1.7.2 Adipose tissue biology and HIV

HIV infection and ART cause alterations to adipose tissue distribution and biology, with broad effects on cytokine and hormone expression, lipid storage, and the composition of adipose-resident immune cells [162]. Mechanisms of adipose tissue alterations in people LWHIV are complex and result from the direct effects of HIV proteins, antiretroviral agents and the immune response on adipocyte health. However, these mechanisms are not fully elucidated [148]. Whereas the effect of ART on adipose tissue has been proposed since the mid-1990s, the role of HIV itself is an area of increasing study. Many ART-naive people LWHIV have lower overall fat mass, lean mass, and can have fat alterations, including fibrosis, reduced adipogenesis, decreased production of adiponectin and leptin, and increased production of proinflammatory cytokines, affecting both SAT and VAT compartments, suggesting that HIV infection can affect adipose tissue and induce metabolic disturbance [148].

Furthermore, HIV-infected T cells and macrophages can secrete viral proteins within the adipose tissue that affect proximal adipocytes. In *in vitro* studies, viral protein Nef, has been

shown to alter adipogenesis and may contribute to the onset of insulin resistance in adipocyte [163]. Although HIV impacts adipose tissue function and metabolic profile, these changes are mainly attributable to ART. The mechanisms underlying the effects of individual drugs on adipose tissue have been investigated *ex vivo* and *in vitro*. It is well documented that ART induces mitochondria dysfunction, and in some cases, alters adipogenesis in both adipocyte stem cells and adipocytes [164].

First generation ART such as NRTIs (stavudine and zidovudine, some PIs boosted by ritonavir) and the NNRTI (efavirenz) are known to induce alterations in adipose tissue such as fibrosis, altered adipogenesis, and local immune dysfunction as observed in figure 1.7a. Furthermore, these drugs have lipotoxic effects that resulted in lipoatrophy with long-term cardiometabolic consequences including T2D [164]. Furthermore, adipocytes treated with these first generation ARTs have been shown to present with oxidative stress and insulin resistance [164]. However, new generation INSTIs (dolutegravir (DTG) and bictegravir) are less toxic but are associated with fat and weight gain, adipogenesis, fibrosis, and adipocyte hypertrophy/dysfunction, with increased insulin resistance *in vitro* (figure 1.7b). It is thus important to determine differences in adipose tissue biology between women LWHIV and those without HIV to better understand the metabolic and inflammatory dysfunctions in adipose that contribute to disease risk.

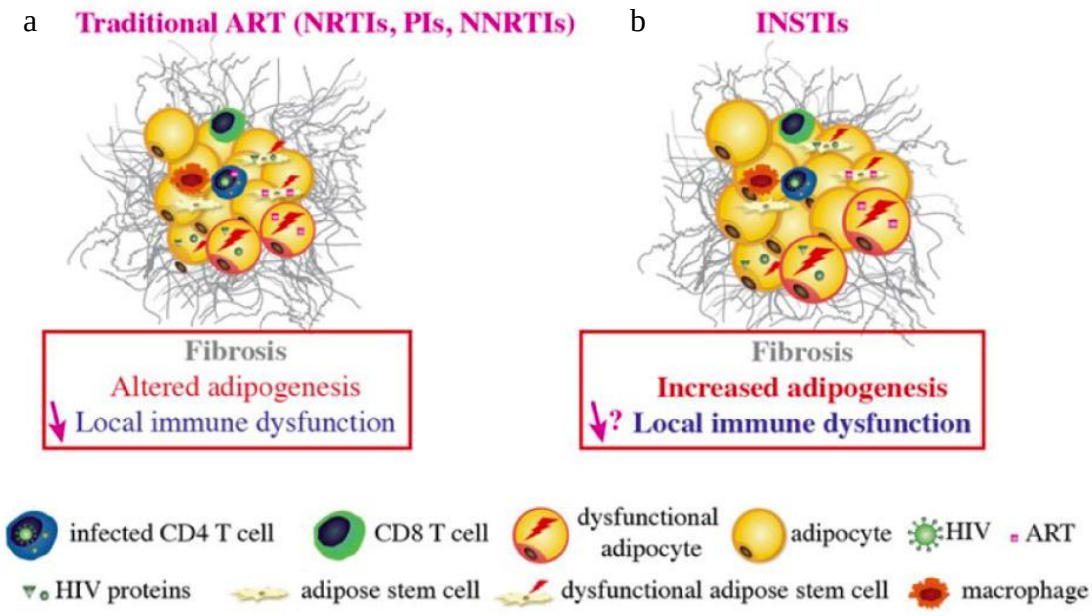


Figure 0.7: The effect of different ART regimens on adipose tissue; adapted from Bourgeois et al., (2021)[164]. a) conventional ARTs induce alterations in adipose tissue such as fibrosis, altered

adipogenesis and local immune dysfunction. b) the INSTIs also induce increased adipogenesis and collagen production, mitochondrial dysfunction, and oxidative stress in both adipocyte stem cells and adipocytes, which might promote fibrosis. Adapted from Bourgeois et. al., 2021.

1.8 Summary, gaps in literature and motivation for research

South Africa has specific health challenges, particularly high obesity and HIV rates in women. As more women continue to live longer due to the wide distribution and availability of ART, HIV infection has become a manageable chronic disease. However, HIV itself, HIV treatments and the ageing of population and menopause transition may potentially increase the risk of non-communicable diseases in people LWHIV [126]. It is therefore of great importance to understand the association between HIV infection and T2D risk.

The pathophysiology of T2D in black African women is complex and differs to that of their white European counterparts. Black African women have been shown to present with hyperinsulinaemia and are at greater risk of T2D, despite having lower VAT and greater peripheral adiposity compared to their White European counterparts, however the mechanism underlying the pathogenesis of T2D in black women remain poorly understood and requires further investigation.

Furthermore, the majority of literature on insulin resistance and T2D risk in African women has been conducted in premenopausal women, and it is not known how the menopausal transition may affect adipose tissue metabolism and insulin sensitivity. Postmenopausal women are known to have greater centralisation of fat, particularly VAT, which is one of the major determinants of insulin resistance [10]. However, the majority of studies that have examined the effects of the menopausal transition on T2D risk have been completed in populations of European descent. There is therefore a need for detailed analyses of the effect of menopause on glucose-insulin homeostasis in post-menopausal African women whose body fat distribution and diabetes risk differs to Europeans. Only one study in Africa has shown differences in body fat distribution between menopausal groups, but none have explored associations between body fat distribution and body composition and insulin sensitivity and response. One study in black SA population showed that with increasing age, there is a decrease in insulin response, but no changes in insulin sensitivity (80). However, it is not known if these changes correspond with the menopausal transition in women. Studies are required to determine whether menopausal status is associated with insulin sensitivity and response in African women.

Additionally, gold standard techniques to measure body composition and body fat distribution and insulin sensitivity and response are not always available, especially in resource poor settings such as in SA. Thus, it is important to determine the agreement between conventional anthropometric measures of body fat distribution, which is more available in our setting, and DXA which is strongly correlated with more gold standard methods like CT and MRI in this population with obesity. It is also important to determine the agreement between FSIGT-derived, which has been shown to correlate with gold standard techniques and OGTT- derived measures of insulin sensitivity and response and beta cell function. Although both OGTT and FSIGT have been used to determine insulin sensitivity and response in a African population [35], they have not been explored in the context of HIV and menopause.

Adipose tissue plays an important role in glucose metabolism as it secretes hormones that alter lipid storage, inflammation, and adipokine signalling which affect insulin sensitivity and T2D risk. Comparing the expression of adipokines, inflammatory, glucocorticoid and lipid metabolism genes between abdominal and gluteal depots in this population, and how they are associated with insulin sensitivity, and the influence of menopause and HIV, will aid in understanding the role of adipose tissue in glucose metabolism.

In conclusion, comparing the key components of the pathogenesis of T2D, insulin sensitivity, insulin secretion and clearance, between black pre- and postmenopausal South African women LWHIV and without HIV, and the role of adipose tissue biology in T2D risk is of particular interest.

1.9. Overall Aim

The aim of the study was to examine the differences in body fat distribution and body fat composition, glycaemia insulin sensitivity and response between pre- and post-menopausal women living with and without HIV; particularly to explore how abdominal and gluteal adipose tissue expression of adipokines, inflammatory, glucocorticoid and lipid metabolism genes differ by menopause and HIV and how these gene associate with insulin sensitivity. This aim was addressed in three results chapter (chapters 3-5) described below.

Chapter 3

The aim of this chapter was to compare conventional body composition and insulin sensitivity and response assessment methods to criterion measures in pre- and post-menopausal women, with and without HIV, with the specific objectives:

- To determine the correlation and agreement between conventional anthropometric and DXA measures.
- To determine the correlation and agreement between OGTT- and FSIGT-derived measures of insulin sensitivity, insulin response and beta cell function.

Chapter 4

The aim of this chapter was to investigate the effect of menopause and HIV status on glycaemia, insulin sensitivity and response in black African women with the specific objectives:

- To determine the differences in body fat distribution and sex hormones between pre- and post-menopausal women with and without HIV.
- To determine the differences in glycaemia, insulin sensitivity and response between pre-and post-menopausal women with and without HIV.

Chapter 5

The aim of this chapter was to determine the difference in adipokines, inflammatory, glucocorticoid and lipid metabolism gene expression between abdominal and gluteal SAT depots, in pre- and post-menopausal women with and without HIV, and their association with insulin sensitivity. The specific objectives were:

- To determine the association between regional body fat and insulin sensitivity in pre- and post-menopausal women LWHIV with and without HIV.
- To explore the differences in adipokines, inflammatory, glucocorticoid and lipid metabolism gene expression in abdominal and gluteal SAT depots, between pre- and post-menopausal women LWHIV with and without HIV.
- To examine the association between adipokines, inflammatory, glucocorticoid and lipid metabolism genes with insulin sensitivity in pre- and post-menopausal women LWHIV with and without HIV.
- To determine abdominal and gluteal SAT adipocyte cell size in a sub-sample of pre- and post-menopausal women LWHIV with and without HIV.

Chapter 2: Materials and methods

2.1 Study design

This cross-sectional study was conducted at the South African Medical Research Council (MRC)/University of the Witwatersrand (WITS) Developmental Pathways for Health Research Unit (DPHRU) at the Chris Hani Baragwanath Hospital in Soweto Johannesburg. A total of 527 women and 501 men were recruited between 2017 and 2018 as part of the larger study "Determinants of type 2 diabetes mellitus (T2D) risk in middle-aged black South African (SA) men and women: dissecting the role of sex hormones, inflammation and glucocorticoids" (Human Research Ethics Committee (HREC) clearance certificate number: M6160604).

Ethical considerations

Ethical approval for this study was obtained from the of the HREC of the University of the Witwatersrand, (HREC clearance certificate number:: M161103). The experiments were thus conducted in accordance with Good Clinical Practice.

There were no appreciable risks associated with the blood sampling, OGTT and FSIGT, other than those associated with routine blood sampling, including discomfort, bruising, swelling and local infection. These tests are used routinely in research to accurately determine insulin secretion and insulin sensitivity. There was also the risk of hypoglycaemia associated with FSIGT, however, blood glucose levels were monitored regularly and if the participant developed hypoglycaemia, 50% dextrose was administered and the test terminated. The risks associated with the infusion of dextrose included fluid effusion into the surrounding tissue, which may cause pain, swelling and redness. All procedures were supervised and carried out by a nursing sister and medical doctor using sterile techniques to minimise any risks of infection, no infections were reported in this study.

DXA scan was associated with minimal radiation exposure, which is less than half that of a chest x-ray (11.3 microSieverts). Adipose tissue biopsies were associated with local stinging for a few seconds after the local anaesthetic was administered. Thereafter the some participants may have experienced mild discomfort during the biopsy and after the biopsies some participants experienced bruising, which generally resolved within 2-3 days. Moreover, none of the participants were allergic to the anaesthetic.

The participants of this study are from a larger cohort of Birth-to-Twenty Caregivers and are a sub-sample of 1007 women on whom baseline data was collected between 2011 and 2014 as part of the African WITS-INDEPTH Partnerships for 125 Genomic Research (AWI-Gen) Collaborative Centre which is a Human Heredity and126 Health in Africa (H3A) Consortium study [165], which provides extensive data on reproductive health in middle age. The study was divided into 2 parts. Participants for Part 1 of the study were recruited and if they had been part of the African WITS-INDEPTH Partnerships for 125 Genomic Research (AWI-Gen) in 2011 and 2014. During part 1, participants completed an informed consent, a series of questionnaires including demographic, food frequency questionnaire (FFQ) and underwent various measurements including an HIV test, anthropometry & dual energy x-ray absorptiometry (DXA), fasting blood sampling and an oral glucose tolerance test.
The following inclusion criteria were applied to the participants collected for part 1 to select the sub-sample for Part 2, which is included in this thesis. The inclusion criteria: (1) age and menopause status: pre-menopausal women 40-45 years and post-menopausal women 55-65 years old; (2) HIV status: HIV negative women or women LWHIV; (3) BMI 20-40 kg/m^2. After applying the inclusion criteria based on HIV and menopause status, a sample of n=360 women were eligible. The following exclusion criteria were then applied: (1) T2D as determined by self-report or OGTT (2) use of hormone replacement therapy; hormonal contraceptives, oral cortisone treatment or treatment with anti-inflammatory drugs; (3) peri-menopausal (irregular periods); (4) currently pregnant or lactating; (5) current smoker; (6) known thyroid dysfunction, inflammatory, hepatic and renal diseases, earlier cardiovascular events. A total of 92 women were recruited and divided into four groups based on HIV and menopause status, and included: (1) pre-menopausal HIV-negative (PRE-; n=21); (2) pre-menopausal women LWHIV (PRE+; n=11); (3) post-menopausal HIV-negative (POST-; n=42); (4) post-menopausal women LWHIV (POST+; n=18). A schematic representation of the selection of participants is presented in figure 2.1. The time period between part 1 and part 2 was not more than 6 weeks, to allow sufficient time to analyse samples from part 1 to inform selection for part 2.

Adipose tissue biopsies were obtained from the abdominal and gluteal SAT depots of 92 participants to determine adipose tissue gene expression and adipose tissue cell size. These procedures are described in detail in section 2.11 and 2.12, respectively. Of the 92 women, only 82 participants completed the FSIGT due to time restrictions on study completion. The

FSIGT was performed to determine indices of insulin sensitivity (S_I), insulin response (AIR_g), disposition index (DI) and glucose effectiveness (S_g), as described in detail section 2.7. Although this thesis includes data from Part 1 (descriptive data and OGTT), the focus of the thesis is on part 2 (FSIGT and biopsy data) of this study.

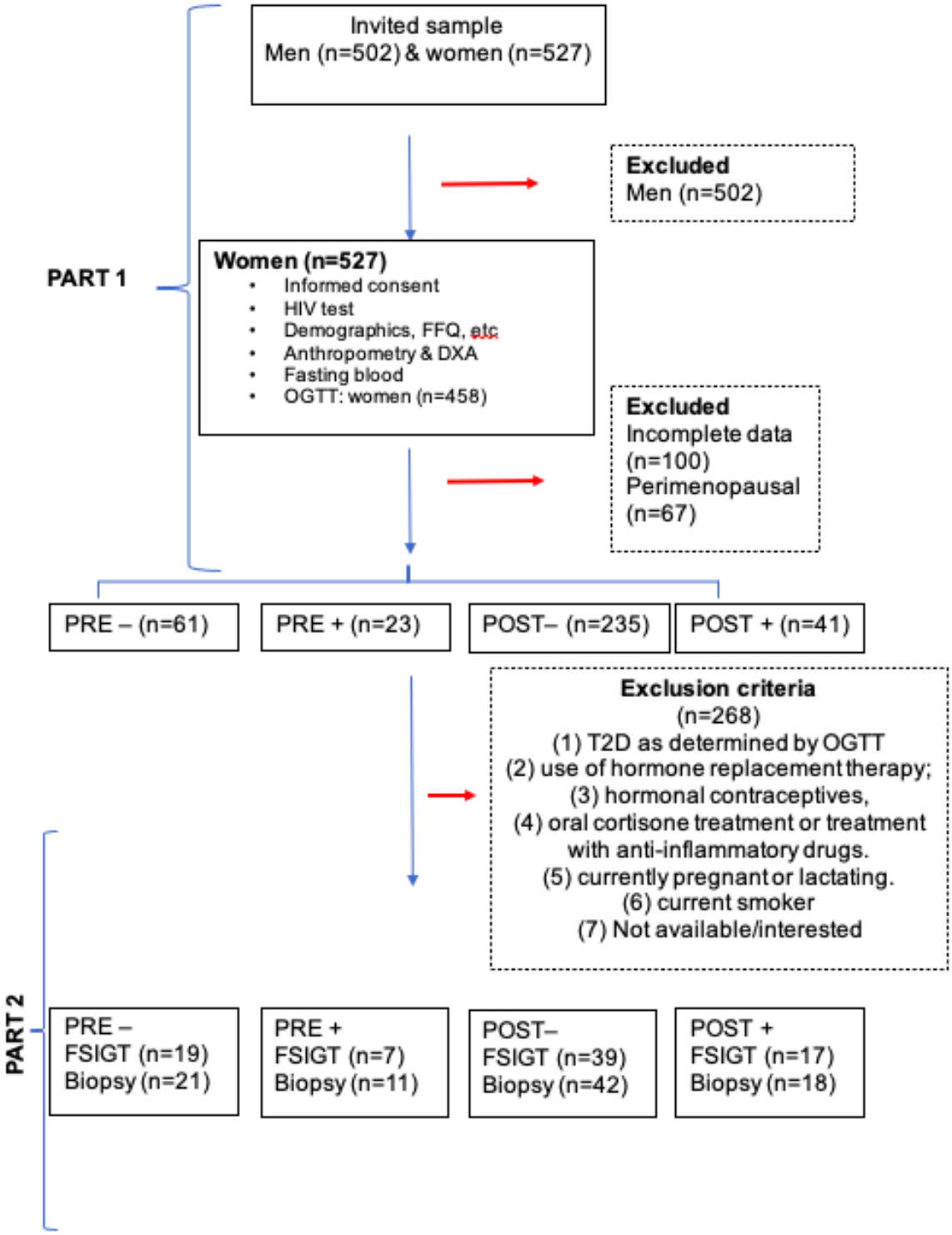


Figure 0.1: A schematic representation of the selection of participants. FFQ, Food frequency questionnaire; DXA, dual energy x-ray absorptiometry; OGTT, oral glucose tolerance test; FSIGT, frequently sampled intravenous glucose tolerance test.

The study was approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (M161103). The procedures and risks associated with the study were explained to the participants and they all signed the consent form prior to participation in the study.

2.2 Demographic and socio-economic factors

A demographic questionnaire was administered in the participants' language of choice to obtain information on marital status, employment, education level, household assets and housing density. Marital status was categorised as (i) either being married or cohabiting, or (ii) unmarried, which included being single, divorced, separated, or widowed. Participants were categorised as employed (formally or informally) or not. Education was categorised as the highest level of education achieved as follows (i) no formal education or primary education, (ii) secondary education (iii) tertiary education. Household assets were a count of the major household amenities in the household out of a possible 12 items (electricity, television, radio, motor vehicle, fridge, washing machine, telephone, video machine, microwave, MNET television channel, DSTV satellite television, cellular telephone); which was categorised as (i) low (1-4 assets), (ii) medium (5-8 assets), (iii) high (9-12 assets). This index has been described as a useful method for determining socio-economic status[166]. Housing density was defined as the number of persons per room living in the household.

2.3 Dietary Intake and Physical activity

A validated Quantitative Food Frequency Questionnaire (QFFQ) for South Africa was used to assess dietary intake[128]. Intake of local foods and convenience food products was collected for the past 7 days during the QFFQ interview, and the frequency and quantity was determined using a combination of household measures, two-dimensional life-size drawings of foods, utensils and three-dimensional food models, as previously described [167]. These were then converted to an average intake in grams per day of each food consumed and converted to nutrients using the nutrient analysis software FoodFinder3 which is based on South African food composition tables hosted by SAMRC [168]. The dietary energy and macronutrients for carbohydrates, proteins and fats were quantified in grams and %.

Physical activity and sedentary behaviour were measured over a 7-day period using an activPAL device (activPAL3c, PAL Technologies Ltd., Glasgow, UK). The activPAL device was worn on the thigh for 24 hours for 7 days to record the daily time in minutes spent sitting, standing and walking. The minimum wear time was 4 days (n=68).

2.4 Menopausal staging

Menopausal status was assessed using the self-reported date of final menstrual period [169]. Pre-menopause was defined as having a regular menstrual cycle, while post-menopause was defined as cessation of the menstrual cycle more than 12 months previously.

2.5 HIV testing and CD4 count

An HIV antibody test (Alere Determine HIV-1/2; Alere San Diego Inc, San Diego, CA), using whole blood collected from a fingerstick, was performed on participants who were not previously confirmed to be HIV positive. If the test result was positive, the participant was retained in the study and referred to a local HIV clinic for confirmatory serological testing. The CD4 count was measured for women LWHIV using flow cytometry (Beckman Coulter, Berea, CA, USA). In addition, the number of years since diagnosis as well as the number of years on HIV treatment, and the medication used, were recorded.

2.6 Body composition assessment

Basic anthropometry including weight, measured to the nearest 0.1 kg (TANITA digital scale; model: TBF-410, TANITA Corporation, US) and height, measured to the nearest 0.1 cm (wall–mounted stadiometer (Holtain, UK), were measured. Waist circumference was measured with a measuring tape placed midway between the lowest rib and iliac crest during gentle exhalation and hip circumference was measured at the largest gluteal area. Waist and hip circumference were measured in triplicate and the mean used for statistical analyses. The waist-to-hip ratio (WHR) was calculated by dividing waist circumference by hip circumference. Body mass index (BMI) was calculated as weight (kg)/height $(m)^2$ and classified according to WHO criteria; normal weight (18.5–24.9 kg/m^2), overweight (25–29.9 kg/m^2), or obese (≥30 kg/m^2)[170].

Whole-body composition was measured using dual energy x-ray absorptiometry (DXA; Hologic Discovery-W (S/N 71201), Bedford, MA, USA). Subtotal body fat (whole-body

minus head) was measured to avoid measurement artefacts that may affect the DXA reading (wigs, hair weaves and piercings etc.) because they have similar densities to that of soft tissue [171]. Regional body fat, namely trunk, leg, android, and gynoid fat mass (expressed in kg and as a percentage of subtotal fat mass, % FM) were also measured using DXA cut off lines positioned at standard anatomical positions, as defined in the software (software version 13.4.2:7) [133] (figure 2.2). The trunk included the region between the neck (line below the bottom of the jaw) and waist (line above the iliac crest) cuts with the lateral boundaries positioned to achieve separation of the upper arm and trunk at the glenoid fossa, and the inclusion of vertical lines on either side of the spine were positioned in order to exclude the spine[172]. The legs were separated by the central vertical line at the region where the oblique lines meet (from the underside of the foot to the iliac crest cut-off, along the femur and lower leg) [172]. Vertical lines extending downward from the waist cut-off were positioned to separate thigh from hands, and oblique lines were positioned to pass through the femoral neck and join the central vertical line between the legs, in order to isolate the legs. The line through the glenoid fossa to the hand formed the anatomical markers for the arm. In addition, abdominal VAT and SAT were estimated using algorithms included in the DXA software, which corresponds very well with similar regional fat measurements using computed tomography[173]. Body composition of participants exceeding the scan field limits was calculated using the arm-replacement[173].

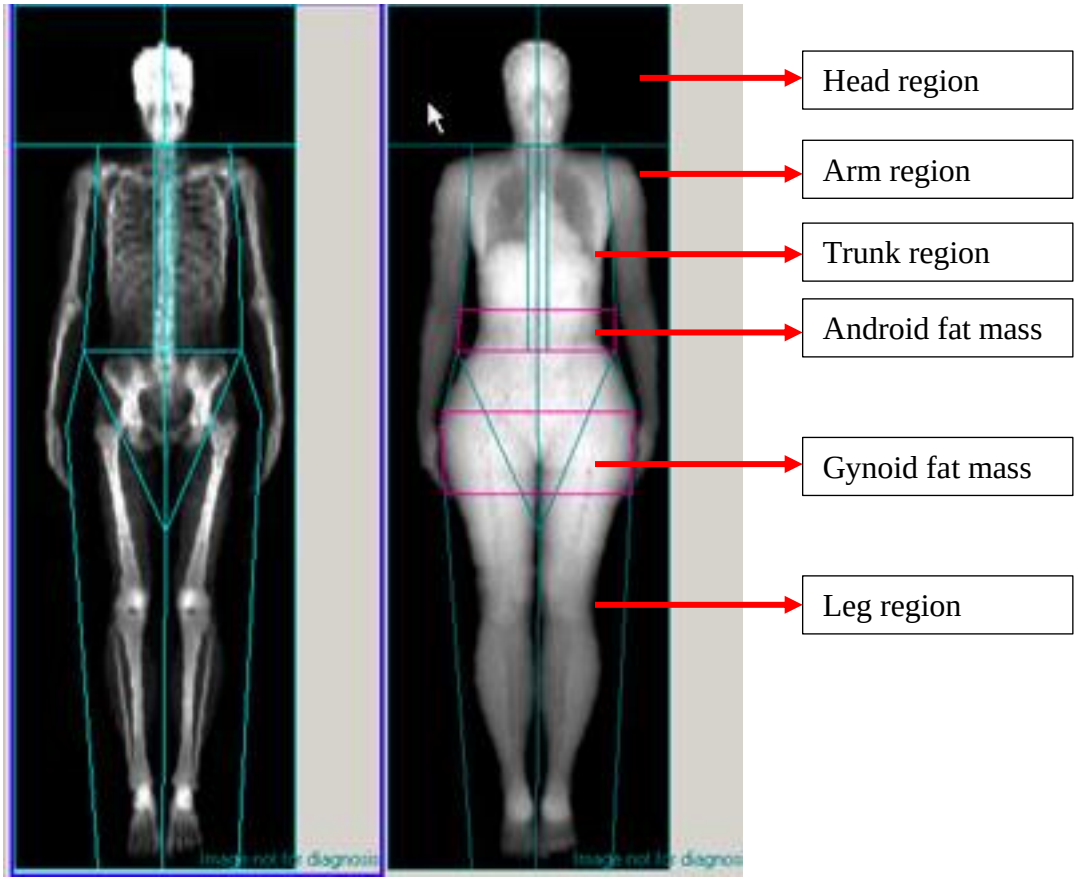


Figure 0.2: Regional body composition measurement by dual-energy X-ray absorptiometry (DXA). Regions of interest (ROI) in head, arm, trunk, leg, android and gynoid are shown in the right panel.

2.7 Fasting blood sampling and OGTT

Participants were requested to fast for 10-12 hours prior to visiting the laboratory and venous blood samples (15ml) were drawn into Serum Separator Tubes (SST), Sodium Fluoride/Potassium Oxalate tubes, Lithium Heparin tubes and EDTA tubes for the determination of plasma glucose, serum insulin, C-peptide, oestrogen (E_2), follicle stimulating hormone (FSH), luteinizing hormone (LH), sex hormone binding globulin (SHBG), lipids (total cholesterol, high density lipoprotein cholesterol (HDL-C) and triglycerides concentrations (TG)), glucocorticoids (cortisol and corticosterone), adipokines (adiponectin and leptin), lipoprotein lipase (LPL), IL-6 and C-Reactive Protein (CRP) concentrations. The fasting blood samples were taken between 7:00 AM and 8:30 AM. All participants then completed a standard oral glucose tolerance test (OGTT). During the OGTT, participants ingested 75 g of anhydrous glucose dissolved in 250 ml water within 5 min. Blood samples (5 ml) were then drawn at 30, 60, 90, and 120 min following glucose ingestion into SST tubes and Sodium Fluoride/Potassium Oxalate tubes. The samples were centrifuged at 2214g for

10 min at 4°C, the plasma was stored at −20°C for subsequent analysis of glucose concentrations, and the serum was stored at −80°C for all other analyses.

2.8 Frequently sampled intravenous glucose tolerance test

On a separate day, an insulin-modified frequently sampled intravenous glucose tolerance test (FSIGT) was completed by a medical doctor. All pre-menopausal women were tested during the follicular phase of their menstrual cycle (days 1-10). After an overnight fast (10-12 hours), cannulae were inserted into the antecubital veins of each arm. One arm was used for intravenous glucose and insulin infusions and the contralateral arm was used for blood sampling. The arm used for blood sampling was heated to arterialize venous blood into SST for serum and Sodium Fluoride/Potassium Oxalate tubes. Baseline samples were taken at −5 and −1 min before a bolus of glucose (50% dextrose; 11.4 g/m^2 body surface area) was infused intravenously over a 60 second period beginning at time 0 min. At 20 min, human insulin (0.02 unit/kg, Actrapid; Novo Nordisk) was infused over 5 min at a constant rate. Samples for determination of plasma glucose and serum insulin concentrations were drawn at 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 220, and 240 min. The samples were centrifuged at 2214g for 10 min at 4°C, the plasma was stored at −20°C for subsequent analysis of glucose concentrations, and the serum was stored at −80°C for the subsequent analysis of insulin concentrations.

2.9 Biochemical analysis

Plasma glucose, and serum total cholesterol, HDL-C and TG) were analysed on the Randox RX Daytona Chemistry Analyzer using enzymatic methods (Randox Laboratories Ltd., London, UK) (CVs were 1.3%, 1.3%, 1.8% and 1.5% respectively). The low density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald formula (LDL cholesterol (mg/dL) = total cholesterol - HDL cholesterol - (triglycerides/5) [174]. Serum insulin and C–peptide concentrations were analysed using the Immulite® 1000 Immunoassay System (Siemens Chemiluminescent Healthcare GmbH, Henkestr, Germany) (CVs were 1.4% and 2.7%, respectively). HbA1c levels were measured on whole blood samples using the D-10™ Hemoglobin Analyzer (Bio-Rad Laboratories, Inc., CA, USA) (CV was 1.8%). Serum FSH, LH and SHBG were analysed using chemiluminescent microparticle immunoassays (Architect assays, Abbott Laboratories, IL, USA) at Lancet Laboratories. Serum E_2 was analysed using mass spectrometer (Acquity® Xevo TQ-XS mass spectrometer (Waters,

Manchester, UK). C-Reactive Protein (CRP) was analysed using an immunoturbidimetric assay (Alinity hsCRP Vario assay, Abbott Laboratories, IL, USA). Cortisol and corticosterone were measured using Ultra-high Pressure Liquid Chromatography (UPLC) Mass Spectrometry (aXevo-TQS MS, Waters Corporation, Milford, United States) (CVs were 9.6% and 2.9%, respectively). Adiponectin was measured using ELISA (BMG Labtech FLUOstar Omega and analysed using the Mars software) and leptin, LPL and IL-6 proteins analysis were performed with the multiplex immunoassays (Olink Proseek Multiplex Metabolism, CVD II and CVD III, Olink, Uppsala, Sweden).

2.10 Calculations

2.10.1 Calculations of fasting measures of glucose and insulin sensitivity, response and clearance

Fasting glucose and insulin concentrations were used to calculate indices of insulin resistance (HOMA-IR), as well as insulin sensitivity (HOMA2S) and secretion (HOMA-2B) using the Homeostasis Model Assessment (HOMA2) calculator v2.2.3 [175]. Basal insulin clearance (ng/mIU) was calculated as the ratio of fasting C–peptide/fasting insulin concentrations [58].

2.10.2 Calculations from the OGTT

Participants were classified according to the WHO glucose tolerance categories[176]: normal glucose tolerance (NGT) if fasting glucose was <6.1 mmol/l and 2-h post glucose load was < 7.8 mmol/l; impaired fasting glucose (IFG) if fasting glucose ≥ 6.1 and < 7.0 mmol/L; impaired glucose tolerance (IGT) if 2-h post glucose load ≥ 7.8 mmol/l and < 11.1 mmol/l).

The integrated area under the curve (iAUC) of insulin, glucose and C-peptide, were calculated using the trapezoidal method, and the ratio of insulin and C-peptide was used as an estimate of postprandial insulin clearance [58].

Insulin sensitivity was estimated using the Matsuda Index and the composite measures of Matsuda index were used for participants with missing data points [177]:

$$\text{ISI (Matsuda)} = \frac{10\,000}{\sqrt{Glucose\ 0\text{min}\ x\ Insulin\ 0min\ x\ Glucose\ mean\ x\ Insulin\ mean}}$$

Insulin response was estimated using the insulinogenic index [73];

$$Insulinogenic\ Index\ (IGI) = \frac{Insulin\ 30min - Insulin\ 0min}{Glucose\ 30min\ - Glucose\ 0min}$$

The estimate of insulin secretion, c-peptide index was calculated as [178];

$$C - peptide\ \ index = \frac{C - peptide\ 30min\ - C - peptide\ 0min}{glucose\ 30min\ - Glucose\ 0min}$$

The oral disposition index (DI_O), which is often used as a proxy for beta-cell function was calculated using;

$$DIo = Matsuda\ Index\ \times\ Insulinogenic\ Index$$

The general consensus is that the physiological relationship between insulin sensitivity and insulin response is hyperbolic. The data was log transformed, and regression analysis was used to explore this relationship. A hyperbolic relationship was assumed if the slope of the linear relationship (β) was approximately equal to−1, and the 95% confidence interval (95% CI) excluded 0 [51]. Using the data from this study in pre and post-menopausal women living with and without HIV there was a hyperbolic relationship between ln(Matsuda Index) and ln(insulinogenic index) (β =-0.783; [95% CI -1.222 to -0.345]), (Figure 2.3), such that at low levels of insulin sensitivity, a small change in insulin sensitivity was associated with a large insulin response, and at high levels of insulin sensitivity, a small change in insulin sensitivity was associated with a small insulin response.

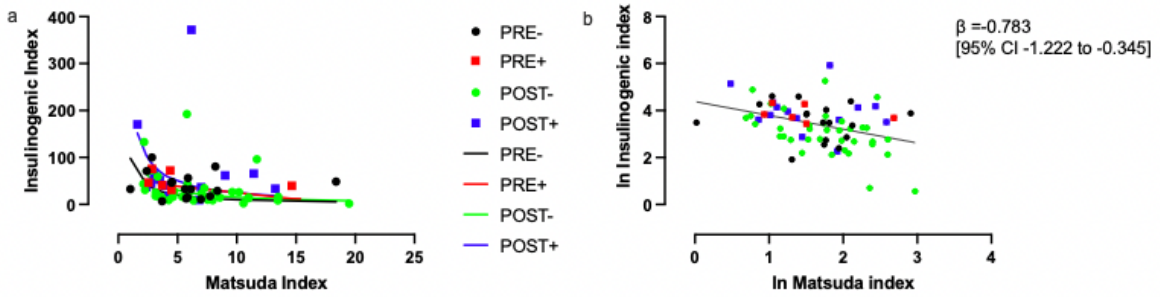


Figure 0.3: The hyperbolic relationship between OGTT derived insulin response (IGI) and insulin sensitivity (Matsuda Index) in pre and post-menopausal women living WHIV and without HIV. a) the scatter plot of Insulinogenic index and Matsuda index. b) the correlation between the log transformed insulinogenic index and Matsuda index (β =-0.783; [95% CI -1.222 to -0.345]). The relationships did not differ by groups.

2.10.3 Calculations from FSIGT

Glucose and insulin data from the FSIGT were used to calculate indices of insulin sensitivity (S_I) and glucose effectiveness (S_g), which is the ability for glucose to stimulate its own uptake and to supress its own production under the steady state, using Bergman's minimal model (MINMOD) of glucose kinetics [179]. Acute insulin response to glucose (AIR_g), was calculated as the mean incremental insulin response above basal between 2 and 10 min after the intravenous glucose bolus was started. The disposition index (DI) which is the insulin response for the level of insulin sensitivity was calculated by calculating the product of AIR_g and S_I [180]. This is illustrated by the hyperbolic relationship between ln(AIRg) and ln(S_I) (β = -0.669; [95% CI = -0.937 to -0.401]), (Figure 2.4), in pre and post-menopausal women LWHIV and without HIV.

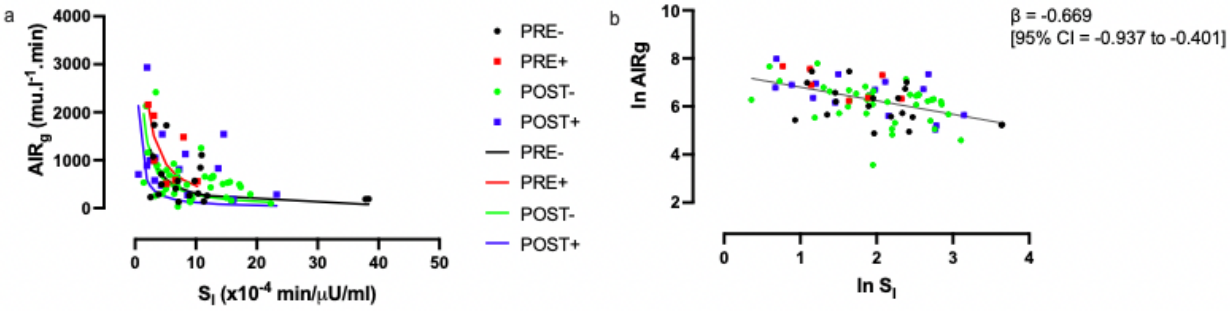


Figure 0.4: The hyperbolic relationship between FSIGT derived insulin response (AIRg) and insulin sensitivity (S_I) in pre and post-menopausal women LWHIV and without HIV. a) the scatter plot of AIRg and S_I. b) the correlation between the log transformed AIRg and S_I (β = -0.669; [95% CI = -0.937 to -0.401]). The relationships did not differ by groups.

2.11 Abdominal and gluteal adipose tissue biopsies and analysis

Adipose tissue biopsies were obtained from the abdominal and gluteal subcutaneous adipose tissue (SAT) depots after an overnight fast (10-12 hours). Abdominal SAT was taken at the level of the umbilicus (4-7 cm lateral to the umbilicus), and gluteal SAT was collected from the right upper outer quadrant of the buttock. After administration of local anaesthesia (5–10 ml of 1% lignocaine without adrenaline), a 14g biopsy gun needle was used to obtain a biopsy sample for histology. Thereafter, a 14G sterican aspiration needle attached to a 60ml syringe, was used to aspirate fat using the mini liposuction technique for subsequent RNA analysis [181]. Approximately 300 mg of fat was obtained from each depot. Samples from the gun biopsy were washed with saline and stored in 4% formalin for histology. Samples from the liposuction were washed with saline to remove the blood and snap frozen in liquid nitrogen and stored at -80 °C for subsequent RNA extraction.

Total RNA was extracted using the RNeasy Mini lipid kit (Qiagen Ltd, Germantown, MD, USA), and cDNA reverse transcribed using the Qiagen RNeasy system (Qiagen Ltd, Crawley, UK). Yield was determined by measuring the absorbance at a wavelength of 260 nm (A_{260}) using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, USA) and quality was determined using the Agilent RNA 6000 Nano and small RNA kits (Agilent Technologies) according to the manufacturer's instructions.

Real-time polymerase chain reaction (RT-PCR) was performed in triplicate using the Applied Biosystems QuantStudioTM^3 Real-Time PCR system with predesigned Taqman assays from Thermo Fisher Scientific (Warrington, UK). A standard curve was constructed for each primer probe set using a serial dilution of cDNA pooled from all samples. Adipose tissue expression of adipokines, genes involved in inflammation, adipogenesis and lipid biology and sex hormones were quantified by real-time PCR using TaqMan gene expression assays (Applied Biosystems) and normalized to the mean expression of the three endogenous control transcripts. The three (phosphoglycerate kinase 1 (PGK1), ribosomal protein lateral stalk subunit P0 (RPLP0) and low-density lipoprotein receptor-related protein 10 (LRP10)) endogenous "housekeeping" genes were selected after being identified amongst five (Ubiquitin C (UBC), Peptidylprolyl isomerase A (PPIA), PGK1, RPLP0 and LRP10) as the most stable combination of transcripts using the NormFinder algorithm (v0.953, Denmark). The Normfinder algorithm ranks the set of candidate normalization genes according to their

expression stability in a given sample set and given experimental design. A detailed description of these transcripts is available in table 2.1.

Table 0.1: A description of transcripts and the assay ID

Gene name	Gene symbol	Assay ID
Housekeeping genes		
phosphoglycerate kinase 1	PGK1	Hs99999906_m1
ribosomal protein lateral stalk subunit P0	RPLP0	Hs99999902_m1
low-density lipoprotein receptor-related protein 10	LRP10	Hs00204094_m1
Adipokines		
adiponectin	ADIPOQ	Hs00605917_m1
leptin	LEP	Hs00174877_m1
Inflammation		
Integrin, alpha X	CD11c(ITGAX)	Hs00174217_m1
mannose receptor 1	CD206 (MRC1)	Hs00267207_m1
interleukin-6	IL6	Hs00174131_m1
Lipid biology		
lipoprotein lipase	LPL	Hs00173425_m1
peroxisome proliferator-activated receptor gamma	PPAR γ	Hs01115729_m1
glucocorticoid receptor alpha	NR3C1	Hs00353740_m1
E_2 receptors		
estrogen receptor alpha	ESR1	Hs00174860_m1))
estrogen receptor beta	ESR2	Hs00230957_m1

2.12 Adipose tissue cell size

Adipose tissue biopsies were fixed in paraformaldehyde, embedded in paraffin wax, cut into 5 μm sections, and stained with hematoxylin and eosin. Sections at 3 different levels of depth were selected and viewed at ×10 magnification, and adipocyte cross-sectional area was calculated using automatic recognition and manual validation for three sections including a

minimum of 200 cells (in total) using Adobe Photoshop 5.0.1 (Adobe Systems, San Jose, CA) and Image Processing Tool Kit (Reindeer Games, Gainesville, FL) ([182]). Adipose tissue cell size was measured on a sub-sample of participants that was not different to the main cohort as shown in Table 2.2. The cell size distribution was calculated. The AUC for adipose tissue cell size was determined using Graphpad Prism version 8.4.3. (GraphPad Software, San Diego, California USA).

Table 0.2: Comparison of main cohort and cell size samples size.

Variable	Without Cell Size (n=74)	With Cell Size (n=18)	P value
Age	53 (50- 57)	49 (46- 54)	0.060
BMI (kg/m^2)	31.6 (28.1- 34.8)	32.9 (27.1- 35.9)	0.694
BF %	44.4 ± 4.8	44.5 ± 4.4	0.944
FMI(kg/m^2)	14.1 ± 3.6	13.9 ± 3.4	0.884
VAT (cm^2)	89.9 (64.8-113.5)	93.9 (69.9-120.6)	0.482
SAT (cm^2)	437.8 ± 129.1	440.6 ± 128.2	0.936

2.13 General statistics

The Shapiro–Wilk test was used to assess the distribution of continuous variables. Normally distributed data are presented as mean ± standard deviation (SD) while skewed data are presented as median and interquartile range (25^{th} to 75^{th} percentile). Normal distribution for skewed variables was approximated through log transformation or Box-Cox transformation where applicable. Significance was set as $P < 0.05$. Data were analysed using STATA (Version 13.1, Statcorp, College Station, Texas). The statistics relating to each results chapter can be found within the chapter.

2.14 My contribution

I was responsible for writing my PhD proposal, applying and obtaining ethical approval from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand.
I was responsible for obtaining participant informed consent and conducting interviews for demographic questionnaires, for both Part 1 and Part 2 of the study, as well as selecting and recruiting participants for Part 2. I was trained and set up the FSIGT and adipose tissue biopsy techniques that had not previously been done at DPHRU, and assisted the medical doctor with conducting the tests. I was responsible for processing, storing and analysing the glucose and

insulin samples from the OGTT for Part 1 (n=92) of the study and for the FSIGT, as well as computing the analysis using MINMOD (Chapter 2.10.3).

In addition, I was responsible for processing and cleaning of adipose tissue samples and storing biopsy samples in formalin and fixing in wax. I extracted RNA from adipose tissue biopsy samples at the Biomedical Research and Innovation Platform (BRIP) at the South African Medical Research Council in Cape Town. I also analysed the RNA using qPCR and performed histology analysis at the Oxford Centre for Diabetes, Endocrinology and Metabolism (OCDEM), University of Oxford under the guidance of Professor Fredrik Karpe. I was responsible for the conceptualisation, statistical analysis, interpretation of the data, and drafting and editing of this thesis.

Chapter 3: Comparison of conventional body composition and T2D risk methods to criterion methods in black African women

3.1 Introduction

Body fat distribution is an important determinant of T2D risk [77]. Previous studies have shown that anthropometric measures such as waist circumference and the waist-to-hip ratio are stronger predictors for T2D than overall adiposity [78] . Moreover, Cameron et al., have shown that considering both waist and hip circumference simultaneously identified almost 20% more people at greater risk of death compared to using waist circumference only [183]. However, these measures reflect abdominal adiposity, and cannot distinguish visceral fat from abdominal subcutaneous adipose tissue (SAT).

Studies that have directly measured adipose tissue depots by magnetic resonance imaging (MRI) or computed tomography (CT) have consistently reported that visceral adipose tissue (VAT) is strongly associated with insulin resistance and T2D [80,81]. These gold standard techniques are not always available, especially in resource poor settings such as in SA.

DXA has often been used as an objective measure of whole body and regional distribution of fat and lean tissue and has minimal radiation exposure. Moreover, studies have shown strong correlations between body composition parameters obtained by DXA and those obtained by CT or MRI in adults of normal weight [85]. Furthermore, DXA is able to predict fat mass with greater accuracy than waist circumference since waist circumference has shown weaker correlation with VAT in individuals with obesity [184]. This weak correlation is considered to be due to poor reproducibility due to large intra- and inter-rater variations [185]. Thus, it is important to determine the agreement between conventional anthropometric measures of body fat distribution, which is more available in our setting, and DXA which is strongly correlated with more gold standard methods like CT and MRI in this population with obesity.

Insulin sensitivity and response are important determinants of T2D [186]. Several quantitative methods have been developed to measure insulin sensitivity with the EHC considered the gold standard [48].

However, clamp procedures are invasive, costly and time-consuming, and thus not feasible for large studies or resource poor settings. The FSIGT provides an estimate of and insulin sensitivity that correlate with the gold standard [50] and is able to measure first and second phase insulin response. However, it is laborious and costly and may also not be feasible in resource poor settings. The oral glucose tolerance test (OGTT) is a clinically useful test for detecting glucose tolerance and can provide proxy measures of insulin that correlate with gold

standard measures [55]. Although limited by a variable endogenous insulin response, the OGTT may be more suitable in under-resourced settings like SA, and also represent a more physiological response as the glucose is ingested rather than infused as in the clamp and FSIGT. However the OGTT-derived indices of insulin sensitivity have been shown to be less likely to detect differences in insulin sensitivity between different ethnic groups, including Africans [47].

Indeed, compared to white Europeans, black Africans present with a distinctive gynoid body fat distribution and a phenotype of low insulin sensitivity and hyperinsulineamia [35,38]. Further, for the same waist circumference, black Africans have less VAT and more abdominal and gluteal SAT than white European women. While these conventional and criterion measures of body composition and insulin sensitivity and response have been extensively compared in European populations [47,54,83], there are only a few studies that have been performed in black Africans [35,71], and none have explored the association between these measures in the context of HIV and menopause.

This chapter aimed to 1) examine the correlation and the agreement between conventional and DXA-derived measures of total and regional adiposity in pre-menopausal and post-menopausal women with and without HIV, 2) examine the correlation and the agreement between OGTT and FSIGT derived indices of insulin sensitivity, response and beta cell function to determine their ability to detect differences between pre-menopausal and post-menopausal women with and without HIV who may be at risk for T2D.

3.2 Statistical analysis for chapter 3

The Shapiro–Wilk test was used to assess the distribution of continuous variables. For non-parametric data, spearman correlations were completed to determine the association between anthropometric and DXA measures of total and regional adiposity, and between OGTT- and FSIGT-derived measures of insulin sensitivity (Matsuda Index and S_I), insulin response (Insulinogenic Index and AIRg) and disposition indices (DI_O and DI). Regression analyses was done to determine if associations differed by menopause status, HIV status and interaction terms.

Bland-Altman plots were used to determine the level of agreement between anthropometric and DXA derived measures of central (waist circumference, VAT and android fat) and peripheral adiposity (hip circumference and gynoid fat) which were standardized using z-scores. The z-score is a standardised variable that has been rescaled to have a mean of zero and standard deviation of one, and this ensures that all variables of different units can contribute evenly to a scale when compared to each other. To calculate the z-score, the mean is subtracted from the value for each case, resulting in a mean of zero. Then the difference between the individual scores and the means are divided by the standard deviation, which results in a standard deviation of one. To elaborate, correlation refers to the presence of a relationship between two different variables, whereas agreements refer to the concordance between the measurements of one variable. This is important in when determining inter-rater variability or to decide whether one technique for measuring a variable can substitute another[187].

To determine the level of agreement between OGTT- and FSIGT-derived measures of insulin sensitivity, insulin response and beta cell function all measures were standardized using z-scores and the Bland-Altman plots were completed. Homoscedasticity and Heteroscedasticity were determined using *Pearson's* correlation. Homoscedasticity was assumed when the scatter of the values for the difference between the two measures was uniform across all values of the average of the two measures, suggesting that there is no proportional bias and the p value >0.05. In contrast, heteroscedasticity is assumed when the scatter of values for differences between the measures changes progressively as the average values increase or decrease, suggesting that there is proportional bias.

3.3 Results

3.3.1 Association between anthropometry and dual-energy X-ray absorptiometry derived measures of regional adiposity

The correlations between measures of regional adiposity using anthropometry and DXA are presented in Figure 3.1. Waist circumference was positively correlated with VAT (r_s =0.665; p<0.001), SAT (r_s=0.743; p<0.001) and android fat (r_s=0.834; p<0.001), while hip circumference was positively correlated with gynoid fat mass (r_s =0.929, p<0.001). The strengths of these associations did not differ by menopausal or HIV groups (p>0.05).

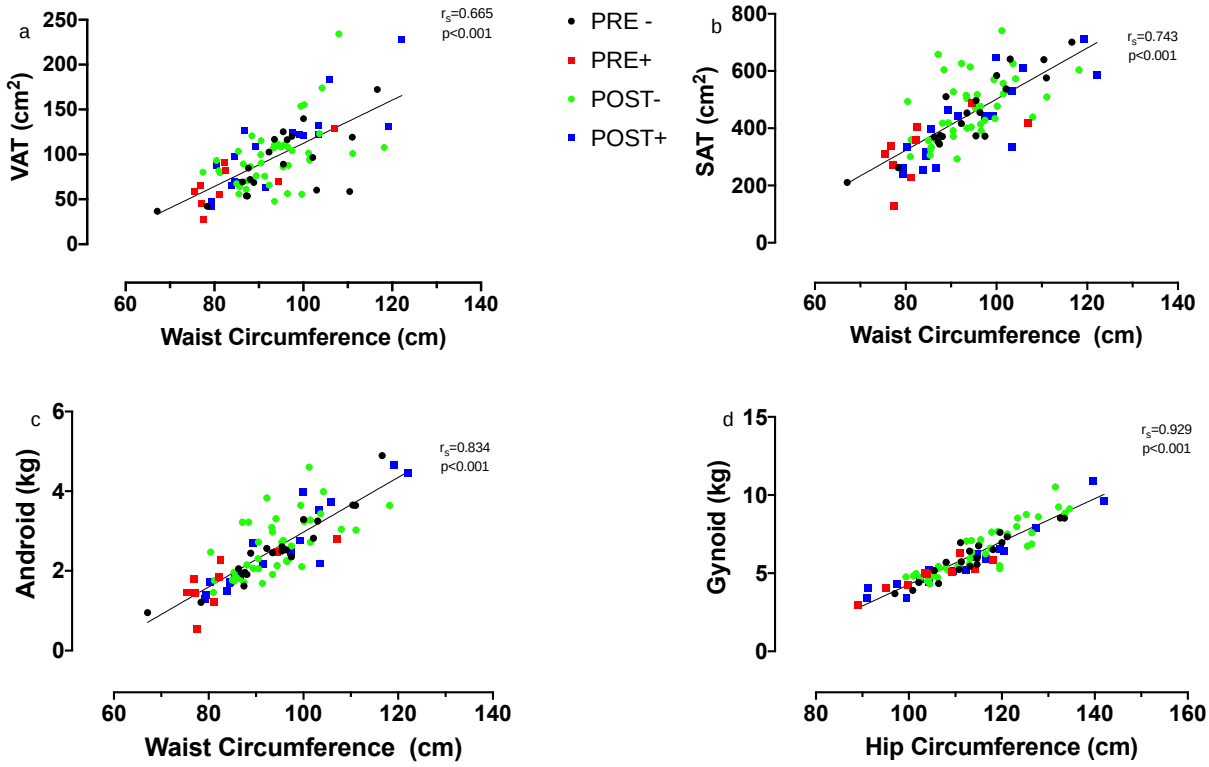


Figure 0.1: Correlations between waist circumference and VAT, SAT and android fat, and hip circumference and gynoid fat.

The Bland-Altman agreement plots (figure 3.2) and *Pearson* correlation showed that there was homoscedasticity between the waist circumference and VAT (r= -0.058, p= 0.588), SAT (r= -0.064, p= 0.549), and android fat (r= -0.078, p= 0.468) and between hip circumference and gynoid fat (r= -0.044, p= 0.680). The limits of agreement (LOA), as indicated by -2SD

for waist and VAT and SAT are far greater than that of the android and gynoid and hip. These agreements did not differ by groups (p>0.05).

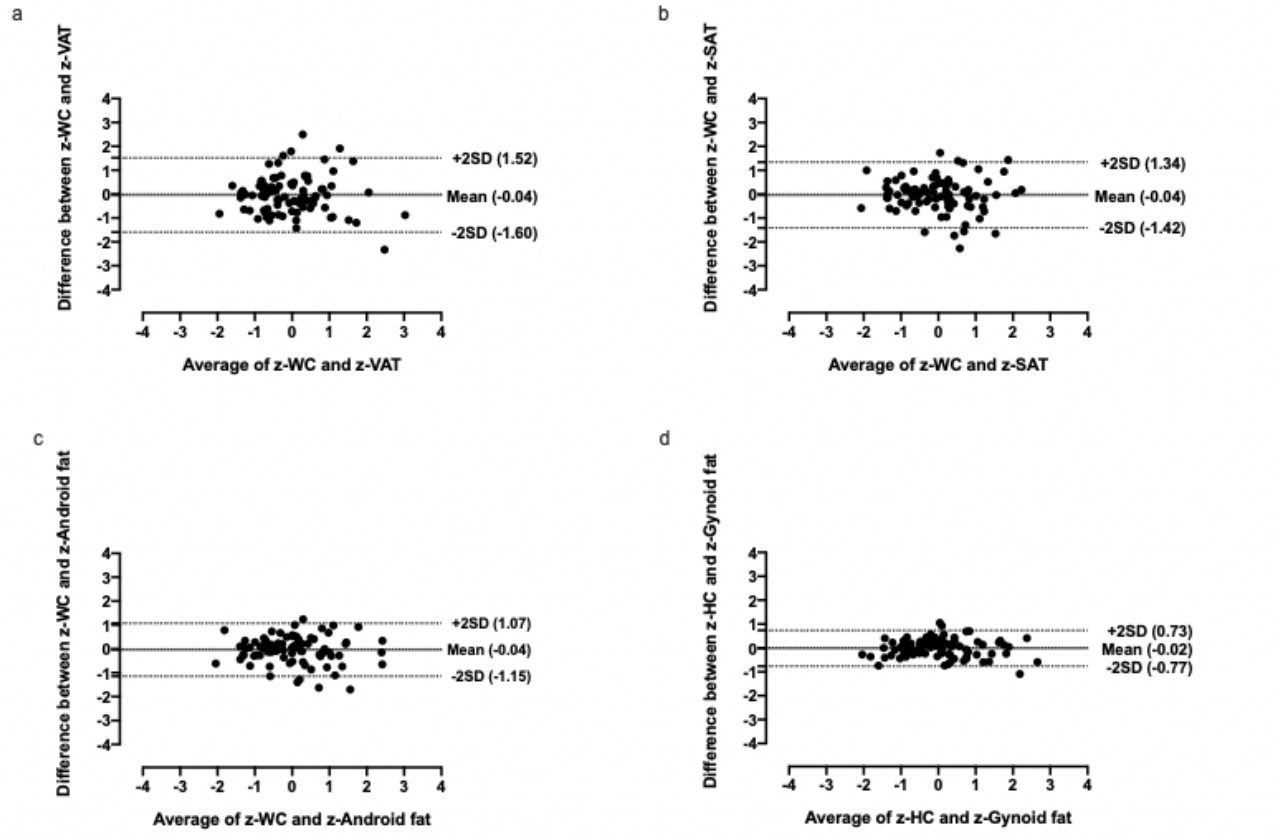


Figure 0.2 : The agreement between conventional anthropometric and DXA-derived measures of regional adiposity z-scores in pre- and post-menopausal women, with and without HIV. a) Bland Altman plot of WC and VAT, b) Bland Altman plot of WC and SAT, c) Bland Altman plot of WC and Android fat, d) Bland Altman plot of HC and gynoid fat. WC, waist circumference; VAT, visceral adipose tissue; SAT, subcutaneous adipose tissue; HC, hip circumference;

3.3.2 Comparison of OGTT- and FSIGT-derived measures of insulin sensitivity, response, and beta cell function in pre- and post-menopausal women, with and without HIV.

Both OGTT- and FSIGT-derived measures of insulin sensitivity ((r_s =0.518, p<0.001) and insulin response (r_s =0.517, p<0.001) were positively correlated. However, the OGTT-derived measures only explained 26% of the variation in the FSIGT-derived measures (Figure 3.3).

There was a weak but significant positive correlation between DI_O (OGTT) and DI (FSIGT) (r_s = 0.336, p= 0.004) (Figure 3.3). These associations did not differ by groups (p>0.05).

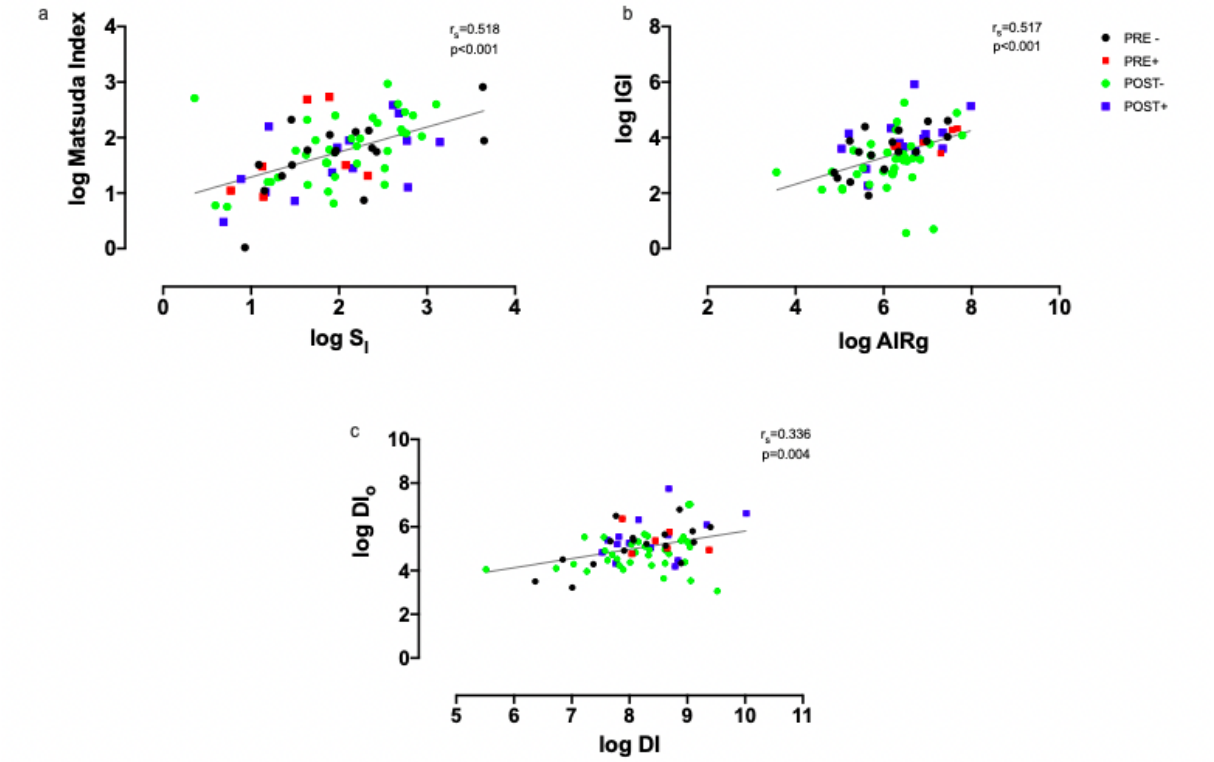


Figure 0.3: Correlation between OGTT-derived and FSIGT-derived indices of insulin sensitivity (a), insulin response (b) and beta cell function (c) in pre- and post-menopausal women, with and without HIV. S_I, insulin sensitivity; IGI, insulinogenic index; AIRg, acute insulin response, DI_O, oral disposition index; DI, disposition index

The Bland-Altman agreement plots (figure 3.4) indicated that there was no proportional bias and that there was homoscedasticity between the S_I and in Matsuda index (r= 0.006, p= 0.960), AIRg and IGI (r= -0.052, p= 0.662), and DI_O and DI (r= -0.017, p= 0.884). The LOA is narrow for insulin sensitivity compared to the other measures of insulin response and beta cell function. These agreements did not differ by groups (p>0.05).

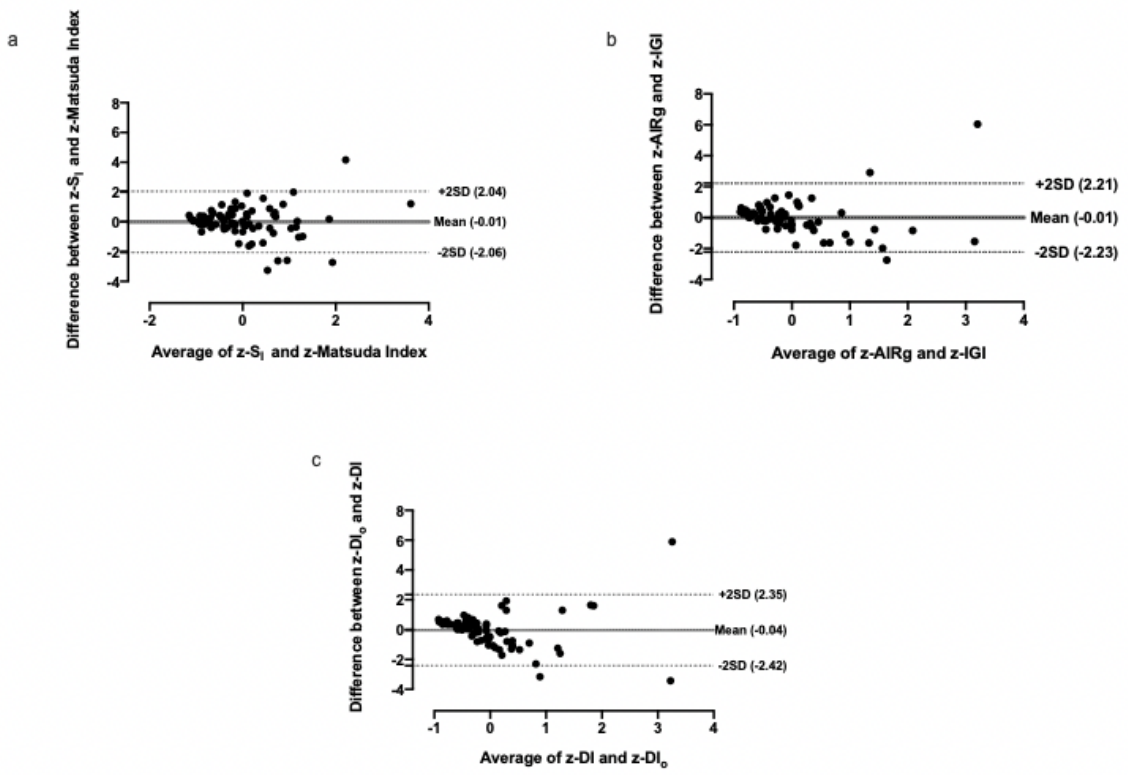


Figure 0.4: The agreement between OGTT-derived and FSIGT-derived for insulin sensitivity, insulin response and disposition index z-scores in pre- and post-menopausal women, with and without HIV. S_I, insulin sensitivity; IGI, insulinogenic index; AIRg, acute insulin response, DI_O, oral disposition index; DI, disposition index;

3.4 Discussion

In this chapter, I showed that in this sample of middle-aged black SA women, anthropometric and DXA derived measures of regional adiposity were correlated and that there was good agreement between the two methods. Similarly, the OGTT- and FSIGT-derived measures of insulin sensitivity, response and beta cell function were correlated and there was agreement between the two methods, with no evidence of proportional bias.

We showed a strong correlation between waist circumference and android fat, which comprises both VAT and SAT. Moreover, only 44,2 % of the variation in waist circumference could be explained by the linear relationship between VAT and waist circumference, whereas 55.2% of the variation in waist circumference can be explained by the relationship between SAT and waist circumference, possibly because waist circumference does not distinguish between visceral and subcutaneous fat depots. SAT is a larger depot than VAT, and African women have been shown to have larger SAT depots compared to white women, even after adjustment for differences in total body fatness [188]. The correlation and agreement between hip circumference and gynoid fat was stronger than those of abdominal fat measures, as indicated by the narrow limits of agreement. Notably, black Africans typically present with a phenotype of high peripheral SAT deposition [35,72], which may be accurately reflected in the hip circumference measures.

Correlation between the OGTT and FSIGT derived measures of insulin sensitivity and response and beta cell function were moderate (r=0.518, r=0.517 and r=0.336 respectively), whereas other studies have shown a stronger correlations between Matsuda index and S_I (r=0.71), AIRg and IGI (r=0.72) and [189,190]. These differences could be attributable to differences in study populations, particularly because our study consists of women of African ancestry. It is important to note that OGTT-derived measures could be confounded by physiological processes including gastric emptying, glucose absorption rate and incretin response [47], whereas FSIGT derived measures are more comparable to the gold standard (EHC) due to the intravenous glucose administration followed by serial glucose and insulin measures. Moreover, studies exploring insulin response in Black Africans using intravenous glucose administration show consistent evidence of hyperinsulineamia, whereas those using oral glucose tolerance test have more variable results [35,38,45,46]. In black Africans, hyperinsulieamia has been shown to be due to both increase insulin secretion and reduced

clearance which may influence measures of insulin sensitivity, thus a tendency to underestimate insulin sensitivity in black Africans [46].

This study has some limitations. This study only included women; thus, the results should be validated in men since men have a different body fat distribution that may alter the correlations and agreements. Additionally, DXA provides estimates of VAT, whereas CT is more accurate, as such DXA-derived estimates of VAT should be interpreted with caution. Another strength is the inclusion of C-peptide measures in the OGTT thus I was able to distinguish between hepatic insulin clearance and insulin secretion. Unfortunately, I did not have these measures for the FSIGT, and thus could not determine insulin clearance using FSIGT, which would have improved the understanding of the relationship between insulin secretion and insulin clearance.

In conclusion, the conventional anthropometry measures perform similarly to DXA-derived measures, and this could be useful in resource-constrained settings. Although I showed a correlation between WC and VAT, caution should be applied when interpreting waist circumference, as it is a better measure of abdominal SAT (55%) than it is of VAT (44%). Furthermore, OGTT- derived measures of insulin sensitivity, response and beta cell function performed similarly to FSIGT-derived measures, and thus may be practical measures in black African women in resource-constrained setting.

In chapter 4, both OGTT and FSIGT- derived measures of insulin sensitivity and response were used to explore associations with menopause and HIV status, and in chapter 5, the OGTT-derived insulin sensitivity measures were used to examine the association between insulin sensitivity and adipose tissue function. The OGTT-derived Matsuda Index was used because it showed a strong correlation and agreement with the FSIGT-derived S_I, and there was a greater number of participants who had completed OGTT than FSIGT, which increased the power of the statistics.

Chapter 4: The differences in insulin sensitivity and response between pre- and post-menopausal women, with and without HIV and their association with body composition, body fat distribution

4.1 Introduction

Obesity is a major public health concern globally and is a risk factor for a variety of chronic conditions including type 2 diabetes (T2D) and cardiovascular disease [191,192]. In South Africa, obesity affects more women than men; 68% of women are considered obese or overweight compared with 31% of men[193]. Central fat mass is associated with increased risk of T2D, while female-patterned (gluteo-femoral) peripheral subcutaneous adipose tissue (SAT) has a neutral, or even a protective role against insulin resistance[194]. Interestingly, black African women have been shown to have greater gluteo-femoral fat and less VAT than white European women for the same BMI [34,35,154]. Despite this, black African women are paradoxically more insulin resistant [35] and this may be explained by the notion that black African women may be more sensitive to the effects of VAT accumulation on insulin resistance than white European women [195].

Aging is associated with weight gain and an increase in body fat, particularly in women as they transition through menopause [196–198]. Notably, alterations in body fat redistribution observed during the menopause transition have been associated with changes in the levels of reproductive hormones, particularly oestrogen (E_2)[199] and a reduction in insulin sensitivity, a major risk factor for T2D [9–11]. However, the majority of studies that have examined the effects of the menopausal transition on T2D risk have included populations of European descent. There is therefore a need for detailed analyses of the effect of menopause on glucose-insulin homeostasis in post-menopausal African women whose body fat distribution and diabetes risk differs to Europeans.

In addition to the burden of non-communicable diseases (NCD), South Africa also has an additional challenge of a high burden of infectious diseases, particularly HIV. The prevalence of HIV in Africa is 13.5%, with South Africa reporting a national prevalence of 19% in adults (ages 25-49) in 2019 [121]. The improved availability and roll out of antiretroviral therapy (ART) [200] means people are living longer with the virus and what was once a life-threatening condition is now a chronic disease [201]. However, several ART drugs are associated with dyslipidaemia, lipodystrophy and T2D [202]. Many women living with HIV (LWHIV) in South Africa are living with obesity, and the use of ART may exacerbate the risk of T2D [203]. Given that a large majority of women living with HIV will transition through menopause, the interaction between HIV infection and menopause on the risk of T2D should

be investigated. Indeed, issues pertaining to age-related comorbidities including T2D risk, represent an emerging aspect of HIV care [204].

Thus, the aim of this chapter was to investigate the effect of menopause and HIV status on glycaemia, insulin sensitivity and response in black African women.

4.2 Statistical analysis for chapter 4

A total of 82 participants were included in the analysis. They included participants with complete OGTT and FSIGT outcome variables. The selection is described in the consort diagram in chapter 2.

The Shapiro–Wilk test was used to assess the distribution of continuous variables. Normally distributed data are presented as mean ± standard deviation (SD) while skewed data are presented as median and interquartile range (25^{th} to 75^{th} percentile). Normal distribution for skewed variables was approximated through log transformation or Box-Cox transformation where applicable. Two-way ANOVA was used to compare the participant characteristics, body fat distribution, glycaemia, insulin sensitivity and response and beta-cell function between pre- and post-menopausal women, with and without HIV infection, including the menopause*HIV interaction. Post-hoc pairwise comparisons were conducted when there was a significant interaction effect. Significance was set as $P < 0.05$. Data were analysed using STATA (Version 13.1, Statcorp, College Station, Texas).

4.3 Results

4.3.1 Participants characteristics

Characteristics of the study sample according to menopause and HIV status are presented in Table 4.1. Postmenopausal women were older than the premenopausal women ($p<0.0001$), but age did not differ between HIV groups within each menopausal stage. Menopause and HIV groups did not differ in terms of marital status, education level, asset index, housing density and employment status. There were no differences in CD4 count, the number of years since HIV diagnosis, and the number of years on HIV treatment between the premenopausal and postmenopausal women LWHIV. The majority of the participants were on a combination of nucleotide analogue reverse transcriptase inhibitor (NtRTI), non-nucleoside reverse transcriptase inhibitor (NNRTI) and nucleoside reverse transcriptase inhibitor (NRTI). CRP levels were higher in women LWHIV compared to the HIV-negative women. Lifestyle factors, including dietary intake and minutes per day spent sitting, standing and walking were not different between the groups.

The concentrations of LH and FSH were higher in the post- compared to premenopausal women and did not differ by HIV status. In contrast, E_2 concentrations were lower in post- compared to premenopausal women and were lower in women LWHIV compared to HIV-negative women ($p=0.001$), while SHBG concentrations were higher in women LWHIV compared to their HIV-negative counterparts ($p=0.004$). TG and HDL-C concentrations did not differ between groups, however total cholesterol and LDL-C were higher in postmenopausal compared to premenopausal women ($p=0.001$ and $p=0.005$, respectively).

Table 0.1: Characteristics of pre- and post-menopausal women with and without HIV

	Pre-menopausal		**Post-menopausal**		**P-value**		
	HIV Negative (n=19)	**HIV Positive (n=7)**	**HIV Negative (n=39)**	**HIV Positive (n=17)**	**Menopause**	**HIV**	**Menopause * HIV**
Age	49 (46-51)	46 (43-47)	55 (52-60)	53 (53-59)	<0.0001	0.070	0.345
Married, n (%)	8 (42%)	2 (29%)	14 (36%)	7 (41%)	0.648	0.532	0.466
Employed n (%)	10 (53%)	2 (29%)	13 (33%)	5 (29%)	0.162	0.284	0.463
Highest Education level							
Primary Education	0	0	6 (15%)	2 (12%)	0.108	0.362	0.590
Secondary Education	16 (84%)	7 (100%)	30 (77%)	15 (88%)			
Tertiary Education	3 (16%)	0	3 (8%)	0			
Asset Index							
Low (1-4)	0	1(14%)	1 (3%)	0	0.511	0.086	0.050
Medium (5-8)	6 (32%)	4 (57%)	18 (46%)	8 (47%)			
High (9-12)	13 (68%)	2 (29%)	20 (51%)	9 (53%)			
CD4 count		437 ± 231		707 ± 316		0.070	-
Years since HIV diagnosis		8 ± 4		8 ± 7		0.956	-
Years on HIV treatment		5 ± 5		6 ± 4		0.592	-
CRP (mg/L)	2.6 (1.25-4.1)	4.55 (2.8-22.5)	3.55 (1.3-6.8)	8.3 (4.5-20.9)	0.533	0.022	0.398

Physical activity							
Sitting (min/day)	419.2 ± 100.7	431.5 ± 93.4	443.1 ± 86.5	412.2 ± 114.0	0.932	0.740	0.439
Standing (min/day)	333.0 (279.4-420.7)	327.2 (306.7-355.4)	346.5 (295.3-364.2)	296.0 (207.5-383.2)	0.700	0.244	0.316
Walking (min/day)	125 ± 44	115 ± 34	132 ± 37	133 ± 44	0.274	0.698	0.591
Dietary Intake							
Energy Intake (kJ/day)	6137 (4641-8303)	7624 (5434 - 10261)	6644 (4607 - 8675)	6717 (6145-7803)	0.867	0.289	0.486
CHO (% energy intake)	55.1 ± 6.2	56.3 ± 3.6	55.9 ± 8.8	57.2 ± 6.5	0.655	0.538	0.994
Protein (% energy intake)	11.3 ± 1.8	12.0 ± 2.3	11.5 ± 3.0	11.2 ± 1.8	0.643	0.777	0.467
Fat (% energy intake)	32.5 (28.7-35.7)	31.0 (27.3-32.5)	30.7 (27.5-34.30)	31.2 (27.2-35.3)	0.901	0.692	0.455
Sex Hormones							
E_2 (pg/mL)	213 (153- 750)	128 (96- 353)	23 (14-36)	8 (6-11)	<0.001	0.001	0.108
LH (IU/L)	5.7 (4.0-10.1)	3.5 (2.4-5.1)	22.3 (18.4-32.6)	22.2 (18.8-38.1)	<0.001	0.490	0.253
FSH (mIU/ml)	9.82 (4.7-15.1)	8.25 (6.4-11.1)	59.7 (48.9-71.55)	69.3 (50.3-75.3)	<0.001	0.693	0.372

SHBG (nmol/L)	57 (48-82.8)	98.6 (52.6-150.2)	57.3 (43.4-69.6)	66.3 (48.3-113.95)	0.183	0.004	0.865
Lipids							
Total cholesterol (mmol/l)	4.0 ± 0.8	3.7 ± 0.9	4.6 ± 0.9	4.6 ± 0.7	0.001	0.659	0.493
HDL-C (mmol/l)	1.2 (1.1-1.5)	1.3 (0.9-1.5)	1.2 (1.1-1.5)	1.4 (1.1- 1.5)	0.209	0.667	0.223
LDL-C (mmol/l)	2.4 ± 0.8	2.2 ± 0.9	2.9 ± 0.8	2.9 ± 0.7	0.005	0.622	0.749
TG (mmol/l)	0.5 (0.5-.8)	0.7 (0.6-0.9)	0.71 (0.6-0.9)	0.8 (0.7-0.9)	0.254	0.206	0.733

Values are presented as mean ± standard deviation or median (25-75th percentile). P-value in Menopause colum indicates the menopausal effect of the variable; P-value in HIV colun indicates the HIV status effect on the variable; P-value in in the Menopause*HIV indicates the interaction effect of menopause and HIV status effects on the variable. CRP, C-reactive protein; CHO, carbohydrates, E_2, Oestrogen; LH, Luteinizing Hormone; FSH, Follicle Stimulating Hormone; SHBG, Sex Hormone Binding Globulin; LDL, low density lipoprotein cholesterol ; HDL, high density lipoprotein cholesterol ; TG, triglycerides.

4.3.2 Body fat distribution

The obesity prevalence was 60% for all the women combined, with none of the conventional anthropometric measures being significantly different between the groups (Table 4.2). The mean waist circumference was 93.5cm and the mean hip circumference was 112.8 cm. While there were no differences in median BMI, there were differences in BMI categories by HIV status, such that there was a greater proportion of HIV negative women in the obese category than women LWHIV ($p=0.023$).

There were no differences in DXA-derived measures of total body fatness or fat free soft tissue mass (FFSTM) between HIV and menopausal groups. In terms of body fat distribution, there were no differences in regional adiposity between any of the groups, except for VAT and VAT:SAT ratio, which were significantly higher in the post- compared to pre-menopausal women ($p=0.027$ and $p=0.046$ respectively).I also sought to determine whether body composition and fat distribution in women LWHIV were correlated with HIV treatment and exposure (time since diagnosis), but there were no significant correlations between these variables.

Table 0.2: Anthropometry, body fat and fat distribution measures in pre- and post-menopausal women with and without HIV

	Pre-menopausal		**Post-menopausal**		**P value**		
	HIV Negative (n=19)	**HIV Positive (n=7)**	**HIV Negative (n=39)**	**HIV Positive (n=17)**	**Menopause**	**HIV**	**Menopause * HIV**
Anthropometry							
Height (m)	1.58 ± 0.07	1.58 ± 0.06	1.59 ± 0.06	1.56 ± 0.05	0.613	0.331	0.434
Weight (kg)	81.0 ± 14.8	81.5 ± 20.7	81.3 ± 11.4	77.8 ± 15.6	0.646	0.679	0.592
BMI (kg/m^2)	31.9 (28.2-34.8)	28.6 (26.6-38.2)	31.9 (28.3-34.9)	31.2 (28.1-37.0)	0.912	0.827	0.859
Waist circumference (cm)	94.0 ± 11.9	92.5 ± 18.0	93.7 ± 8.6	93.7 ± 13.2	0.752	0.942	0.671
Hip circumference (cm)	111.6 ± 8.4	112.2 ± 12.8	114.9 ±9.5	112.5 ± 14.6	0.540	0.758	0.589
WHR	0.84 ± 0.08	0.82 ± 0.12	0.82 ± 0.07	0.85 ± 0.08	0.979	0.857	0.272
BMI Categories							
Normal weight % (n)	0	0	0	18% (3)	0.374	0.023	
Overweight % (n)	42% (8)	57% (4)	38% (15)	29% (5)			

Obese (n (%))	58% (11)	43% (3)	62% (24)	53% (9)			
DXA-derived body fat							
Body fat (%)	43.9± 3.6	42.1 ± 3.0	45.4 ± 4.2	44.5 ± 5.7	0.130	0.291	0.708
Trunk %FM	44.7 ± 7.1	41.4 ± 4.2	42.4 ± 4.7	43.9 ± 6.5	0.942	0.593	0.152
Leg %FM	42.8 ± 7.1	45.8 ± 4.8	45.8 ± 4.9	43.5 ± 7.0	0.831	0.857	0.132
Android %FM	7.3 ± 1.5	7.0 ± 0.8	7.2 ± 1.1	7.5 ± 1.3	0.641	0.991	0.358
Gynoid %FM	17.4 ± 2.2	18.8 ± 4.0	18.2 ± 1.9	18.0 ± 2.5	0.986	0.393	0.218
VAT (cm^2)	84.4 (58.7-119.2)	69.7 (65.2-81.9)	93.6 (76.1-108.3)	121.1 (69.9 - 126.9)	0.027	0.676	0.218
SAT (cm^2)	454 ± 137	373 ± 81	457 ± 109	423 ± 151	0.466	0.116	0.522
VAT: Leg (cm^2 /kg)	5.6 (3.5-9.2)	5.4 (4.7-6.9)	5.7 (4.9-7.3)	7.61 (5.8-9.8)	0.229	0.544	0.217
VAT/SAT	0.191 (0.155-0.246)	0.193 (0.169-0.203)	0.204 (0.181-0.255)	0.258 (0.199-0.302)	0.046	0.319	0.265

Values are presented as mean ± standard deviation or median (25-75th percentile). P-value in Menopause column indicates the menopausal effect of the variable; P-value in HIV column indicates the HIV status effect on the variable; P-value in in the Menopause*HIV indicates the interaction effect of menopause and HIV status effects on the variable. BMI, Body mass index; WHR, Waist-to-Hip ratio; VAT, Visceral Adipose Tissue; SAT, Subcutaneous Adipose Tissue; BMI categories- Normal weight 18.5 – 24.9kg/m^2, Overweight 25.0 – 29.9 kg/m^2, Obese ≥30.0 kg/m^2.

4.3.3 Glycaemia

There was no difference in fasting blood glucose, but postprandial glycaemia (iAUC glucose in response to OGTT) was higher in post- compared with pre-menopausal women (Table 4.3). Since VAT was higher in post-menopausal women, I adjusted the glucose iAUC for VAT, but the level of significance was maintained (p=0.039), suggesting that the higher glucose response in the post-menopausal women was independent of VAT. Corresponding with the raised postprandial glucose concentrations in post-menopausal women, there was a tendency for raised HbA1c in post-menopausal women compared with pre-menopausal women (p=0.057). However, the 2 hour post prandial glucose was not different between the groups.

Insulin sensitivity

Whilst there were no statistically significant differences between the groups for fasting and OGTT-derived measures of insulin sensitivity (HOMA and Matsuda index), the FSIGT-derived S_I showed a tendency (p=0.052) to be lower in women LWHIV compared to HIV-negative women. There were no differences in insulin sensitivity between the menopausal groups.

Insulin response and clearance

The OGTT-derived insulinogenic index (IGI) and C-Peptide index did not differ by menopause status. Insulin response (IGI) (p=0.015) and secretion (C-peptide index) (p=0.058) were higher in women LWHIV than HIV negative women. In contrast, the fasting and AUC for insulin and c-peptide did not differ between the groups. Accordingly, neither fasting, nor postprandial insulin clearance differed between HIV and menopausal groups.

Corresponding to the OGTT-derived insulin response findings, the FSIGT-derived measure of the first phase insulin response, AIRg, was nearly twice as high in women LWHIV compared to HIV negative women (p=0.005). Neither DI_O or FSIGT-derived DI did not differ between the groups, suggesting that the insulin response was appropriate for the level of insulin sensitivity (Matsuda Index).

Table 0.3: Measures of glycaemia and insulin dynamics in the fasted state, in response to an OGTT and FSIGT in pre- and post-menopausal women with and without HIV

	Pre-menopausal		**Post-menopausal**		**P value**		
	HIV Negative (n=19)	**HIV Positive (n=7)**	**HIV Negative (n=39)**	**HIV Positive (n=17)**	**Menopause**	**HIV**	**Menopause * HIV**
HbA1c	5.6 (5.1-6.1)	5.5 (5.3-5.7)	6.0 (5.5-6.2)	5.8 (5.4-6.3)	0.057	0.757	0.760
Hb	12.4 (10.6-14)	13 (11.8- 14.4)	14.3 (13-15.3)	15 (12.25- 15.6)	0.027	0.634	0.696
Fasting							
Fasting Glucose (mmol/l)	4.9 ± 0.6	4.8 ± 0.5	4.9 ± 0.5	4.8 ± 0.8	0.870	0.460	0.834
Fasting Insulin (mU/l)	7.9 (5.2-9.8)	12.6 (3.7-14.4)	6.7 (4.3-12.1)	10.3 (6.0-14.9)	0.911	0.224	0.399
Fasting C-peptide (ng/ml)	1.5 (1.3-1.9)	1.8 (1.0-2.2)	1.6 (1.2-1.9)	2.0 (1.4-2.5)	0.564	0.510	0.256
HOMA-IR	1.7 (1.2-2.2)	2.7 (0.7-3.4)	1.4 (1.0-2.9)	2.4 (1.5-3.5)	0.908	0.326	0.412
Basal insulin clearance (ng/mlU)	0.206 (0.157-0.259)	0.169 (0.134-0.283)	0.237 (0.176-0.289)	0.190 (0.162-0.233)	0.438	0.151	0.759
HOMA-2%S	692.35 (534.5-1005.2)	420.4 (360.1-1503.1)	746 (432.2-1211.9)	521 (350.4- 850.4)	0.908	0.249	0.404

HOMA-2%B	29.3 (20.1- 41.1)	33.8 (22.5- 46.3)	24.2 (19.2- 35.6)	39.5 (25.0-49.5)	0.941	0.083	0.456
OGTT							
2 hr glucose (mmol/l)	5.2 ± 0.8	5.3 ± 0.7	5.7 ± 1.2	5.1 ± 1.3	0.679	0.366	0.272
Glucose iAUC (mmol/l)	87.6 (30.9-137.0)	89.1 (15.9-141.3)	141.0 (105.3-180.6)	104.0 (36.1-146.3)	0.032	0.144	0.381
Insulin iAUC (mU/l)	4775 (3502-8516)	4497 (2651-9746)	4742 (3406-7250)	4707(4137-8510)	0.579	0.648	0.556
C-peptide iAUC (ng/ml)	546 (499-739)	562 (382-709)	670 (491-950)	747 (483-1058)	0.128	0.810	0.947
OGTT Insulin clearance (ng/mlU)	0.126 (0.097-0.148)	0.110 (0.100-0.144)	0.127 (0.108-0.162)	0.126 (0.096-0.154)	0.171	0.534	0.675
Matsuda Index ($(mgl^2/mUmin)$)	5.8 (4.1-7.8)	4.4 (2.9-14.6)	5.8 (3.6-9.6)	4.3 (3.0-7.0)	0.921	0.486	0.458
c-peptide index (ng/mmol)	3.4 (1.7-6.0)	4.2 (3.2-4.8)	2.8 (1.8-3.9)	4.7 (3.5-6.2)	0.748	0.058	0.533
IGI (mU/mmol)	32.7 (17.5-56.2)	43.5 (39.8-72.1)	23.6 (14.5-33.2)	48.8 (36.7-65.5)	0.439	0.015	0.309
DI_O (mmol/l)	199 (90- 284)	183 (140- 316)	121 (71- 204)	205 (125- 442)	0.673	0.080	0.404
FSIGT							

Sg (min^{-1})	0.029 ± 0.013	0.031± 0.013	0.030 ± 0.011	0.028 ± 0.009	0.572	0.918	0.622
S_I $x10^{-4}$ $(mU/l)^{-1}$ min^{-1}	7.1 (4.3-10.9)	5.2(3.1-8.0)	7.1 (5.1-12.8)	6.8 (3.2-13.7)	0.717	0.052	0.713
AIRg ($mu.l^{-1}.min$)	409 (230-844)	991 (558-1928)	532 (302-688)	813 (476-1048)	0.433	0.005	0.279
DI	3158 (2139-7081)	4658 (3103-5950)	3895 (2207-7551)	3485 (2346-6558)	0.777	0.325	0.564

Values are presented as mean ± standard deviation or median (25-75th percentile). P-value in Menopause column indicates the menopausal effect of the variable; P-value in HIV column indicates the HIV status effect on the variable; P-value in in the Menopause*HIV indicates the interaction effect of menopause and HIV status effects on the variable. HbA1c, glycated haemoglobin; Hb, haemoglobin; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance ; ISI, Insulin secretion index= C-peptide(30-0)/glucose (30-0); IGI, Insulinogenic Index=insulin(30-0 min)/glucose (30-0min) ; DI_O, Oral Disposition Index=IGI x Matsuda Index; DI, Disposition Index (FSIGT)= AIRg x SI; Sg, glucose effectiveness; S_I, Insulin sensitivity index; AIRg, Acute insulin response to glucose ; tAUC, total area under curve; iAUC, incremental area under curve. OGTT Insulin clearance= iAUC c-peptide/iAUC insulin during the OGTT.

Finally, I explored whether factors associated with HIV disease status could explain the higher insulin response within the group of women LWHIV. However, I found no correlations between AIRg and IGI with circulating levels of CRP, the number of years since HIV diagnosis, years on medication and CD4 count.

4.4 Discussion

The main findings of our study were that irrespective of menopause status, I observed a greater insulin response, measured using both the OGTT and FSIGT, and a tendency for lower insulin sensitivity in women LWHIV. Despite these differences in insulin response, measures of glycaemia did not differ by HIV status. In contrast, insulin sensitivity and response did not differ by menopausal status, but postprandial glycaemia was higher in post-menopausal women compared to their premenopausal counterparts and this was independent of the higher VAT in postmenopausal women.

To our knowledge the finding that the first phase insulin response to glucose is greater in women LWHIV compared to HIV- negative women, has not been previously reported. Further, iAUC for insulin and c-peptide did not differ between the groups, indicating that the differences are only in the first phase insulin response. As I did not observe differences between HIV groups in basal or postprandial insulin clearance in response to the OGTT, this suggests that the higher insulin response in the women LWHIV was due to increased insulin secretion rather than insulin clearance. Indeed, there was a tendency for the C-Peptide index, which is a marker of insulin secretion, to be greater in women LWHIV compared to HIV negative women, which further supports that the higher insulin response relates to insulin secretion rather than clearance. Although I did not measure gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), GIP secretion has previously been associated with increased prehepatic insulin secretion in nondiabetic patients LWHIV on HAART [205]. Additionally, Dave et. al., showed no differences in HOMA-IR, HOMA-β, but greater insulinogenic index and DI_O in normoglycaemic South African men and women LWHIV on NNRTI-based ART compared to ART naïve patients [206], suggesting that it may be the effect of the ART rather than the HIV infection. However, in this study, I cannot separate the effect of the disease and the treatment, as I did not have a treatment naïve group and this would require further investigation.

Despite differences in insulin response, there were no differences in glycaemia between those with and without HIV. Commensurate with the increased insulin response, I found a tendency for lower insulin sensitivity in women LWHIV when using the FSIGT but not when using the OGTT, which may perhaps be due to the high insulin response in the FSIGT, resulting in a lower insulin sensitivity measure. This could explain why women LWHIV were able to

maintain normal plasma glucose concentrations with a tendency for lower insulin sensitivity compared to their HIV negative counterparts. Of note, studies have shown that disproportionately greater insulin response may lead to insulin resistance and increased risk of T2D [207–209]. Further investigations are therefore needed to understand whether the greater capacity to secrete insulin precedes development of T2D among South African women including putative mediators of increased insulin response. Importantly, this study is the first to suggest that hyperinsulinaemia in African women may be exacerbated by HIV and it's treatment. Indeed, the role of insulin resistance in the development of T2D is well documented. It has been suggested that diabetes risk in people LWHIV is to a major extent associated with insulin resistance [210] substantially driven by obesity [211,212]. There were no differences in whole body BF% or regional fat distribution between the HIV groups, suggesting that the tendency for lower S_I in women LWHIV observed in the current study is not due to body fat or regional fat distribution. Earlier HIV treatment options (nucleoside reverse transcriptase inhibitors (NRTIs) and first-generation protease inhibitors (PIs)) have been shown to be associated with visceral adiposity and fat loss from the limbs and face (lipoatrophy), that contribute to insulin resistance and T2D[213–215]. However the current treatment regimens, (combination of nucleotide analogue reverse transcriptase inhibitor (NtRTI), non-nucleoside reverse transcriptase inhibitor (NNRTI) and nucleoside reverse transcriptase inhibitor (NRTI)), which came into effect in April 2013, appear to have little impact on body fat distribution [216]. The majority (80%) of the participants in this study were using this regimen. In accordance with this, I showed no differences in in body fat distribution associated with HIV status.

A number of studies have demonstrated that menopause is associated with an increase in adiposity and the redistribution of fat from the periphery to the central regions [217,218]. Redistribution of fat to the central regions, particularly the increase in VAT is a major determinant of insulin resistance and T2D risk in post-menopausal women, and this has been shown in African American and African women [75,219]. I found higher DXA-derived VAT in post-menopausal compared to pre-menopausal women. Despite differences in VAT, insulin sensitivity and response were not different between the menopausal groups. However, I did show greater postprandial glycaemia in post-menopausal compared to premenopausal women, but this was not associated with differences in VAT between pre- and postmenopausal women. Similarly, studies have confirmed that the post-menopausal

state is a risk factor for elevated fasting plasma glucose [220] and impaired glucose tolerance [221], but I only show postprandial elevation, which suggests that there may be some degree of reduced glucose tolerance without differences in insulin sensitivity. A cross-sectional study in back South African women showed that obese women had lower insulin sensitivity compared to normal weight women [222], suggesting an already altered insulin sensitivity in our population of obese women. This could explain why we did not show differences between menopause groups, as they are already obese. Furthermore, there was a tendency for higher HbA1C in post- compared to pre-menopausal women, however this could be attributed to reduced red blood cell turnover in post-menopausal women as after adjusting for differences in Hb, differences in HbA1C were no longer significant.

The difference in sex hormones between pre- and post-menopausal women is well documented, however there is limited information on these differences in women LWHIV. The finding that women LWHIV had lower circulating levels of E_2 and greater levels of SHBG has also been previously reported by Karim et al., 2013 [223]. Although they did not expand on this finding, there have been inconsistent finding in literature regarding levels of E_2 in women LWHIV, with some showing low levels while others do not show differences [224]. However, the literature appears to be consistent with high levels of SHBG in those LWHIV, and irrespective of ART [225].

This is the first study in Africans to explore differences in glycaemia, insulin sensitivity and response between pre and post-menopausal women and explore the interaction with HIV. This is important as black African women present with a different phenotype to white Europeans i.e. less VAT and hyperinsulinaemia. A weakness of our study is the limited sample size in the group of premenopausal WLHIV, which may introduce type 2 error. However, I was able to extensively characterise the participants using FSIGT, which is an accurate measure of insulin sensitivity and response, and has been shown to be highly correlated with the gold standard, the hyperinsulinaemic euglycaemic clamp [50]. I could only calculate insulin clearance for OGTT, but unfortunately, I did not measure C-peptide during the FSIGT which would have given a more accurate measure of insulin response and clearance during this test. Another limitation is that I only recruited participants with NGT; in order to study the disease process, it is of major interest to study participants with varying degrees of glucose levels. Despite the age difference of only 6 years, there were significant

sex hormone differences between the premenopausal and the postmenopausal women, however one may expect to see greater differences if younger women were selected for the premenopausal group these differences could be explored in younger premenopausal populations in future.

In conclusion, insulin response to glucose, derived from OGTT and FSIGT was higher in middle-aged African women LWHIV, irrespective of their menopausal status. The cause and significance of this higher insulin response in women LWHIV requires further investigation, including prognostic implications for glucose tolerance. Postprandial glycaemia was higher in post-menopausal women compared to their premenopausal counterparts and this was independent of the higher VAT in postmenopausal women.

Chapter 5: Adipose tissue adipokine, inflammatory and glucocorticoid gene expression and the association with insulin sensitivity in pre- and post—menopausal women with and without HIV

5.1 Introduction

Obesity, in particular central obesity, characterised by increased abdominal fat, is associated with increased risk of insulin resistance (IR), type 2 diabetes and cardiovascular disease [226–228]. The abdominal adipose tissue is compartmentalized into subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT), with both being positively correlated with insulin sensitivity. However, VAT has been shown to be the most significant determinant of insulin sensitivity in studies including predominately White women of European descent [7,229]. Black women have less VAT but are paradoxically more insulin resistant than their white European counterparts. Some studies have shown that abdominal SAT is more closely associated with insulin sensitivity than VAT in black African women, which may be largely due to its size and increased capacity to release free fatty acids and adipokines [7,72,219,230]. In contrast, lower body fat, for example gluteo-femoral fat (gSAT), is associated with a lower risk of T2D, due to its ability to act as a metabolic sink by trapping excess fatty acids and preventing prolonged exposure of insulin sensitive organs to fatty acids [194,231].

To date, most studies exploring body fat distribution in black women have focused on premenopausal women with no studies to our knowledge focusing on postmenopausal women. Premenopausal women demonstrate a distinctive gluteo-femoral body fat distribution [194,232], but the menopausal transition is associated with a preferential increase in abdominal adiposity, which is independent of the effect of age and total body adiposity [217]. In the previous chapter (chapter 4), this was confirmed where I showed that despite similar BMI, postmenopausal black African women had more VAT than their premenopausal counterparts. Sex hormones are important determinants of regional body fat distribution, as evidenced by sex differences in body fat distribution [233]. Further, the decline in E_2 levels, experienced during the menopause transition, is associated with increased abdominal fat [234]. It is also noteworthy that the effects of E_2 are mediated by binding to its receptors alpha (ERα) and beta (ERβ), which are present in most tissues including SAT, with their relative ratios regulating the biological response [235]. Studies have shown that ERα knockout mice have increased adiposity, significantly higher VAT, and are more insulin resistant compared to their wild type litter mates [236]. Notably, compared to white European premenopausal women, black premenopausal women had higher gluteal ERα that was

associated with a more favourable body fat distribution, but it is not known if these associations persist past the menopausal transition [155]. The menopausal decline in E_2 is likely to impact on regional adipose tissue and thus impact on whole body metabolism.

It is widely recognised that adipose tissue produces and secretes biologically active proteins, such as adipokines, cytokines and proinflammatory factors [237,238], with adiponectin and leptin being the most well-researched ones [239]. Leptin plays an important role in the regulation of energy balance, metabolism and neuroendocrine function, while the role of adiponectin is less well defined, but statistically strongly associated with glucose and lipid metabolism [240,241]. Leptin levels are markedly increased with obesity and may predict the risk of T2D [242], whereas serum levels of adiponectin decrease with increased abdominal obesity and higher levels are associated with lower risk of insulin resistance and T2D [243], but this association is unlikely to be causal [244]. Notably, other studies have shown that leptin in men and adiponectin in both sexes were independent predictors of T2DM, suggesting that there are clear sex differences that should be taken into consideration [245,246] .Among African Americans, adiponectin was inversely associated with incident type 2 diabetes, whereas leptin's direct association with diabetes was mediated by insulin resistance [247].

Adipokines are also involved in the regulation of inflammatory responses that may contribute to the development of insulin resistance [248,249]. Chronic low-grade adipose tissue inflammation, is a distinct characteristic of obesity and has been associated with insulin resistance [250].The enlargement of adipose tissue is associated with an increase in adipose tissue-associated macrophages as a result of monocyte recruitment. The macrophage-1-related marker (M1), CD11c (integrin αX chain) is a beta-2 integrin with prominent functions in cell adherence of monocytes and macrophages to vascular endothelial tissue and VAT, and has been shown to correlate with insulin resistance in humans [251]. Furthermore, monocytes have been shown to express macrophage-2-associated marker (M2) CD206 (mannose receptor). This macrophage subtype has anti-inflammatory properties and is associated with improved insulin sensitivity in humans and mice [252]. Accordingly, Wentworth et al. concluded that the modulation of CD11c and CD206 expression in monocyte-macrophage lineage cells is crucial in obesity and the development of insulin resistance [251]. Adipose tissue inflammation is not only mediated by immune cells, but also by inflammatory

cytokines such as interleukin-6 (IL-6). High secretion of IL-6 from adipose tissue may contribute to obesity-induced insulin resistance [156,249,253]. Furthermore, it has been shown that SAT inflammatory gene expression was higher in black premenopausal South African women than their white counterparts and this correlated with the down-regulation of the expression of peroxisome proliferator activated receptor γ (PPARγ) and PPARγ-responsive genes, including lipoprotein lipase (LPL), in the gluteal depot, and was associated with reduced insulin sensitivity [74]. LPL activity is necessary for adipocytes to take up fatty acids from triglycerides in the circulation, and hence regional differences in LPL activity may determine regional fat storage [254]. Notably, it is important to understand how depot differences in these genes relate to the depot-specific differences in insulin sensitivity and how they differ by menopause status.

Glucocorticoids have anti-inflammatory functions and its action on target tissues depends not only on the circulating concentrations of cortisol but also on the expression and function of the glucocorticoid receptor α isoform (GRα) and 11β-hydroxysteroid dehydrogenase type 1 (11HSD1), which catalyses the activation of cortisol from cortisone [255]. Indeed, it has been shown that the higher inflammatory SAT profile of obese premenopausal black South African women compared to European women was associated with down-regulation of GRα expression in black SA women [156].

In addition, SA has a high prevalence of HIV, particularly in women. HIV is associated with changes in fat distribution and/or insulin sensitivity [130], so it is important to understand the role of HIV on immune and metabolic function in adipose tissue. Despite improvements (reducing side effects and improving health outcomes) in antiretroviral therapy (ART), HIV patients may remain at increased risk of metabolic comorbidities, including insulin resistance and T2D [148].

Since adipose tissue is an endocrine organ that secrete hormones that may affect lipid storage, inflammation, and adipokine signalling, it is important to understand how the expression of these hormones/proteins in abdominal and gluteal fat depots are altered by menopause and HIV in black South African women. While I did not show differences in insulin sensitivity between HIV and menopausal groups in the previous chapter, differences in the transcript levels and their association with insulin sensitivity may explain these findings. Therefore, the

aim was to determine the difference in adipokines, inflammatory, glucocorticoid and lipid metabolism gene expression in abdominal and gluteal SAT depots, between pre- and post-menopausal women with and without HIV, and their association with insulin sensitivity.

Based on previous findings I postulate that the opposing relationships between abdominal and gluteal fat mass on whole body metabolism and insulin sensitivity is underpinned by functional differences between the tissues that are complex as they may depend on age, overall adiposity, HIV status and menopause.
I have therefore analysed adipose tissue obtained from fat biopsies of abdominal and gluteal SAT, with the aim of addressing the following objectives:

- To determine the association between regional body fat and insulin sensitivity in pre- and post-menopausal women LWHIV and without HIV.
- To explore the differences in adipokines, inflammatory, glucocorticoid and lipid metabolism gene expression in abdominal and gluteal SAT depots, between pre- and post-menopausal women LWHIV and without HIV.
- To examine the association between adipokines, inflammatory, glucocorticoid and lipid metabolism genes with insulin sensitivity in pre- and post-menopausal women LWHIV and without HIV.
- To determine abdominal and gluteal SAT adipocyte cell size in a sub-sample of pre- and post-menopausal women LWHIV and without HIV.

5.2 Statistical analysis for chapter 5

A total of 92 participants were included in the analysis. They included participants with OGTT outcome variables and adipose tissue biopsy transcript results. The OGTT-derived insulin sensitivity was selected because there was relatively good agreement between the measures of insulin sensitivity and that OGTT represents a more physiological response the compared to the FSIGT. There were a greater number of participants who completed the OGTT (n=92) than FSIGT (n=82). The selection is described in the consort diagram in chapter 2. A sub-sample of these women (n=18) were selected for adipose tissue cell size analysis, and these women were representative of the whole sample size as shown in table 2.2. A candidate gene approach was used to select genes that have been shown to be differentially associated with insulin sensitivity in the abdominal and gluteal depots of black

African women compared to White European women [222]. The mean Ct and SD Ct for each of these genes has now been included as Appendix A.

The Shapiro–Wilk test was used to assess the distribution of continuous variables. Normally distributed data are presented as mean ± standard deviation (SD) while skewed data are presented as median and interquartile range (25^{th} to 75^{th} percentile). Normal distribution for skewed variables was approximated through log transformation or Box-Cox transformation where applicable. DXA-derived measures of regional adiposity (android fat and gynoid fat, and VAT and SAT, and leg fat) were standardized using z-scores, allowing for direct comparison of risk magnitude per 1 SD change difference in the adiposity measurement [256]. Regression analysis were done to determine the association between insulin sensitivity and android and gynoid fat, VAT and SAT, and leg fat. These associations were adjusted for FMI, to account for overall adiposity.

Differences between menopause and HIV groups were analysed using two-way ANOVA. Depot-specific differences in gene expression were assessed using Wilcoxon sign-rank test. The differences in cell size ratio between depots, BMI, menopausal and HIV groups were analysed using two-way ANOVA. Statistical analysis was performed using STATA 14 (StataCorp).

Principal component analysis (PCA) was used to summarize the largest variation in measures of body composition and insulin sensitivity, and SAT transcript levels in pre and post-menopausal women LWHIV and without HIV. Further multivariate analysis, by means of orthogonal partial least squares (OPLS) analysis, was performed to assess the relationship between insulin sensitivity and the included variables. All multivariate analyses were carried out using SIMCA version 13.0.3.0 (Umetrics AB, MKS, Umeå, Sweden).

Of note, the participants characteristics of this chapter may differ to those of Chapter 4, due to the differences in sample size.

5.3 Results

5.3.1 Participants characteristics

The characteristics of the participants included in this chapter are described in table 5.1. The postmenopausal women were on average 6 years older than the premenopausal women ($p<0.001$), and the HIV negative women were marginally older than those LWHIV ($p=0.021$). The majority of the women (56%) were classified with obesity, but there were no differences in anthropometric measures between the four groups. Despite the average BMI being similar between groups, a greater proportion of HIV negative women presented with obesity compared to the women LWHIV (62% vs. 43%). A detailed analysis of body composition using DXA revealed that BF%, FMI and SAT were lower in women LWHIV compared to HIV negative women ($p=0.019$, $p=0.028$ and 0.008), respectively. However, regional adiposity, characterised by android, gynoid and leg fat mass, did not differ by HIV status. Only BF% and VAT were different between the menopausal groups with both being higher in the postmenopausal compared to the premenopausal women ($p=0.026$ and 0.005, respectively). Android fat %, gynoid fat % or leg fat % did not differ between menopausal groups. Despite the apparent significant interaction effect of HIV and menopause ($p=0.046$) on leg fat %, post-hoc analysis revealed that there were no differences by HIV or menopause status.

Clinical biomarkers, including sex hormones, glucocorticoids, adipokines, inflammatory markers, were compared between the groups and are presented in Table 5.2. Expectedly, serum E_2 concentrations were higher and LH and FSH concentrations were lower in premenopausal compared to postmenopausal women ($p<0.001$). (Table 5.2). Additionally, E2 ($p<0.001$) and SHBG ($p=0.004$) levels were higher in women LWHIV compared to HIV negative women ($p=0.004$). Conversely, free androgen index, free testosterone and testosterone bioavailability were lower in women LWHIV compared to HIV negative women ($p<0.001$). Total testosterone concentrations showed an interaction effect ($p=0.033$), but this was not independent of FMI ($p=0.160$).

The circulating levels of glucocorticoids, cortisol and corticosterone, did not differ between the menopausal or HIV groups. The circulating levels of CRP were determined to assess the levels of non-specific inflammation between the groups, and as expected, this was greater in

women LWHIV compared to those without HIV (p=0.045), and was independent of differences in FMI (p=0.003). In contrast, the circulating levels of IL-6 did not differ between the groups. Serum leptin concentrations were lower in women LWHIV compared to HIV negative (p=0.004) and this was independent of differences in FMI (p=0.024). Circulating levels of adiponectin and LPL were not different between the groups.

There were no differences in insulin sensitivity between the groups.

Table 0.1: Characteristics of pre- and post-menopausal women with and without HIV

	Premenopausal		**Postmenopausal**		**P value**		
	HIV Negative (n=21)	**HIV Positive (n=11)**	**HIV Negative (n=42)**	**HIV Positive (n=18)**	**Menopause**	**HIV**	**Menopause x HIV**
Age (years)	49 (46-51)	46 (44-47)	55 (53-59)	53 (51- 59)	0.000	0.021	0.397
Anthropometry, body fat and fat distribution							
Weight (kg)	81.6 ± 14.4	75.2 ± 19.5	81.8 ± 11.7	76.9 ± 15.5	0.759	0.091	0.820
BMI (kg/m^2)	32.6 (28.6-34.8)	28.6 (26.1-35.9)	31.9 (28.7-34.9)	30.5 (27.1-37.0)	0.508	0.125	0.430
BMI Categories							
Normal weight (n (%))	0	2 (18%)	0	3 (17%)	0.884	0.003	0.583
Overweight (n (%))	8 (38%)	5 (46%)	16 (38%)	6 (33%)			
Obese (n (%))	13 (62%)	4 (36%)	26 (62%)	9 (50%)			
WHR	0.83 ± .09	0.83 ± 0.10	0.82 ± 0.07	0.85 ± 0.08	0.891	0.524	0.343
Body fat (%)	44.3 ± 3.6	40.4 ± 5.4	45.6 ± 4.1	44.1 ± 5.9	0.026	0.019	0.279
FMI (kg/m^2)	14.37 ± 3.20	11.13 ± 3.10	14.56 ± 3.20	13.99 ± 4.43	0.077	0.028	0.121
Android %FM	7.2 ± 1.5	6.6 ± 1.4	7.2 ± 1.1	7.6 ± 1.3	0.092	0.696	0.099

Gynoid %FM	17.6 ± 2.2	19.2 ± 3.1	18.2 ±1.9	17.9 ± 2.4	0.474	0.236	0.075
Leg %FM	43.6 ± 7.2	47.0 ± 6.2	45.8 ± 4.9	43.2 ± 6.9	0.571	0.807	0.046
VAT (cm^2)	87.9 (59.5-118.0)	65.2 (55.8-81.9)	94.4 (76.1-109.7)	115.0 (68.5-126.9)	0.005	0.286	0.096
SAT (cm^2)	452 ± 131	327 ± 109	462.8 ± 112	422 ± 147	0.088	0.008	0.173
Insulin sensitivity							
Matsuda Index ((mgl^2/mUmin)	5.7 (3.6- 7.36)	4.4 (2.8- 15.4)	5.9 (3.7- 9.6)	4.3 (3.0- 7.0)	0.959	0.856	0.173

Values are presented as mean ± standard deviation or median (25-75th percentile). BMI, Body mass index; WHR, Waist-to-Hip ratio; BF%, Subtotal Body Fat %; FMI, Fat Mass Index; VAT, Visceral Adipose Tissue; SAT, Subcutaneous Adipose Tissue; BMI categories- Normal weight 18.5 – 24.9kg/m^2, Overweight 25.0 – 29.9 kg/m^2, Obese ≥30.0 kg/m^2.

Table 0.2: Circulating levels of clinical Biomarkers of pre- and post-menopausal women with and without HIV

	Premenopausal		**Postmenopausal**		**P value**		
	HIV Negative (n=21)	**HIV Positive (n=11)**	**HIV Negative (n=42)**	**HIV Positive (n=18)**	**Menopause**	**HIV**	**Menopause x HIV**
Sex Hormones							
E_2 (pg/mL)	207 (115.5-728.5)	313 (96- 483)	23.5 (14-32)	8 (6-11)	0.000	0.001	0.192
LH (IU/L)	5.7 (3.2-10.8)	3.7 (2.4-5.1)	22.3 (18.0-30.6)	22.5 (18.8-38.1)	0.000	0.606	0.089
FSH (mIU/ml)	9.8 (4.4-16.5)	8 (4.5-11.1)	59.7 (48.9-71.6)	70.3 (50.3-83.3)	0.000	0.494	0.164
SHBG(nmol/L)	60.8 (48.0-94.9)	98.6 (52.6-165.3)	56.9 (44.0-65.7)	69.2 (49.4-114.6)	0.059	0.004	0.911
Total Testosterone (nmol/L)	0.53 (0.35-0.69)[a]	0.23 (021-0.25)[a,b]	0.47 (0.31-0.68)[b]	0.35 (0.29,0.47)[b]	0.049	0.445	0.033
Albumin (g/L)	40.4 ± 2.5	40.3 ± 3.5	41.1 ± 2.4	41.7 ± 2.5	0.090	0.709	0.612
Free androgen index (nmol/L)	0.76 (0.63-1.20)	0.20 (0.12-0.41)	0.87 (0.64-1.39)	0.41 (0.30-0.48)	0.077	<0.001	0.108
Free Testosterone (nmol/L)	0.006 (0.004-0.008)	0.002 (0.001-0.003)	0.006 (0.005-0.010)	0.003 (0.003-0.004)	0.089	<0.001	0.070
Testosterone Bioavailability (nmol/L)	0.124 (0.089-0.176)	0.040 (0.023-0.065	0.133 (0.101-0.196)	0.071 (0.063-0.084)	0.071	<0.001	0.072

Glucocorticoids							
Cortisol (nmol/L)	272.8 (99.8-409.8)	167.8 (83.3-411.0)	174.0 (83.8-309.4)	162.9 (105.5-274.4)	0.707	0.589	0.659
Corticosterone (nmol/L)	6.4 (4.0-10.1)	4.2 (3.8-11.8)	6.2 (5.0-9.1)	4.6 (2.3-12.3)	0.825	0.528	0.564
DHEA (nmol/L)	5.9 (4.7-7.4)	9.9 (6.9-11.2)	6.2 (5.0-8.0)	7.0 (5.5-10.3)	0.311	0.369	0.230
Androstenedione (nmol/L)	1.8 (1.2-2.4)	1.7 (1.5-2.7)	1.1 (0.8-1.4)	1.2 (0.9-2.1)	0.082	0.199	0.785
Inflammatory marker							
CRP (mg/L)	2.9 (1.3-4.7)	3.1 (1.8-19.4)	4.2 (1.4-8.3)	8.0 (3.5-20.9)	0.177	0.045	0.569
IL-6 (pg/mL)	3.6 ± 0.6	3.7 ± 0.8	3.9 ± 0.6	4.0 ± 0.6	0.074	0.420	0.798
Adipokines							
LEP (pg/mL)	7.5 (6.8-7.5)	6.7 (6.5-7.1)	7.2 (7.0-7.7)	7.0 (6.5-7.6)	0.144	0.004	0.296
Adiponectin (ug/mL)	6.4 (5.3- 7.4)	7.6 (3.5- 9.0)	7.0 (5.2- 8.3)	7.8 (5.2- 11.4)	0.427	0.312	0.917
LPL (pg/mL)	10.6 (10.3-10.7)	10.5 (10.1-10.7)	10.6 (10.3-10.8)	10.5 (10.4-10.8)	0.186	0.370	0.966

Values are presented as mean ± standard deviation or median (25-75th percentile). E_2, Oestrogen; LH, Luteinizing Hormone; FSH, Follicle Stimulating Hormone; SHBG, Sex Hormone Binding Globulin; CRP, C-reactive protein; IL-6 , Interleukin-6; LEP, Leptin; LPL, Lipoprotein Lipase.

5.3.2 Body fat and regional adiposity association with insulin sensitivity

Since I found no differences in insulin sensitivity between the groups and there were no interactions between HIV status and menopause status, (i.e. the associations did not differ by menopause or HIV status) the groups were merged to examine the hypothesis that regional fat masses have opposing associations with insulin sensitivity, i.e. that measures of central fat storage (android fat, VAT area) were inversely associated with insulin sensitivity, whereas measures of lower-body fat mass (gynoid and leg fat) are positively associated with insulin sensitivity in middle-aged black African women, independent of whole-body fatness. Insulin sensitivity was regressed against z-score android and gynoid fat, VAT and SAT, and leg fat, with adjustment for FMI. Using adjusted regression analyses, only the measures of central adiposity, android fat mass and VAT area, were inversely associated with insulin sensitivity (p=0.012 and p=0.010, respectively) (Table 5.3).

Table 0.3: Associations between regional adiposity z-scores and insulin sensitivity

	β coefficient	**CI**	**p**	**R^2**
z-Android	-0.32	-0.57 to -0.07	0.012	0.10
z-VAT	-0.21	-0.36 to -0.05	0.010	0.10
z-SAT	-0.16	-0.44 to 0.12	0.254	0.04
z-Gynoid	0.04	-0.21 to 0.28	0.766	0.03
z-Leg	0.04	-0.13 to 0.34	0.364	0.10

5.3.3 Abdominal and gluteal SAT depot differences in mRNA expression

To determine whether the differential association between insulin sensitivity and abdominal and gluteal fat in pre- and post-menopausal women LWHIV and without HIV are presented in Table 5.3, may be associated with differences in transcript levels, the levels of genes involved in inflammation (IL6, ITGAX (CD11c) and MRC1 CD206), sex hormones (ESR1

(Erα) and ESR2 (ERß)), adipokines (ADIPOQ and LEP) adipogenesis (PPARγ) and LPL) and GRα in abdominal and gluteal SAT were compared (Figure 5.1). As there were no depot differences by HIV or menopause status in any of the genes, the groups were combined for subsequent analysis.

mRNA expression of ADIPOQ was greater in abdominal compared to gluteal SAT (0<0.001), while leptin mRNA expression was greater in gluteal compared to abdominal SAT (p<0.001). In terms of inflammatory gene expression, CD11c expression did not differ by depot, but the expression of CD206 and IL6 were greater in the gluteal compared to abdominal depot. When exploring the correlations between the inflammatory genes within each depot, it was found that abdominal CD11c was positively correlated with CD206, but gluteal expression of CD11c was not correlated with gluteal expression of CD206, suggesting an independent regulation in macrophage subspecies in the gluteal depot. In terms of genes that regulate lipid biology, the expression of LPL and PPARγ were greater in the gluteal compared to the abdominal depot, whereas abdominal GRα expression was greater than gluteal GRα. Similarly, both the expression of ERα and ERβ were greater in the abdominal than the gluteal depot. Overall, this shows substantial differences comparing the abdominal and gluteo-femoral transcriptome indicating functional differences between the tissues.

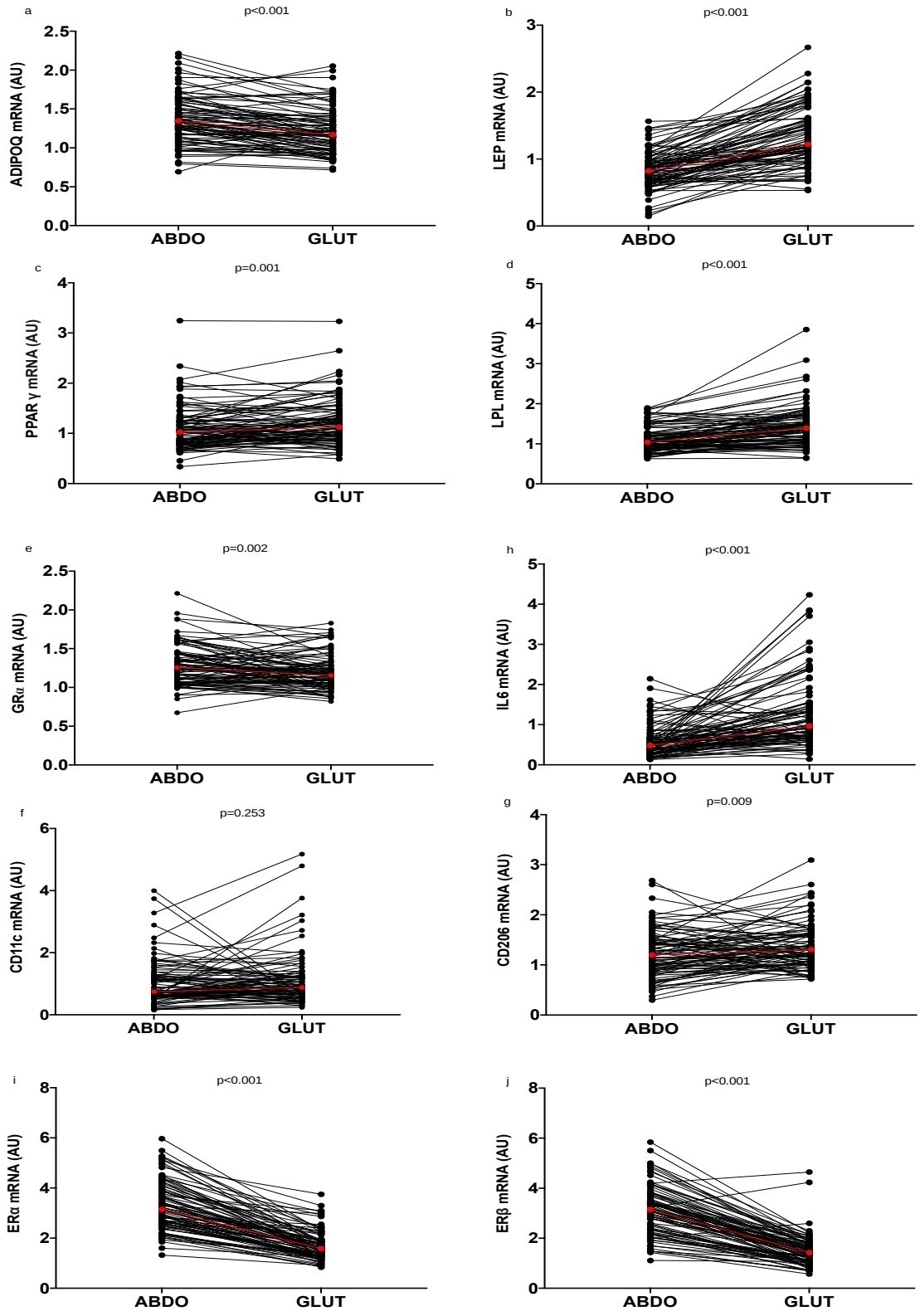


Figure 0.1: Differences in mRNA expression between abdominal and gluteal depots. ABDO, Abdominal; GLUT, Gluteal; ADIPOQ, adiponectin; LEP, leptin; PPARγ, Peroxisome proliferator-activated receptor gamma; LPL, lipoprotein lipase; GRα, glucocorticoid receptor alpha; IL6, interleukin-6; CD11c, Integrin, alpha X; CD206, mannose receptor 1; Erα, Estrogen receptor alpha; ERß, Estrogen receptor beta.

5.3.4 The effect of menopause and HIV status on mRNA expression in the abdominal and gluteal SAT

I then explored whether SAT gene expression differed by menopausal or HIV status within each depot (Table 5.4). I found a menopause x HIV status interaction for ADIPOQ for both depots, such that ADIPOQ expression was greater in premenopausal women LWHIV compared to premenopausal HIV negative women (abdominal: p=0.057; gluteal: p=0.007). This difference was not mediated by differences in FMI (adjusted p= 0.0130 and p=0.0015, respectively). LEP expression in both depots was lower in women LWHIV compared HIV negative women (abdominal: p=0.005; gluteal: p=0.002), and these were also not mediated by differences in FMI (adjusted p= 0.004 and p= 0.005, respectively). When comparing the expression of inflammatory markers, I found no differences in CD206 and IL6 expression between the groups in either depot, however CD11c expression was greater in postmenopausal compared to premenopausal women in both depots (abdominal: p=0.040; gluteal: p=0.018), which may suggest a degree of adipose tissue macrophage-1 activation in postmenopausal women. When comparing the expression of transcripts of lipid biology, I found that there were no differences in expression of either LPL or PPARγ between the groups at either depot. While there were no differences in abdominal GRα between groups, there was a significant menopause status x HIV status interaction for gluteal GRα expression (p=0.048), such that GRα expression in the gluteal depot was greater in premenopausal women LWHIV than postmenopausal women LWHIV and premenopausal HIV negative women, but this interaction was dependent of differences in FMI (adjusted p=0.072). When comparing the E_2 receptor expression, there were no differences in abdominal or gluteal expression of ERβ, but gluteal ERα expression was greater in women LWHIV compared to their HIV negative counterparts (p=0.017), and this was independent of FMI (adjusted p=0.020).

Table 0.4: Abdominal and gluteal gene expression of pre- and post-menopausal women with and without HIV

	Premenopausal		**Postmenopausal**		**P value**		
	HIV Negative (n=21)	**HIV Positive (n=11)**	**HIV Negative (n=42)**	**HIV Positive (n=18)**	**Menopause**	**HIV**	**Menopause x HIV**
Abdominal							
Adipokines							
ADIPOQ	1.20 ± 0.27[a]	1.55 ± 0.29[a]	1.38 ± 0.30	1.46 ± 0.31	0.517	0.002	0.057
LEP	0.92 ± 0.32	0.70 ± 0.30	0.86 ± 0.22	0.72 ± 0.29	0.715	0.005	0.582
Inflammation							
CD11c(ITGAX)	0.63 (0.56-0.76)	0.77 (0.38-1.54)	0.77 (0.61-1.24)	1.08 (0.56-1.61)	0.040	0.673	0.979
CD206 (MRC1)	1.13 ± 0.49	1.14 ± 0.54	1.27 ± 0.42	1.28 ± 0.56	0.226	0.912	0.998
IL6	0.50 (0.38-0.67)	0.48 (0.26-0.78)	0.40 (0.26-0.66)	0.54 (0.43-0.67)	0.790	0.624	0.167
Lipid biology							
LPL	0.98 (0.82-1.08)	1.02 (0.82-1.44)	1.06 (0.93-1.27)	1.10 (0.88-1.64)	0.226	0.170	0.818
PPAR γ	1.00 (0.80-1.10)	0.97 (0.78-1.45)	1.06 (0.80-1.29)	0.98 (0.73-1.26)	0.690	0.459	0.294
GRα (NR3C1)	1.19 (1.03-1.43)	1.34 (1.30-1.38)	1.23 (1.06-1.43)	1.27 (1.11-1.60)	0.705	0.104	0.773
E_2 receptors							
ESRα	3.43 (2.61-4.25)	3.41 (2.91-4.37)	2.90 (2.51-3.37)	3.46 (2.69-4.12)	0.081	0.134	0.965
ESRβ	3.26 ± 1.11	3.34 ± 0.97	2.90 ± 0.81	3.31 ± 1.16	0.397	0.285	0.491

Gluteal							
Adipokines							
ADIPOQ	1.05 (0.92-1.21)[a]	1.41 (1.01-1.90)[a]	1.15 (1.03-1.39)	1.23 (1.05-1.36)	0.786	0.004	0.007
LEP	1.49 ± 0.53	1.19 ± 0.39	1.40 ± 0.40	1.09 ± 0.30	0.330	0.002	0.945
Inflammation							
CD11c(ITGAX)	0.68 (0.43-0.97)	0.93 (0.42-1.56)	0.94 (0.72-1.38)	1.16 (0.64-2.00)	0.018	0.478	0.964
CD206 (MRC1)	1.47 (1.13-1.78)	1.19 (1.08-1.39)	1.31 (1.02-1.58)	1.22 (0.96-1.59)	0.331	0.472	0.577
IL6	0.96 (0.74-1.32)	0.66 (0.61-2.40)	0.96 (0.72-1.46)	1.25 (0.72-1.56)	0.865	0.844	0.973
Lipid biology							
LPL	1.32 (1.11-1.53)	1.52 (1.20-1.85)	1.47 (1.12-1.63)	1.22 (0.99-1.67)	0.483	0.441	0.162
PPAR γ	1.18 (0.95-1.29)	1.28 (0.93-1.47)	1.14 (0.95-1.47)	1.05 (0.85-1.78)	0.831	0.260	0.419
GRα (NR3C1)	1.14 (0.98-1.28)[a]	1.44 (1.19-1.65)[a, b]	1.16 (1.04-1.25)	1.07 (1.01-1.30)[b]	0.015	0.013	0.048
E_2 receptors							
ESRα	1.54 (1.26-1.71)	1.94 (1.41-2.30)	1.57 (1.26-1.95)	1.54 (1.31-2.41)	0.449	0.017	0.239
ESRβ	1.42 (1.11-1.73)	1.60 (1.00-1.82)	1.31 (1.08-1.75)	1.47 (1.01-1.69)	0.869	0.767	0.859

Values are presented as mean ± standard deviation or median (25-75th percentile). ADIPOQ, adiponectin; LEP, leptin; PPARγ, Peroxisome proliferator-activated receptor gamma; LPL, lipoprotein lipase; GRα, glucocorticoid receptor alpha; IL6, interleukin-6; CD11c, Integrin, alpha X; CD206, mannose receptor 1; Erα, Estrogen receptor alpha; ERß, Estrogen receptor beta

5.3.5 Abdominal and gluteal SAT mRNA expression in relation to circulating proteins

I then explored correlations between depot-specific transcript expression and the circulating levels of the respective proteins. There were no significant correlations between GRα expression in either depot and circulating levels of cortisol and corticosterone, or between ERα and ERβ expression in either depot and circulating levels of E_2. However, LPL expression at the abdomen, but not at the gluteal region, was positively and significantly correlated with circulating levels of LPL ($r= 0.353$, $p= 0.001$). As leptin is primarily secreted from adipose tissue, expectedly there was a correlation between abdominal LEP expression ($r= 0.509$, $p<0.0001$) and gluteal LEP expression ($r= 0.496$, $p<0.0001$) with circulating levels of leptin. Similarly, both abdominal and gluteal ADIPOQ expression ($r= 0.560$, $p<0.0001$ and $r= 0.502$, $p<0.0001$, respectively) were correlated with circulating adiponectin. Lastly, gluteal IL6 expression ($r=0.357$, $p=0.001$), but not abdominal IL-6 expression ($r= 0.115$, $p= 0.286$), was correlated with circulating levels of IL-6.

5.3.6 SAT gene expression and the association with insulin sensitivity

To extract the maximum information reflecting the variation in this complex and multivariable data set, a PCA model based on general measures of body composition (SAT and VAT) and insulin sensitivity, as well as abdominal and gluteal SAT transcript levels, in all 4 groups was performed (Figure 5.2). Two outliers were identified, but when explored in detail I found no evidence of data error and they were included in the analysis. No other data were excluded from this analysis. The results reflect no separation by HIV group, but rather a separation between pre- and post-menopausal women that was driven by expected differences in oestrogen, age, LH and FSH (Figure 5.3). The largest variation (P1) was due to adiposity and insulin sensitivity measures. As the PCA showed that there were no distinct variables that separated the groups (Figure 5.3), it justifies combining them to run a multivariate Orthogonal Partial Least Squares model (OPLS) to examine associations with insulin sensitivity.

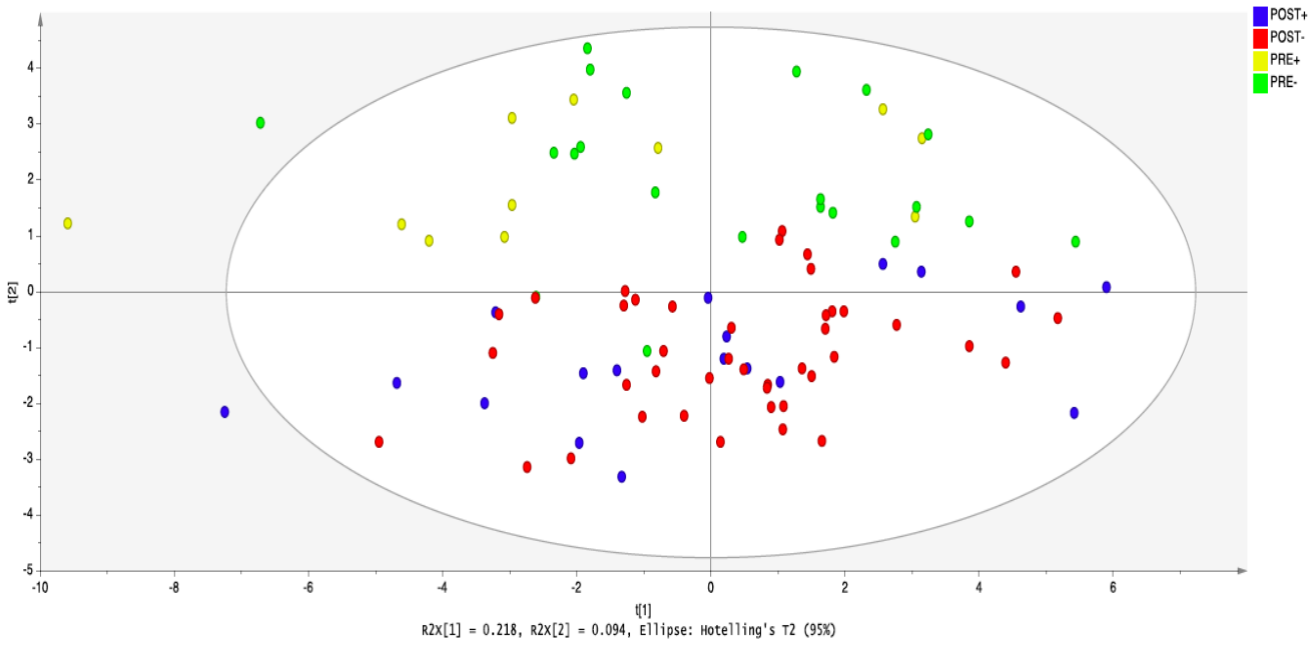


Figure 0.2: Subject plot from principal component analysis (PCA) model based on body composition, insulin sensitivity measures and adipose tissue transcript levels.

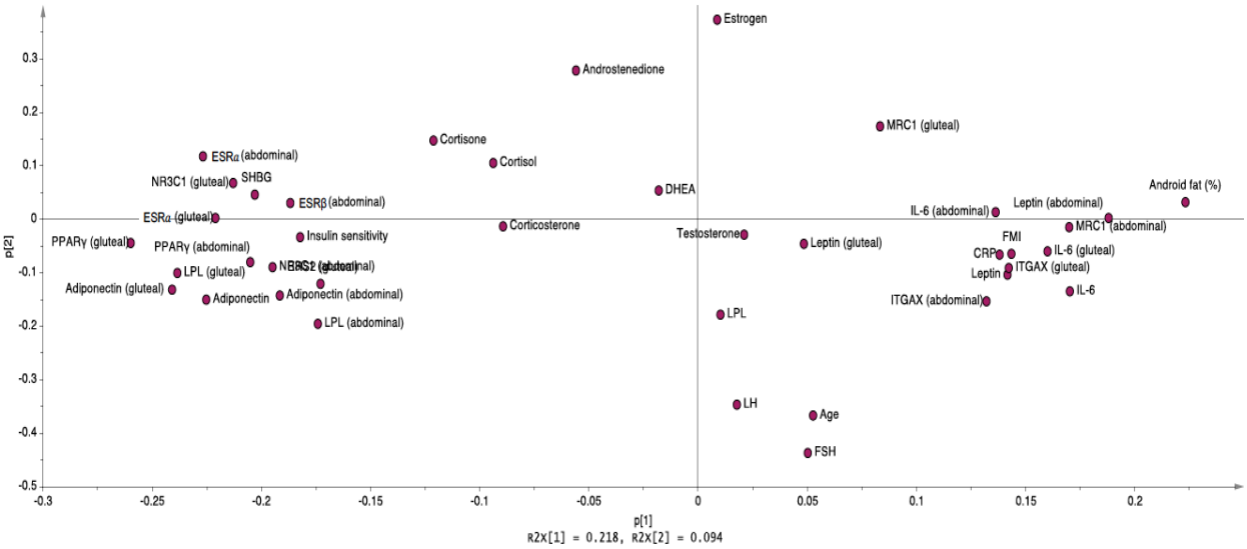


Figure 0.3: Variable plot from principal component analysis (PCA) model based on body composition, insulin sensitivity measures and adipose tissue transcript levels.

OPLS analysis was performed to assess the relationship between whole body fat and regional adiposity, and adipokine, inflammatory, glucocorticoid, lipid metabolism and E_2 receptor mRNA expression, and insulin sensitivity, estimated using the Matsuda Index (Figure 5.4).

The OPLS loadings revealed a strong positive association between circulating levels of adiponectin, SHBG concentration and insulin sensitivity. Clustered together with this are

gluteal mRNA expression of ERα and ERβ and abdominal ERβ which were positively associated with insulin sensitivity, further supporting the role of sex hormones in insulin sensitivity. Gluteal mRNA expression of ADIPOQ, LPL, PPARγ were more strongly associated than their respective abdominal mRNA expression of PPARγ, ADIPOQ suggesting genes of lipid metabolism and glucose regulation in the gluteal depot contributed significantly to insulin sensitivity.

In contrast, higher levels of circulating leptin, which was also mirrored by abdominal LEP expression were associated with lower insulin sensitivity. Leptin is a marker of adiposity and clustered together with markers of increased body fat, in particular android fat, and was significantly correlated with BF% ($r= 0.587$; $p<0.001$). Markers of inflammation (CRP and IL-6) also clustered with adiposity parameters. Higher levels of circulating CRP and IL-6 were associated with lower insulin sensitivity, which was also mirrored by the abdominal IL-6 expression, suggesting that inflammation is associated with low insulin sensitivity.

SHBG, gluteal ADIPOQ and ERα were positively associated with insulin sensitivity. Abdominal and gluteal LEP, circulating CRP and Leptin were negatively associated with insulin sensitivity and were also different by HIV status. So in order to explore this further, regression analyses were done to determine if HIV status altered the relationship between these factors and insulin sensitivity. I showed that all these factors were associated with insulin sensitivity independent of HIV status.

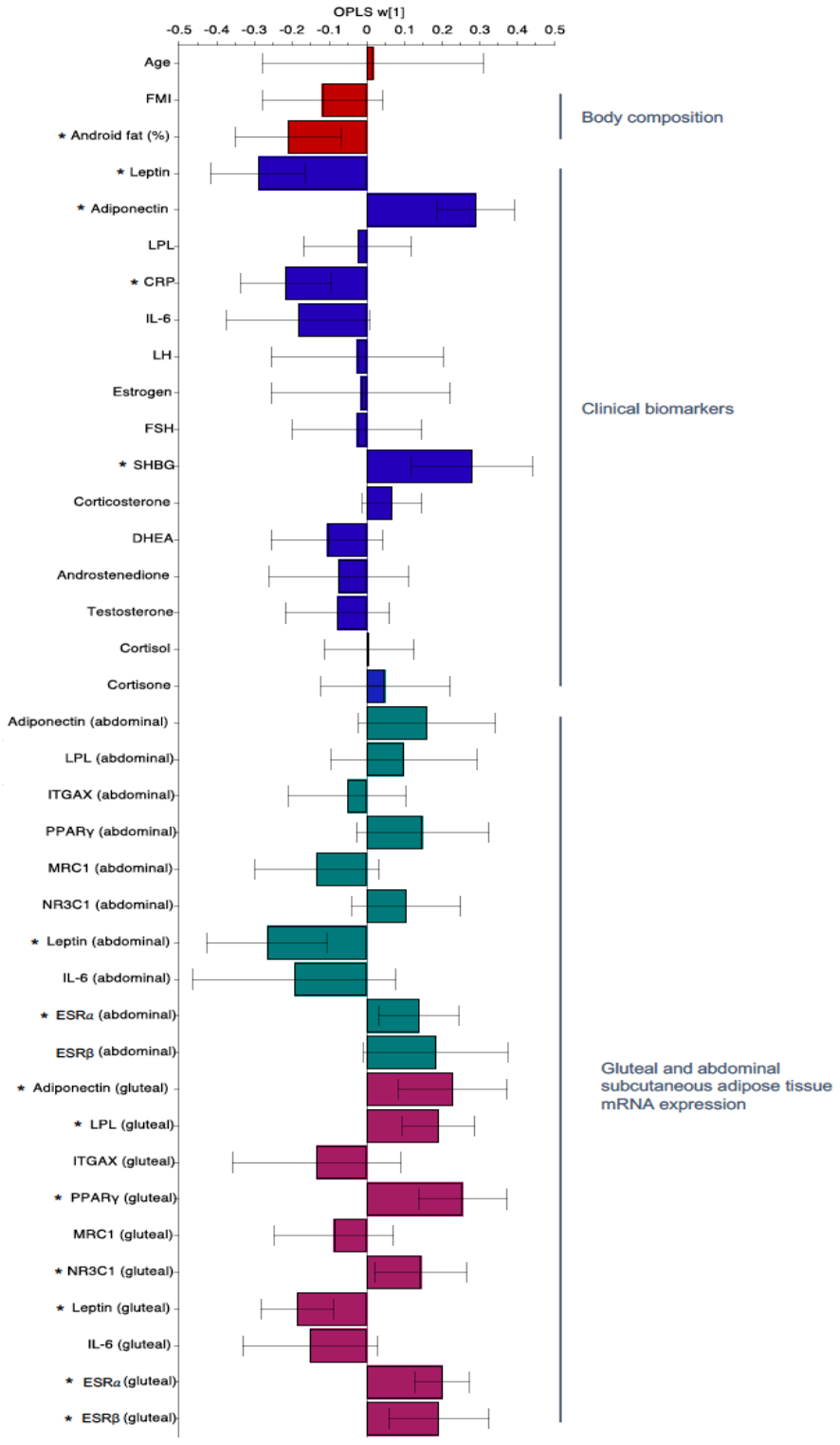


Figure 0.4: Orthogonal Partial Least Squares (OPLS) model describing the correlates of insulin sensitivity (Matsuda Index) including body fat and regional adiposity, as well as SAT adipokine, inflammatory, glucocorticoid, lipid metabolism and E_2 receptor transcript levels (p=0.0000). Above zero indicates a positive association with Matsuda and below zero indicates a negative association. ADIPOQ, adiponectin; LEP, leptin; PPARγ, Peroxisome proliferator-activated receptor gamma; LPL, lipoprotein lipase; GRα, glucocorticoid receptor alpha (NR3C1); IL6, interleukin-6; CD11c, Integrin, alpha X (ITGAX); CD206, mannose receptor 1(MRC1); ERα, Estrogen receptor alpha; ERß, Estrogen receptor beta

5.3.7 Adipocyte cell size

Lastly, the adipocyte cell size was determined in the abdominal and gluteal depots of a sub-sample of women (n=18). The majority (n=10, 56%) of these participants were obese and 61% (n=11) were premenopausal. The groups were combined to determine differences between depots (Figure 5.5). A crossover in the adipocyte size distribution pattern was noted at 4000 μm^2, which was then used as a cut off for large and small cells. There was a greater proportion of smaller adipocytes (0-4000 μm^2) in abdominal compared with gluteal adipose tissue depots (p=0.002), and the opposite was seen for larger adipocytes (>4000 um^2) (p= 0.001). In order to illustrate cell size distribution in a single figure, I calculated a cell size ratio represented by the ratio of large to small cells within each depot. The cell size ratio was higher in the gluteal depot than the abdominal depot (p= 0.002), suggesting a greater number of large to small cells in the gluteal depot compared to the abdominal depot.

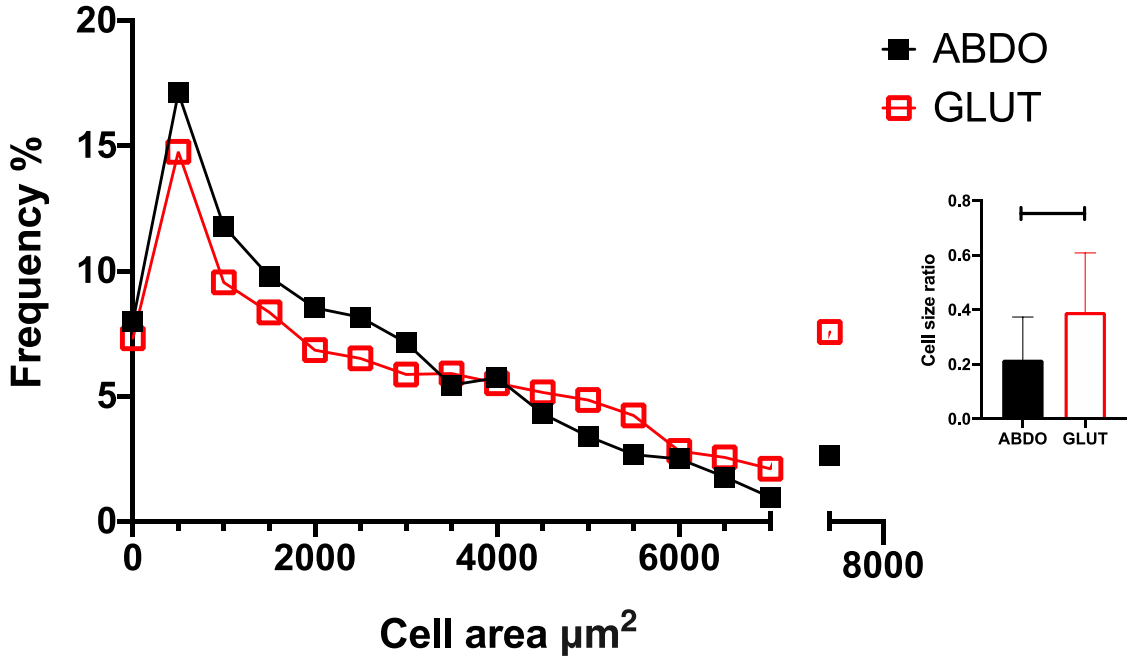


Figure 0.5: Cell size (area mm^2) of gluteal and abdominal adipocytes as determined from hematoxylin and eosin–stained histological sections. The cell size ratio represents the ratio of large to small cells within each depot. A cut off of 4000μm^2 was used to determine large cells and small cells.

I then explored whether adipocyte cell size differed by BMI, by categorising participants into those with overweight (n=8) and obesity (n=10) (Figure 5.6). At the abdominal depot the cell size ratio was higher in the women with obesity (p= 0.043), such that women with obesity

had fewer small adipocytes in the abdominal depot compared to women in the overweight category. There were no differences between the groups for the gluteal depot.

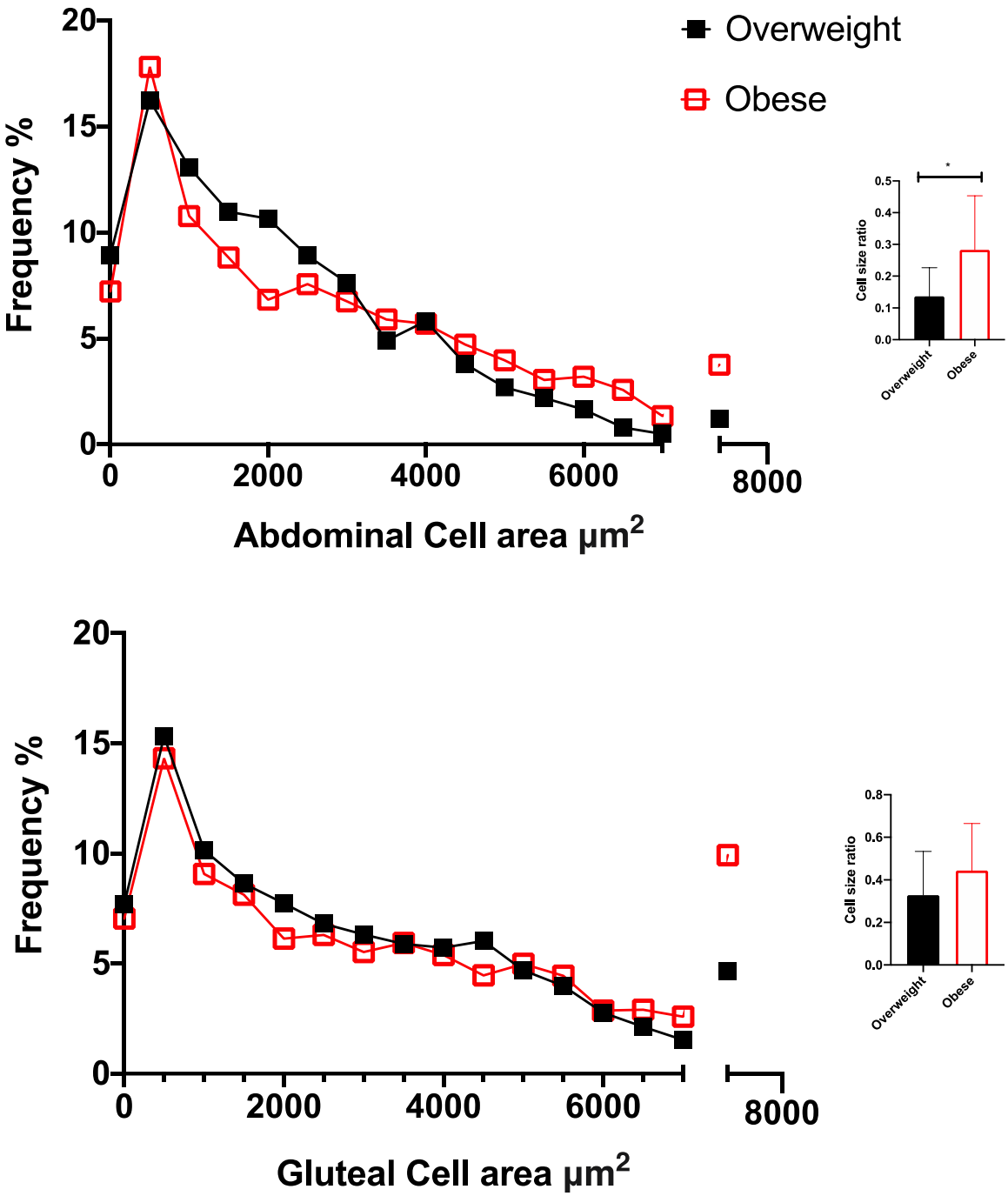


Figure 0.6: Cell size (area mm^2) of gluteal and abdominal adipocyte distribution by BMI. The cell size ratio represents the ratio of large to small cells within each depot. A cut off of 4000μm^2 was used to determine large cells and small cells. Overweight 25.0 – 29.9 kg/m^2, Obese ≥30.0 kg/m^2

I then explored whether there were differences in cell size ratio between pre- and post-menopausal women (figure 5.7). I adjusted for FMI in the analysis of the cell size ratio. There were no differences in adipocyte cell size ratio between pre- and postmenopausal women at either the abdominal or gluteal depots (p= 0.826 and p= 0.564, respectively).

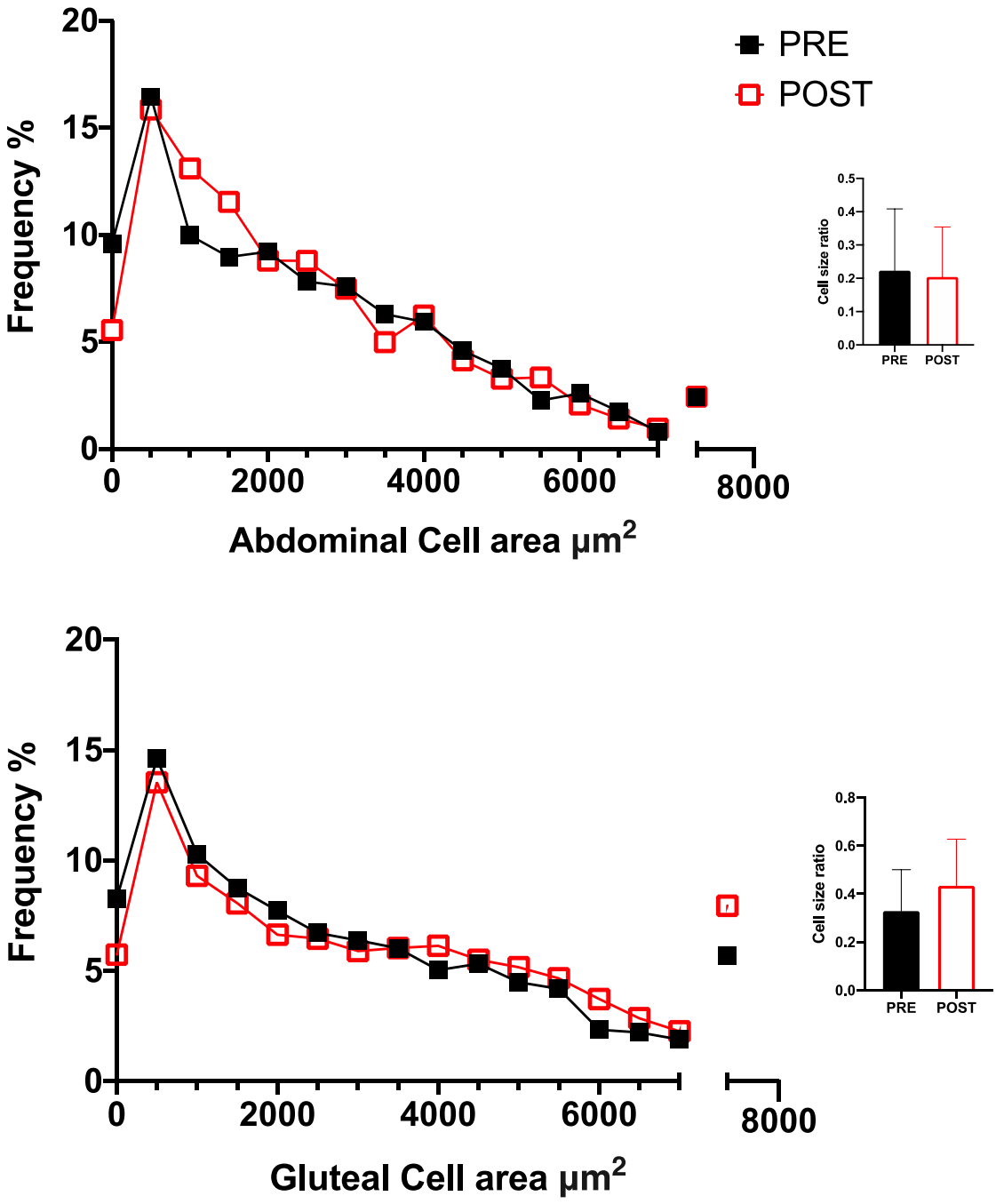


Figure 0.7: Cell size (area mm^2) of gluteal and abdominal adipocyte distribution by menopause status. The cell size ratio represents the ratio of large to small cells within each depot. A cut off of 4000μm^2 was used to determine large cells and small cells. Difference in the cell size ratio between groups was adjusted for differences in FMI.

I compared HIV groups, but due to differences in FMI between the groups, I adjusted for FMI in the analysis of the cell size ratio. The cell size ratio in the abdominal depot was associated with HIV status (p=0.001), such that women LWHIV had a lower cell size ratio compared to HIV negative women, and this remained after adjusting for FMI (p= 0.003) (Figure 5.8). This suggests that women LWHIV have a larger proportion of smaller cells and lower proportion of large cells in the abdominal depot compared to HIV negative women. However, in contrast, the cell size ratio in the gluteal depot did not differ significantly by HIV status.

Of note, there appears to be an a large number of very large adipocytes (>7500um^2) in both the abdominal and gluteal depots for all the groups, However, the reasons are not clear and this requires further investigation.

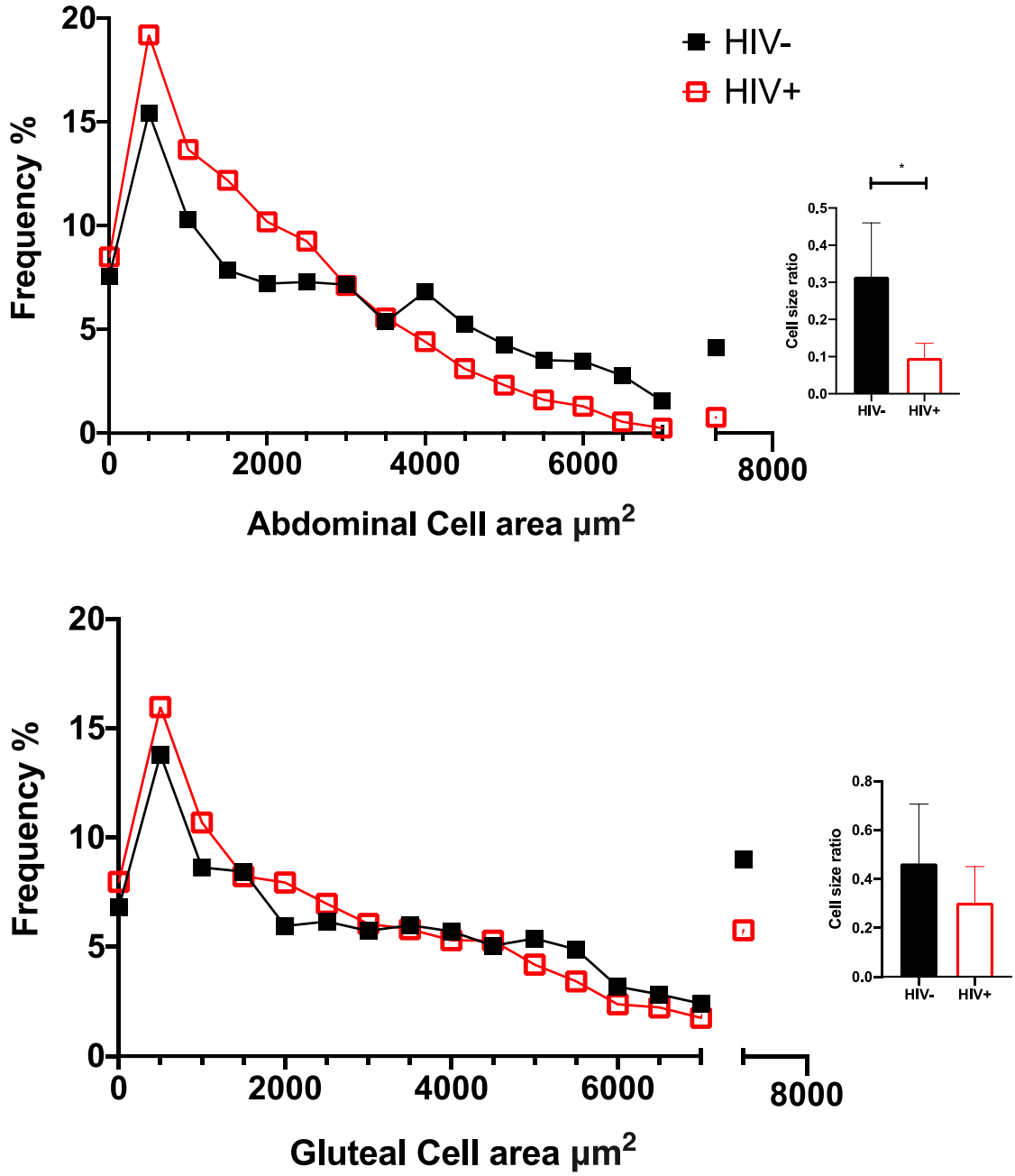


Figure 0.8: Abdominal and gluteal adipocyte distribution by HIV status. The cell size ratio represents the ratio of large to small cells within each depot. A cut off of 4000µm^2 was used to determine large cells and small cells.

5.4 Discussion

In this chapter, I have examined the complex relationship between regional adiposity and insulin sensitivity, and the impact of menopause and HIV status on adipose tissue gene expression, with a specific aim of understanding the differences in function between human AT depots that show divergent relationships with insulin sensitivity. This study has four main findings: I firstly observed that abdominal and gluteal SAT were differentially associated with insulin sensitivity. Importantly, abdominal fat (android fat and VAT area) was associated with lower insulin sensitivity in middle-aged black South African women, independently of total adiposity. Secondly, I showed that there were differences in cell size distribution between the abdominal and gluteal depots, such that gluteal depot had a smaller proportion of small adipocytes, but a larger proportion of large adipocytes compared to the abdominal depot. I observed no differences in cell size between menopause groups, however women LWHIV had a larger proportion of smaller cells and lower proportion of large cells in the abdominal depot compared to HIV negative women. Thirdly, HIV status had a significant association with circulating and adipose tissue expression of adipokines, such that circulating levels and abdominal and gluteal expression of adiponectin were greater and leptin were lower in women LWHIV compared to HIV negative women, and this was independent of differences in total adiposity. Finally, despite the different functional characteristics of the adipose tissue depots, some transcripts seemed to show co-regulation between depots and associations with insulin sensitivity, such that markers of adiposity (android fat, circulating leptin, aLEP and gLEP) were inversely associated with insulin sensitivity, while gluteal mRNA expression of ADIPOQ, LPL, PPARγ, ERα, and ERβ were positively associated with insulin sensitivity, independent of menopause and HIV status.

The finding that VAT and android fat are negatively associated with insulin sensitivity is well documented [7,72,219]. Although, both VAT and abdominal SAT have been similarly and inversely associated with insulin sensitivity in white European women [7], abdominal SAT was more closely associated with insulin sensitivity in a few studies including black SA and African American women [7,219]. In contrast, other studies have showed that VAT but not SAT was associated with insulin resistance and T2D incidence in black women [75,80]. Although I did not show an association between gynoid fat and insulin sensitivity, previous studies in black SA women have shown that leg fat was positively associated with insulin sensitivity, and that greater leg fat mass was associated with approximately 50% reduced risk

of developing IGM/T2D [75,172]. In contrast, Goedecke et al., [74] showed a negative correlation between gynoid fat mass and insulin sensitivity in a small sample of premenopausal black SA women. They hypothesized that increased gluteal fat deposition in Black SA women with obesity is associated with down-regulation of PPARγ and PPARγ-responsive genes and redistribution of fat to the central regions, which is associated with reduced insulin sensitivity [74]. This suggest that these intrinsic differences in adipose tissue depots depend on the differential expression of the transcriptome leading to differences in metabolic regulation and the difference in adipocyte size.

Indeed, there were intrinsic differences in mRNA expression between abdominal and gluteal SAT depots. The abdominal depot had a greater expression of ADIPOQ, GRα, ERα, and ERβ compared to the gluteal depot. This supports previous finding by Evans et. al., who observed higher adiponectin expression in the abdominal depot compared to the gluteal depot in both black and white women [222]. However, other studies in white European women did not show differences in adiponectin gene expression between subcutaneous abdominal and gluteal adipose tissue depots [257,258]. In this sample of women who present with gynoid fat distribution, I noted hypertrophy (increase in size) of the gluteal depot and a hyperplasia (increase in new cells) of abdominal depot. Indeed, over-expression of ADIPOQ has been shown to lead to increased adipose tissue mass by increasing the number of adipocytes rather than cell size [259], which supports our observation of greater abdominal ADIPOQ and greater proportion of small adipocytes in the abdominal depot. Moreover, ADIPOQ expression in both depots was greater in women LWHIV compared to HIV negative women and this was also mirrored by the lower cell size ration in women LWHIV.

Interestingly, HIV status had significant association with circulating adipokines, adipokine adipose tissue expression and adipocyte cell size, such that circulating levels and abdominal and gluteal expression of adiponectin were higher in women LWHIV and circulating levels and abdominal and gluteal expression of leptin were lower in women LWHIV compared to HIV negative women. Considering the afore mentioned association between adiponectin and leptin and insulin sensitivity, the tendency for lower insulin sensitivity and hyperinsuineamia observed in women LWHIV is contradictory and requires further investigation, since HIV status did not modify these associations. Notably, reduced levels of adiponectin and leptin are associated with lipodystrophy in people LWHIV [260]. Although the women LWHIV had

relatively lower FMI compared to those without HIV, there were no differences in regional fat distribution compared to those without HIV, except for abdominal SAT, which was lower in women LWHIV, suggesting that there wass no lipodystrophy in the women LWHIV. Indeed, the women LWHIV had a larger proportion of smaller cells and lower proportion of large cells in the abdominal depot compared to HIV negative women, which would imply a lower risk of insulin resistance since increased adipocyte cell size was shown as an independent marker of type 2 diabetes [261].

Plasma adiponectin levels and both abdominal and gluteal ADIPOQ expressions were positively associated with insulin sensitivity, which is supportive of other studies [262] [263]. This may be explained by adiponectin's insulin-sensitizing abilities [264] [265] such as reducing skeletal muscle triglyceride content and up-regulates insulin signalling, activating protein phosphorylation activator receptor-α and activating adenosine monophosphate activated protein kinase [266].

Concurrently, the overexpression of ADIPOQ has been shown to lead to decreased infiltration of macrophages into adipose tissue [267] and adiponectin acts locally on adipose tissue to suppress the release of a number of pro-inflammatory cytokines including IL-6 from adipocytes [268,269]. This could explain, in part, the lower expression of IL6 in the abdominal depot compared to the gluteal depot. The greater gluteal expression of IL6 suggest a higher inflammatory profile in gluteal compared to abdominal SAT, which supports previous findings by Evans et. al.,[222]. In contrast, the finding that macrophage-2-associated marker (CD206) were higher in gluteal than abdominal SAT suggest that there are considerable anti-inflammatory effects in the gluteal depot.
Additionally, M1 macrophage marker expression, CD11c, which was higher in both depots in postmenopausal women, but was not associated with insulin sensitivity. It is generally accepted that M1 adipose tissue macrophages is associated with insulin resistance by secreting a variety of proinflammatory cytokines, but I did not find an association with insulin sensitivity [270,271].

Furthermore, the gluteal depot had greater expression of LEP, LPL, PPARγ, IL-6 and CD206. These findings are in line with previous findings that found gluteal SAT had higher expression of inflammatory cytokines, macrophage markers and leptin than abdominal SAT

depots [222], which is seemingly in contrast to the hypothesis that gluteal SAT is 'protective' against insulin resistance. Furthermore, circulating Leptin and abdominal LEP expression were inversely associated with insulin sensitivity, and this was independent of adiposity. Elevated leptin levels are positively associated with insulin resistance and T2DM development, primarily due to adiposity. Of note, the majority of the participants in this study were living with obesity.

Notably, most anti-inflammatory effects of GC are mediated through the GRα, which suppresses expression of inflammatory genes through transactivation or transrepression [272]. Indeed, it has been shown that the higher inflammatory SAT profile of obese black South African women was associated with down-regulation of GRα expression [156]. The finding that GRα is greater in the abdominal depot is consistent with a previous study in SA women, which to our knowledge is the only study that has investigated regional differences in GRα expression in abdominal and gluteal depots [156]. Additionally, I showed that gluteal GRα expression was greater in premenopausal women LWHIV than premenopausal HIV negative women and postmenopausal women LWHIV. Furthermore, gluteal GRα expression was positively associated with insulin sensitivity and this is consistent with previous findings [273].

There are conflicting findings regarding the relative expression of ERα and ERβ in abdominal and gluteal depots with some reporting greater ERα and lower ERβ in the abdominal depot [274] and others reporting similarities in ERα between the depots with ERβ isoforms, ERβ-4 and ERβ-5, higher in the gluteal than abdominal depot [275].I have shown that ERα and ERβ expression is greater in the abdominal depot compared to the gluteal depot in our participants. Notably, ERα appears to regulate SAT homeostasis via growth and proliferation of adipocytes, whereas ERβ appears to regulate the sex-specific distribution of SAT [276]. Unexpectedly, in this study, both ERα and ERβ expression did not differ between pre- and post-menopausal women. Similar results were reported amongst Korean women, where there were no difference in the ERα mRNA or ERβ mRNA levels in abdominal SAT between pre- and postmenopausal women [277]. The authors suggested that the distribution of adipose tissue during the menopausal transition is due to the decline of systemic oestrogen concentration, and not due to a change in the ratio of ER subtypes (ERα/ERβ) in adipose tissue [277]. However, contrasting findings by McInnes, showed a

twofold-higher abdominal ERβ mRNA expression in Swedish premenopausal women compared with postmenopausal women of normal weight. The differences in our findings may be because the women in our study had obesity, thereby minimizing the difference between pre- and postmenopausal women, but the exact mechanism by which oestrogen and its receptors affects adiposity and its distribution remains poorly understood. Additionally,I found greater E_2 and gluteal ERα expression in women LWHIV compared to their HIV negative counterparts, though the implication or reasons are not understood and require further investigation. Moreover both abdominal and gluteal ERα and ERβ were positively associated with insulin sensitivity. The importance of ERα for glucose homeostasis and skeletal muscle insulin sensitivity has been well documented in pre-clinical studies [278,279].

Gluteal adipogenic transcription factor PPARγ and PPARγ-responsive genes (LPL and adiponectin) were positively and strongly associated with insulin sensitivity. PPARγ activation in mature adipocytes induces the expression of genes involved in the insulin signalling cascade, thereby improving insulin sensitivity [280].

Notably the levels of SHBG were shown to be positively and strongly associated with increased insulin sensitivity. This is similar to results from previous findings including a meta-analysis, which reported that a higher concentration of SHBG was associated with a lower risk of development of T2D [281,282]. Conversely, low levels of SHBG have been associated with insulin resistance and T2D [283]. It is proposed that the association between SHBG and insulin sensitivity is causal in nature through modulating the biologic effects of sex hormones (testosterone and oestrogen) on peripheral tissues [282].

This study has some strengths and a few limitations. This is the first study that has explored the association between adipokines, inflammatory, glucocorticoid and lipid metabolism genes and insulin sensitivity. The study is limited by a small sample size, and larger studies are required to verify the findings in black pre- and post-menopausal women and men of corresponding age LWHIV. Additionally, a candidate gene approach was used, which may result in biased conclusions compared to non-targeted approaches such as array techniques or total RNA sequencing methods. This study was cross-sectional study; thus I cannot infer causality. Although I could not determine the adipocyte cell size for all the participants, I was

able compare a subsample for BMI, menopause and HIV groups, which has not been done previously.

In conclusion, despite no differences in insulin sensitivity between HIV and menopausal groups, women LWHIV had greater expression of adiponectin in both abdominal and gluteal depots, a corresponding lower expression of leptin and lower abdominal cell size ratio compared to HIV negative women. Postmenopausal women had greater expression of M1 adipose tissue macrophages, which were positively associated with insulin sensitivity. Gluteal adipogenic transcription factor (PPARγ) and PPARγ-responsive genes (LPL and adiponectin) were positively associated with favourable insulin sensitivity, whereas markers of adiposity (android fat, circulating leptin, abdominal LEP and gluteal LEP) were inversely associated with insulin sensitivity.

Chapter 6: Discussion and conclusion

6.0 Overview of research questions and aims of thesis

This thesis includes middle-aged Black African women from Soweto, observed as representing a cross-sectional snapshot of the population. Despite low VAT, Black African women are characterised by low insulin sensitivity, hyperinsulinemia and increased T2D risk compared with their white European counterparts [34,35,72]. Black African women also present with a preferential gynoid fat distribution, which is typically protective against T2D [69–71]. While there is a dearth of studies in African populations, evidence from European populations show that the risk of developing T2D is significantly greater in postmenopausal women than premenopausal women [109–111], and this is hypothesized to be due to changes in reproductive hormones, body fat distribution, and adipose tissue function [92,199].

South Africa has the highest number of people living with HIV in the world, the majority of whom are women. Moreover, SA has the world's largest ART programme, meaning that more women will live longer with the virus [201]. Many women LWHIV in South Africa are living with obesity, and the use of ART may exacerbate the risk of T2D, possibly through their effects on adipose tissue function [203]. Given that a large majority of women living with HIV will transition through menopause, the interaction between HIV infection and menopause on the risk of T2D is important in this context.

Moreover, given the resource constraints in SA, there is a need for low cost and easily accessible tools for identifying individuals at increased risk of T2D. Gold standard techniques to measure body composition and body fat distribution are not always affordable and available in resource poor settings such as in SA. Thus, it is important to determine the agreement between cost effective conventional anthropometric measures of body fat distribution and more precise, DXA-derived measures. In large epidemiological studies, fasting measures are often used to determine diabetes risk, however, to examine glucose tolerance in black Africans it is important to include OGTT-derived measures, as fasting and HbA1c are not ideal measures [284]. Additionally, OGTT provides measures of insulin secretion and sensitivity, but the FSIGT is a more direct measure because a known amount of insulin is infused thus we can objectively measure insulin sensitivity and insulin response. This is unlike the OGTT, which is reliant on endogenous insulin, and this is variable.

OGTT are often used in large epidemiological studies to estimate insulin sensitivity and response, since it is more physiological than the FSIGT as glucose is ingested. There is a need to determine the agreement between OGTT and FSIGT, since FSIGT is more strongly correlated to the gold standard (euglycaemic hyperinsulinaemic clamp) in our setting.

The unique constellation of body composition, menopause and HIV status, all of which may have separate relationships with insulin resistance in black African women give rise to core questions in my thesis on the associations of the high prevalence of type 2 diabetes. Hence, aim of this thesis is twofold, firstly to examine the differences in body fat distribution and insulin sensitivity and response, measured using oral glucose tolerance tests (OGTT) and frequently sampled intravenous glucose tolerance tests (FSIGT), between pre- and post-menopausal women living with and without HIV. Secondly, to explore how abdominal and gluteal adipose tissue expression of adipokines, inflammatory, glucocorticoid and lipid metabolism genes differ by menopause and HIV and how these gene associate with insulin sensitivity

The aim of this thesis was addressed in three results chapters. The research questions and their main findings are summarised in Table 6.1 below.

Table 0.1: Summary of the findings from the thesis

Chapter	Objectives	Key Findings
3	• To compare regional fat measurements by DXA and anthropometry • To compare OGTT- and FSIGT-derived measures of insulin sensitivity and response and beta cell function in pre- and post-menopausal women, with and without HIV.	• WC was associated with DXA-derived VAT and android fat while hip circumference was associated with DXA-derived gynoid fat mass. • There was homoscedasticity (no proportional bias) between these anthropometric and DXA-derived measures of fat distribution. • There were significant correlations and homoscedasticity between measures of insulin sensitivity, insulin response and disposition index measured using OGTT and FSIGT.
4	• To determine the differences in body fat distribution and sex hormones between pre- and post-menopausal women with and without HIV. • To determine the differences in glycaemia, insulin sensitivity and response between pre-and post-menopausal women with and without HIV.	• There were no differences in total body fatness and only VAT was higher in postmenopausal compared to premenopausal women. • Irrespective of menopause status, there was a greater insulin response, measured using both the OGTT and FSIGT, and a tendency for lower insulin sensitivity when using FSIGT-derived S_I in women LWHIV compared to those without HIV. Despite these differences in insulin response, measures of glycaemia did not differ by HIV status. • Insulin sensitivity and response did not differ by menopausal status, but postprandial glycaemia was higher in post-menopausal women compared to their premenopausal counterparts and this was independent of the higher VAT in postmenopausal women.

5	• To determine the association between regional body fat and insulin sensitivity in pre- and post-menopausal women LWHIV and without HIV. • To determine differences in adipokines, inflammatory, glucocorticoid and lipid metabolism gene expression between abdominal and gluteal SAT depots, in pre- and post-menopausal women with and without HIV. • To determine the association between adipokines, inflammatory, glucocorticoid and lipid metabolism transcripts and insulin sensitivity. • To determine abdominal and gluteal SAT adipocyte cell size in a sub-sample of pre- and post-menopausal women LWHIV with and without HIV.	• Abdominal fat (android fat and VAT mass) was associated with lower insulin sensitivity, independent of FMI, while peripheral fat (gynoid and leg fat) was not associated with insulin sensitivity. • Markers of adiposity (VAT, SAT, circulating leptin, aLEP and gLEP) and inflammation (hsCRP and IL-6) were inversely associated with insulin sensitivity, while circulating adiponectin and SHBG, and gluteal mRNA expression of ADIPOQ, LPL, PPARγ, ERα, and ERβ were positively associated with insulin sensitivity. • The gluteal depot had a smaller proportion of small adipocytes, but a larger proportion of large adipocytes compared to the abdominal depot. There were no differences in cell size between menopause groups, however women LWHIV had a larger proportion of smaller cells and a smaller proportion of large cells in the abdominal depot compared to HIV negative women.

6.1 Summary of key findings

A number of studies have demonstrated that menopause is associated with an increase in adiposity and the redistribution of fat from the periphery to the central regions [217,218]. Despite showing the expected differences in sex hormones (lower E_2 and higher LH and FSH in postmenopausal women) between pre- and postmenopausal women, I did not show direct associations between the sex hormones and the difference in body fat in postmenopausal women. I only showed that postmenopausal women presented with higher BF% and VAT but gynoid fat% and leg fat% did not differ compared with women in the pre-menopausal state. This suggests a preferential VAT accumulation in postmenopausal women, which is consistent with observations in other populations [285].

Furthermore, studies in African American and African women have shown that an increase in VAT is a major determinant of insulin resistance and T2D risk in post-menopausal women [75,219]. Although VAT was strongly associated with insulin sensitivity in the whole sample (chapter 5), I did not observe differences in insulin sensitivity and response between pre- and postmenopausal women. However, postprandial glucose only was higher in postmenopausal compared to premenopausal women, but this was independent of the higher VAT in postmenopausal women. I also did not show differences in glucose effectiveness, which may have explained these results. This suggest that there may be some degree of reduced glucose tolerance without effects on insulin sensitivity and response or glucose effectiveness, which requires further investigation.

The notion that menopause alters adipose tissue biology, with an effect on systemic insulin sensitivity is not consistent with the findings of this thesis. In chapter 5, I showed that there were no differences in SAT expression of adipokines, glucocorticoid and lipid metabolism genes in either the gluteal or the abdominal depots, between pre- and postmenopausal women. However postmenopausal women had greater SAT expression of the M1 adipose tissue macrophages marker, CD11c, in both abdominal and gluteal depots, suggesting higher abundance of the inflammatory type of macrophages in adipose tissue of postmenopausal women. However, this 'local' inflammation response was not coupled with greater systemic inflammatory markers such as CRP and IL6 in plasma. Although, M1 adipose tissue macrophages have been shown to be associated with insulin resistance [270,271], I did not

find an association in this study. Additionally, menopause did not affect adipocyte cell size either in abdominal or in gluteal adipose tissue.

Additionally, there were no interaction associations between HIV infection and menopause on measures of glycaemia, insulin sensitivity or response, suggesting that postmenopausal women LWHIV are not at greater risk of developing T2D than the other groups. This contradicts the hypothesis that diabetes risk is exacerbated by HIV infection in postmenopausal women.

The novel and important finding of this thesis was that women LWHIV had hyperinsulieamia compared to those without HIV. I further showed that this observed hyperinsulineamia was due to increased insulin secretion rather than insulin clearance. Commensurate with the increased insulin response, I found a tendency for lower insulin sensitivity in women LWHIV when using the FSIGT but not when using the OGTT and despite these differences, there were no differences in glycaemia between those with and without HIV. Importantly, hyperinsulineamia may lead to insulin resistance and T2D [33], however this requires further investigation.

Women LWHIV had lower whole-body adiposity as indicated by the lower BF%, FMI and SAT compared to HIV negative women. Although HIV infection has been associated with fat distribution changes [286], there were no differences in regional fat distribution between the HIV groups, except for lower abdominal SAT in women LWHIV, suggesting that there were no overt lipodystrophic signs in the women LWHIV. Further, women LWHIV had a larger proportion of smaller adipocyte cells and a lower proportion of large cells in the abdominal depot compared to HIV negative women (chapter 5), which, on the one hand could reflect lower fat mass, but on the other hand has also been suggested to be independently related to a higher insulin sensitivity [261]. However, the findings from chapter 4 showed a tendency for lower insulin sensitivity (FSIGT-derived) in women LWHIV compared to their HIV negative counterparts, which was accompanied by a greater insulin response in women LWHIV (chapter 4). So, if there was a causal association between small adipocytes and insulin sensitivity, the women LWHIV must have another intrinsic insulin-sensitivity dampening mechanism overriding the effect of adipose tissue function. Alternatively, the observation of the smaller adipocytes in women LWHIV could reflect an HIV/ART-dependent effect in adipose tissue disabling the full maturation of the adipocytes. These interesting and opposing hypotheses will require further studies with larger numbers. Moreover, I showed that

abdominal and gluteal SAT expression of adipokines differed between HIV groups such that ADIPOQ was higher and conversely LEP was lower in women LWHIV than HIV negative women (Chapter 5). Furthermore, over-expression of ADIPOQ has been shown to lead to increased adipose tissue mass by increasing the number of adipocytes rather than cell size [259], which supports our observation of greater abdominal ADIPOQ and greater proportion of small adipocytes in the abdominal depot. Additionally, ART drugs such as NNRTIs, may contribute to adipose tissue alterations in antiretroviral-treated patients. In particular, efavirenz was shown to have profound dose-dependent repression of adipocyte differentiation that was associated with down-regulation of the master adipogenesis regulator genes SREBP-1, PPARγ and C/EBPα, and their target genes encoding lipoprotein lipase, leptin and adiponectin, which are key proteins in adipocyte function [287]. Indeed the women LWHIV were using ART that included efavirenz, which could have contributed to the adipocyte size in women LWHIV.

Interestingly, I showed that women LWHIV had higher circulating levels of E_2 and SHBG, lower free testosterone and testosterone bioavailability compared to HIV negative women. There are inconsistent findings in literature regarding levels of E_2 in women LWHIV, with some showing low levels while others showing no differences [224]. In contrast, high levels of SHBG are consistently shown in those LWHIV, and irrespective of ART [225]. High levels of SHBG were shown to be strongly associated with increased insulin sensitivity in this population, thus I would expect insulin sensitivity to be higher. It is proposed that the association between SHBG and insulin sensitivity is causal in nature through modulating the biologic effects of sex hormones (testosterone and oestrogen) on peripheral tissues [282]. However, the results are contradictory as I have showed a tendency for lower insulin sensitivity in the women LWHIV despite higher SHBG, which requires further investigation.

6.2 Strengths, limitations and future research

Due to the age of the cohort and the strict inclusion criteria, there were limited numbers of premenopausal women particularly in the group of those LWHIV. Based on our sample size of 92 participants, I completed a power analysis using 80% power and an alpha of 0.05. I estimated that I required 23 participants per group to have sufficient power to complete a two-way ANOVAs exploring the effects of menopause and HIV status and their interaction.

Based on this analysis, the study was not sufficiently powered to explore interaction effects, however it was sufficiently powered to explore main effects of HIV status and menopause status, for which we found significant associations. Thus, caution should be taken for the interpretation of interaction effects. Additionally, no Bonferroni correction to account for the multiple testing was performed. These analyses can be regarded as pilot testing to guide future research with more powered studies. However, to avoid concerns around multiple testing in Chapter 5 of this thesis, we used multivariate OPLS since large numbers of corelated dependent variables can be modeled against a single independent variable.

Nonetheless, this is a unique study that for the first time explores differences in differences in glycaemia, insulin sensitivity and response between pre- and post-menopausal women and explored the interaction with HIV, using both OGTT- and FSIGT derived indices. Although the EHC is considered a gold standard for measuring insulin sensitivity, in this study I wanted to explore both insulin sensitivity and secretion. Indeed, including FSIGT-derived indices of insulin sensitivity, response and disposition index was a strength of this study as these measures are more precise measures of insulin sensitivity and response, are strongly correlated to gold standards and are able to distinguish subtle differences that may not otherwise be observed when using OGTT. However, the OGTT provides a more physiological response and is influenced by gastric emptying, glucose absorption, insulin secretion and incretin hormones, [47]. Thus, there are benefits to using both OGTT and FSIGT. Another strength is the inclusion of C-peptide measures in the OGTT thus I was able to distinguish between hepatic insulin clearance and insulin secretion. Unfortunately, I did not have these measures for the FSIGT, which would have improved the understanding of the relationship between insulin secretion and insulin clearance. In addition, we only excluded participants with T2D, but included those with IFG and IGT, which could have affected their insulin secretion capacity and their insulin sensitivity thereby impeding our ability to predict diabetes risk.
Thus, future studies should explore these hypotheses in women with NGT.

This study also included DXA measures of body composition and body fat distribution, in addition to basic anthropometric measures of body fat distribution, since DXA provides an estimate of VAT, total and regional adiposity and is objective. Although the MRI is considered the gold standard of measuring VAT, DXA measured VAT has been shown to correspond well.

Although the prevalence of obesity is higher in SA women compared to men, recent research indicates that obesity in men is on the rise [288] which is of concern since the strength of the association between adiposity and diabetes risk is greater in men than women[289]. This study only included women; thus, the results should be validated in men since men have a different body fat distribution that may alter their disease risk.

In addition, this is the first study that has examined both gluteal and abdominal SAT from these four groups of women, however only limited number of selected transcripts were analysed using RT-PCR and did not include latest technologies such as RNA Sequencing. RNA Sequencing would allow for the analysis of differential gene expressions at a much broader dynamic range, which could potentially allow for the exploration of different pathways. Prospective studies should include non-targeted techniques of RNA quantification, such as array techniques or total RNA sequencing methods to explore different biological pathways and genes.

Furthermore, future studies should identify the cause and biological mechanisms by which women LWHIV have hyperinsulieamia, including prognostic implications for future diabetes risk. The majority of the women LWHIV in this study were on an ART regimen that included a combination of nucleotide analogue reverse transcriptase inhibitor (NtRTI), non-nucleoside reverse transcriptase inhibitor (NNRTI) and nucleoside reverse transcriptase inhibitor (NRTI)). Unfortunately, we cannot separate the effect of HIV and ART in this population, as the majority of women were on HIV therapy.

Prospective studies should explore the effects of the new ART regimens that include INSTI on changes in body fat distribution, diabetes risk as well as adipose tissue biology, since they have been shown to cause excessive weight gain and treatment emergent obesity than non-INSTI-based regimens and may increase the risk of weight-related co-morbidities [136]. Lastly, despite the age difference of only 6 years, there were significant sex hormone differences between the premenopausal and the postmenopausal women, however one may expect to see greater differences if younger women were selected for the premenopausal group these differences could be explored in younger premenopausal populations in future. It is also important to note that this study was cross-sectional; thus, causality could not be inferred. Future longitudinal studies may provide better evidence of causality.

6.3 Conclusion

In conclusion, this study has provided evidence that postmenopausal women have greater postprandial glycaemia than premenopausal women, however this was independent of the higher VAT in postmenopausal women. This supports the hypothesis of a preferential increase in abdominal adiposity in postmenopausal women. This study demonstrates for the first time that insulin response to glucose was higher in women LWHIV, irrespective of their menopausal status. However, the cause and significance of this higher insulin response in women LWHIV requires further investigation. Moreover, despite a tendency for lower insulin sensitivity, women LWHIV had greater expression of adiponectin in both abdominal and gluteal depots, a corresponding lower expression of leptin and lower abdominal cell size ratio compared to HIV negative women which requires further exploration. Lastly, irrespective of menopause and HIV status, gluteal adipogenic transcription factor and (PPARγ) and PPARγ-responsive genes (LPL and adiponectin) were associated with favourable insulin sensitivity, whereas markers of adiposity (android fat, circulating leptin, abdominal LEP and gluteal LEP) were associated with low insulin sensitivity. Thus, future research should explore biological pathways involved in SAT expression and insulin sensitivity in this population.

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Appendices

Appendix A : Supplementary tables

Table S.1 Transcripts Ct mean and SD.

Transcript	Ct Mean	Ct SD
ADIPOQ	23.88	0.17
LEP	25.53	0.11
CD11c(ITGAX)	32.04	0.16
CD206 (MRC1)	32.18	0.13
IL6	35.07	0.33
LPL	24.44	0.12
PPAR γ	29.25	0.14
NR3C1	28.77	0.14
ESR1	30.94	0.13
ESR2	32.59	0.15

Appendix B: Ethical Clearance Certificate

R14/49 Miss Maphoko Adelade Masemola et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M161103

NAME: (Principal Investigator)	Miss Maphoko Adelade Masemola et al
DEPARTMENT:	MRC/Wits Developmental Pathways for Health Research Unit Chris Hani Baragwanath Academic Hospital
PROJECT TITLE:	Type 2 Diabetes Mellitus (T2D) Risk in Middle-Aged Black South African (SA) Women: Dissecting the Role of Menopause, HIV Infection and Adipose Tissue Biology
DATE CONSIDERED:	25/11/2016
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof Julia Goedecke and Prof Lisa Micklesfield
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	29/03/2017

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 301, Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, 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 November and will therefore be due in the month of November each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix C: Informed consent

PARTICIPANT INFORMATION SHEET 1

Determinants of type 2 diabetes mellitus (T2D) risk in middle-aged black South African (SA) men and women: dissecting the role of sex hormones, inflammation and glucocorticoids

Invitation

Hello, my name is Lisa Micklesfield and I am an Associate Professor at the MRC/Wits Developmental Pathways for Health Research Unit (DPHRU) from the University of the Witwatersrand.

I would like to invite you to participate in the research study entitled; Determinants of type 2 diabetes mellitus (T2D) risk in middle-aged black South African men and women: dissecting the role of sex hormones, inflammation and glucocorticoids. Before, agreeing to participate, it is important that you understand the purpose of the study, the study procedures, benefits and risks as well as your right to withdraw from the study at any time. If you have any questions, do not hesitate to ask me. You should not agree to take part unless you are satisfied with all the procedures involved. If you decide to take part in this study, you will be asked to sign this document to confirm that you understand the study. You will also be given a copy to keep.

Why is the study being done?

Studies have shown that body composition (the amount of muscle and fat in your body) and body fat distribution (where the fat is located in your body, for example, fat around your stomach) changes as one gets older, and that this is associated with an increased risk of certain diseases such as type 2 diabetes (sugar disease). However, most of these studies were undertaken in white populations from developed countries. It is important to study this in the black African population as many factors such as socio-economic status, physical activity and nutrition, are specific to this population, and may have an important impact on how your body works and how this changes with aging. Further, it is not known how body fat distribution and the risk of diabetes is affected by HIV infection, which is known to change body fat and its distribution. This study will examine different changes that happen in the body with aging and how these influence body fat distribution and type 2 diabetes risk in middle-aged black men and women. The factors that we are interested in examining include male and female sex hormones (for example, testosterone and oestrogen), inflammatory markers (involved in the immunity) and circulating cortisol (stress hormone).

Who can participate?

We are going to be testing women who participated in the Birth to Twenty Study of Women Entering and in Endocrine Transition (SWEET study) between 2011 and 2014, and men who participated in the H3Africa/AWIGEN study in 2014.

You will only be eligible for this follow-up study if you participated in these studies.

What will happen if you decide to take part in the study?

If you meet the criteria and decide to take part in the study, you will be required to complete two testing sessions described below, at DPHRU at Chris Hani Baragwanath Hospital in Soweto.

You are under no obligation to take part in the study and are not required to give a reason if you do not wish to participate. If you decide to take part in the study, you are free to withdraw at any time and without giving a reason and without prejudice. If you decide to withdraw from the study, we will discuss with you what will happen to any information or samples that you have provided. If the incomplete samples and information can usefully contribute to the study, we will ask your permission to store them and use them in our analysis. Alternatively, on your request all your information and samples will be destroyed.

Testing Session 1: Testing at any time of the day and will take 2 hours of your time.

You will be requested to complete a series of questionnaires by interview, which will include questions on measures of socioeconomic status (i.e. housing, employment and income), personal and family history of disease (e.g. diabetes, obesity, high blood pressure, and cardiovascular disease), reproductive history, access to food, and stress. In addition, questions on lifestyle factors, including smoking and alcohol intake, medication and supplement use will be included. Furthermore, your dietary food intake over the past 7 days will be measured using a questionnaire that lists all possible foods. You will also be asked to wear two small devices, one on your hip and one on your thigh, for 7 days to measure your physical activity and sedentary time. Your weight and height will be measured, as well as your waist and hip circumferences. In addition, you be required to undergo a scan to measure your body fat, muscle mass, and bone density, using a special X-ray scan. If there is any risk that you may be pregnant, you will be requested to have a pregnancy test prior to the scan, as the scan will expose you to a small amount of radiation. Your blood pressure will also be measured and you will also be requested to have an HIV test. You will also be required to bring your ID document, clinic card as well as any medication that you are currently taking.

Further details of these procedures are provided below:

Demographic, socioeconomic status and lifestyle questionnaire

You will be asked questions about various measures of social and economic status (e.g. if you are employed or not, what do you do at work, your source (s) of income) and questions about whether there are people in your family with diseases such as high blood pressure or heart problems. You will also be asked questions about your personal health, stress and reproductive history. In addition questions on lifestyle factors including smoking and alcohol intake, medication and supplement use will be included. We will fill in the answers for you. You can skip any questions that make you uncomfortable.

Food frequency questionnaire (FFQ)

You will also be asked to fill in a food frequency questionnaire to measure your usual dietary intake (what you normally eat). This questionnaire will give us a sense of what and how much you have eaten over the last week. In addition, we will ask you a few questions about your food security, in other words, your access to food and if you or your family ever experience periods of hunger.

Body composition and DXA (dual-energy X-ray absorptiometry) measurements

Your weight, height, waist and hip circumference will be measured as part of your body composition assessment. In addition, you will undergo a special X-ray scan (DXA) that will tell

us about your muscle mass, body fat and bone density. The scan will take approximately 20 minutes to perform during which you will lie quietly on the scanning table in a medical gown provided. You will be asked whether or not you are pregnant. If there is any possibility that you may be pregnant please tell the technician and we will perform a pregnancy test. If you are pregnant, you will not have the scan. The only risk associated with the DXA scan is exposure to radiation. However, the radiation exposure with a DXA scan is less than half that of a chest x-ray.

Blood pressure:

After a 5-minute relaxation period, blood pressure will be measured 3 times in a row, separated by 5 minutes of relaxation between readings. A standard blood pressure monitor will be used.

Physical activity

You will be asked to wear two motion sensors (accelerometer and activPAL) for 7 days, to measure your activity and sedentary patterns. They are the size of a small matchbox, and one is worn on the waist with a lightweight belt and the other is attached to the thigh using a waterproof plaster. You will be instructed on how to use the monitors. There are no side-effects associated with wearing the monitors.

Grip strength

Your grip strength will be measured using a hand dynamometer. In a standing position you will be required to squeeze the dynamometer with as much force as possible with your non-dominant hand. You will be required to do this 3 times with a 10-20 second rest in between.

Sleep

To help us understand your personal sleep habits, we will ask you to complete three questionnaires relating to your sleep quality, daytime sleepiness levels and your chronotype (i.e. your personal preference for mornings or evenings). You will also keep a sleep diary for one week. Together these will allow us to measure your sleep habits such as how long you sleep for, when you go to bed and wake up and how efficient your sleep is.

HIV test

We would like to test your HIV status. This is very important as it may have effects on your body composition, risk for diabetes and the factors that we will measure in your blood. The HIV test is voluntary, however If you have previously been tested as HIV positive this is not necessary. If you refuse the test, you will not be able to participate in the study. It is also important to note that if you decide to test, you will be given pre-test counseling. This test is always strictly confidential and can only happen if you agree. There is no way in which anyone can link your HIV status to your name as all results in this study are coded with a number. No one including your doctor, family, or work colleagues will be told about this test without your permission. The advantage of a rapid test is that you do not have to return to get your test result. Results will be available when your check out, after all the other tests, measurements and questionnaires are completed.

You will have your finger pricked with a sterile needle and the drop of blood will be tested on specific HIV testing kits to check for HIV antibodies. Test results will be given to you in private by a registered trained counsellor. If the report states negative it means that there are no antibodies to HIV. The window period will be explained. If the report states positive, it means that you are HIV positive and that there are antibodies to HIV. You will be given a letter to refer you to a clinic specialising in HIV treatment and you will be given a second test at the clinic to confirm this result. Sometimes we cannot clearly tell if the results are negative or positive. We will then refer you to a clinic specialising in HIV treatment and you will be given a second test at the clinic to confirm this result.

There is a chance that some of the questions in the questionnaire might trigger some emotional distress. If this happens, we will refer you to our counselling nures on site and she will make the necessary appointment for you to see a psychologist at the Psychology Unit at Chris Hani Baragwanath Hospital

Testing session 2: Testing in the early morning (between 7:30 and 8:00 am) and will take 2½ hours of your time

You will be requested to visit the DPHRU offices at the Chris Hani Barangwanath Hospital between 7:30 and 8:00 am in the morning approximately 7 days after testing session 1, but after an overnight fast (nothing to eat or drink, except water, from 10pm the night before). You will be asked to donate a sample of blood and then undergo an oral glucose tolerance test (OGTT) to determine whether you are at risk for developing diabetes. You will also be required to return the two physical activity monitors that were given to you in the first testing session.

Blood sampling and oral glucose tolerance test (OGTT)

Blood sampling and the OGTT can only be performed in the morning after an overnight fast. Therefore it is important that you do not eat or drink anything (except water) from 10 PM the night before, or for 10-12 hours before your test begins. You are not allowed to take any medication, food or drink, chew gum or sweets, before your blood test.

Blood sampling will be performed by inserting a small plastic tube into a vein in your arm and a small plastic tap or valve will be attached onto it so that blood samples can be drawn before the 2 hour OGTT test. The first blood sample (50 ml) will be drawn for the measurement of blood lipids (fats), glucose (sugar) and various hormones (e.g. insulin and cortisol) and inflammatory markers in the fasted state (i.e. before you eat anything). You will then be asked to drink a cup of water containing 75 g of glucose (sugar). Thereafter, blood samples (5 ml or 1 teaspoon each) will be drawn from your arm at 30, 60, 90 and 120 min after the glucose ingestion (total 20ml, 4 teaspoons) for the later measurement of changes in glucose and insulin levels. After your test you we will give you something to eat and drink.

What are the risks and discomforts of this study?

There are no risks or discomforts associated with the administration of the questionnaires. Strict confidentiality of results will be maintained. Your name will be removed from all data, and you will be assigned a number, which will be used to identify data relating to you. All records will be kept in a locked room and in a secure computer database in the research unit. Your name will not be used in any publication of the results.

There are no risks associated with the use of the physical activity monitors. The only risk associated with the DXA scan is exposure to radiation. However, the radiation exposure with a DXA scan is less than half that of a chest x-ray (11.3 microSieverts).

There are no appreciable risks associated with the fasting blood sampling and OGTT, other than those associated with routine blood sampling. Sometimes when blood is taken you may feel a prick at the place where the needle enters your body. Afterwards there may be a little bruise, and pain (which are associated with normal blood sampling), and in very unusual circumstances, local infection. Very occasionally participants may 'faint'. This is a stress response to a trigger (e.g. the sight of blood) and has no long-term effects. This test is used routinely for both research and medical purposes. The total amount of blood drawn will be 70 ml, which is substantially less ($1/7^{th}$) than that of a standard blood donation (500 ml). All procedures will be supervised and carried out by a nursing sister and appropriately trained medical personnel using sterile techniques to minimise any risks of infection.

If we discover that you have any health problems based on our tests, you will be contacted and referred to the appropriate clinic for treatment and/or management.

Are there any benefits to you for being in the study?

You will receive your own results from the study, including your body composition (e.g. muscle and fat mass), your blood pressure, lipids (fats in your blood) and glucose tolerance (your risk for diabetes). In addition, you will contribute to our understanding of the how body fat distribution and the risk for type 2 diabetes differs between black South African men and women (pre-menopausal and post-menopausal), with and without HIV. This information can be used to provide evidence for future strategies for the prevention, treatment and management of diabetes risk in middle-aged black South Africans.

What will happen when the study is over?

Detailed analysis of the samples will take some time, but once these analyses have been completed, the final results of the study will be shared with you. In addition, the results of the study will be published in scientific journals, as well as in the local media. Your name will not be used in any publication of the results. You may be contacted for a follow-up study.

Will you receive reimbursement for transport?
You will receive R150 to cover transport costs to DPHRU for each of the two testing sessions. The transport money will be paid to you at the end of each session.

Who will see the information that is collected about you during the study?

Strict confidentiality of results will be maintained. Your name will be removed from all data, and you will be assigned a number, which will be used to identify data relating to you. All records will be kept in a locked room and in a secure computer database in the research unit. Your name will not be used in any publication of the results.

Who do I contact if I have any questions about the study?

If you have any questions or you experience any problems during or after the tests, please contact Associate Professor Lisa Micklesfield or Associate Professor Julia Goedecke, or a representative of the Human Research Ethics Committee.

Associate Professor Lisa Micklesfield (PhD)
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Associate Professor Julia Goedecke (PhD)
Principal Investigator
Honorary Associate Professor, School of Clinical Medicine, Wits University
Address: Room 230 RIND Building, South African Medical Research Council, Francie van Zijl Drive, Parow, 7725, Cape Town
Tel: 021-9380862(w) 0828255616 (cell)
Email : julia.goedecke@mrc.ac.za

Human Research Ethics Committee contact details:
Prof P Cleaton-Jones, Tel 011 717 2301, email peter.cleaton-jones1@wits.ac.za or Ms Zanele Ndlovu/ Mr Rhulani Mkansi/ Mr Lebo Moeng Administrative Officers 011 717 2700/2656/1234/1252 zanele.ndlovu@wits.ac.za; Rhulani.mkansi@wits.ac.za; and Lebo.moeng@wits.ac.za

INFORMED CONSENT 1

Determinants of type 2 diabetes mellitus (T2D) risk in middle-aged black South African (SA) men and women: dissecting the role of sex hormones, inflammation and glucocorticoids

Consent to participate in the study:

"I, _____, hereby give consent to participate in this research trial to be conducted by DPHRU, within the Department of Paediatrics at the University of Witwatersrand.

I understand that the study will involve completion of questionnaires by interview, an HIV test, routine body measurements (i.e. height, weight, hip and waist circumference), an X-ray scan to measure my body fat and muscle mass, and bone density, blood pressure, grip strength, collection of blood samples after an overnight fast (10-12 hours) and during a 2-hour oral glucose tolerance test, as well as wearing an accelerometer and ActivPAL for 7 days to measure physical activity and sedentary time, respectively. The purpose and all the details of this study have been explained to me.

I have read and have had explained to me the procedures described. I have had an opportunity to ask questions and my questions have been answered in a satisfactory way.

I understand that all the information collected during the study will be treated with the strictest confidentiality and will only be used for scientific research purposes. All samples will be kept in a freezer in a secure facility with access limited to research personnel. All records will be kept in a locked room and in a secure computer database in the research unit. My name will not be used in any publication of the results. I understand that for data verification and quality control purposes regulatory authorities and/or members of the Wits Human Research Ethics Committee (Medical) may be allowed access to my personal data under conditions of strict confidentiality.

I understand that I may be contacted for a follow-up study.

I agree to participation in the study on the condition that:

1. I can withdraw voluntarily from the study at any time and that no adverse consequences will follow on withdrawal from the study.

2. I have the right not to answer any or all questions posed in the questionnaire.

3. The University of the Witwatersrand's Human Research Ethics committee has approved the study protocol and procedures.

4. All results will be treated with the strictest confidentiality.

5. Only group results, and not my individual results, will be published in scientific journals and in the media.

6. The study scientific team are committed to treating participants with respect and privacy through interviews conducted in private and follow-up counselling available on request.

7. I will receive a referral note to psychological services if I experience any psychological distress during the study.

I have read the information, or it has been read to me. I have had the opportunity to ask questions about it and my questions have been answered to my satisfaction. I consent voluntarily and understand that I have the right to withdraw my consent without this affecting the current research study or my medical care.

Print Name of Participant _____

Signature of Participant _____

Date _____

I have accurately read or witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

RESEARCH ASSISTANT:

Printed Name Signature/Mark or Thumbprint
Date and Time

Copy provided to participant _____ (initialed by researcher)

WITNESS: (If applicable)

Printed Name Signature/Mark or Thumbprint
Date and Time

PARTICIPANT INFORMATION SHEET 2

Determinants of type 2 diabetes mellitus (T2D) risk in middle-aged black South African (SA) men and women: dissecting the role of sex hormones, inflammation and glucocorticoids

Information sheet:

Will any of your blood samples be stored and used for research in the future?

The researchers will ask your permission to store your blood samples for future research indefinitely. Also, we will be extracting DNA from your blood and this will be stored indefinitely. All samples will be kept in a freezer in a secure facility with access limited to research personnel only; all records will be kept in a locked room and in a secure computer database in the research unit. Your name will not be used in any publication of the results. For data verification and quality control purposes regulatory authorities and/or members of the University of the Witwatersrand Human Research Ethics Committee may be allowed access to your personal data under conditions of strict confidentiality.

Future research analyses on the stored blood samples and DNA will be based on new research that we are at present not aware of, but may be important in our understanding of the risk for disease. Any research done in the future will only be executed once it has been approved by the Human Research Ethics Committee (Medical) of the University of Witwatersrand that is set up to determine that the research is done according to accepted standards. You will not be penalized in any way for not allowing the use of your blood or tissue for future research. If you decide not to donate blood for future research, it will be destroyed on completion of this study.

When entering into the study, you will receive a unique code that will be used for sample and data analysis, which serves to maintain your confidentiality. When storing samples, you may choose that we keep the unique code on the sample so that we can link any new results to your existing data. If any clinically relevant information relating to this sample is found, we will inform you of the results. Alternatively, you can remove the identifying number, so that your information will not be linked to the sample and you will not be informed of any clinical results relating to the new analyses.

Associate Professor Lisa Micklesfield (PhD)
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Soweto Johannesburg
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Associate Professor Julia Goedecke (PhD)
Principal Investigator
Honorary Associate Professor, School of Clinical Medicine, Wits University
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Email : julia.goedecke@mrc.ac.za
Human Research Ethics Committee contact details:
Prof P Cleaton-Jones, Tel 011 717 2301, email peter.cleaton-jones1@wits.ac.za or Ms Zanele Ndlovu/ Mr Rhulani Mkansi/ Mr Lebo Moeng Administrative Officers 011 717 2700/2656/1234/1252 zanele.ndlovu@wits.ac.za; Rhulani.mkansi@wits.ac.za; and Lebo.moeng@wits.ac.za

PARTICIPANT INFORMATION SHEET 2

INFORMED CONSENT 2

Determinants of type 2 diabetes mellitus (T2D) risk in middle-aged black South African (SA) men and women: dissecting the role of sex hormones, inflammation and glucocorticoids

Certificate of Consent:

1) If any of the **BLOOD** that I have provided for this research project is unused or leftover when the project is completed

   ☐ I give permission for my **blood** sample to be stored indefinitely

   AND if my **blood** sample is to be stored:
   ☐ I give my permission for my **blood** sample to be stored and used in future research of any type, which has been properly approved

   AND if any research on my **blood** sample cannot easily be done in South Africa:
   ☐ I give my permission for a portion of my **blood** sample to be sent out of the country for analysis if appropriately approved

I have read the information, or it has been read to me. I have had the opportunity to ask questions about it and my questions have been answered to my satisfaction. I consent voluntarily and understand that I have the right to withdraw my consent without this affecting the current research study or my medical care.

Print Name of Participant _____

Signature of Participant _____

Date _____

I have accurately read or witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

RESEARCH ASSISTANT:

Printed Name Signature/Mark or Thumbprint
Date and Time

Copy provided to participant _____ **(initialed by researcher)**

WITNESS: (If applicable)

Printed Name Signature/Mark or Thumbprint
Date and Time

INFORMED CONSENT 3

Determinants of type 2 diabetes mellitus (T2D) risk in middle-aged black South African (SA) men and women: dissecting the role of sex hormones, inflammation and glucocorticoids

The information around the blood sample taken from me and the DNA that will be extracted from the blood is clear and the purpose of consent is for me to inform the study what they can or cannot do with these samples.

I understand that all procedure/tests on the stored blood and DNA samples will be approved by the Human Research Ethics Committee of the University of the Witwatersrand.

YES ☐ NO ☐

I am in agreement that my DNA may be stored and used for the purposes described above.

YES ☐ NO ☐

I am in agreement that the data generated from my DNA may be made available as stated above.

YES ☐ NO ☐

I am in agreement that the information I have supplied in the list of questions and the information from the tests and measurements taken from me may be used as stated above.

YES ☐ NO ☐

I agree that a small bit of my DNA may be sent out of the country if the research cannot easily be done in South Africa.

YES ☐ NO ☐

I agree that an portion of my DNA may be stored in a biobank (laboratory) and that some data may be stored in a database as stipulated and that these may be shared according to the processes and procedures of the H3Africa initiative by using my study code or another code that de-identifies my sample and data.

YES ☐ NO ☐

I understand that every time a new study is done on my DNA, permission will be obtained from the ethics committee for the study to make sure that it is used only for the purposes stated above.

YES ☐ NO ☐

I understand that I will not benefit directly from the research done on my DNA.

YES ☐ NO ☐

I understand that I may withdraw from the study at any time.

YES ☐ NO ☐

PARTICIPANT

_____

Printed name Signature/Mark or Thumbprint
Date and Time

RESEARCH ASSISTANT:

Printed Name Signature/Mark or Thumbprint
Date and Time

Copy provided to participant _____ (initialed by researcher)

WITNESS: (If applicable)

Printed Name Signature/Mark or Thumbprint
Date and Time

HIV COUNSELLING FORM AND INFORMED CONSENT

Hello! My name is _____. First, we would like to discuss some matters with you. Information collected will be not be used in any identification form outside this facility. Therefore confidentiality will be maintained. We will provide you with information you need to know about HIV and AIDS. This will then be followed by information to help you understand your risk exposure to HIV and then you could be able to take an HIV test.

CLIENTS HIV HISTORY

Have you been tested for HIV before?	Yes	No	If Yes, when did you test? What was the HIV results		Negative	Positive
If positive, do you have a copy of the results	Yes	No	If no, would you like to do another test?		No	Yes
What was your reason for testing	Illness	Insurance	Partner died	Pregnancy (Females Only)	Employment	General Check up
If other please state reason						

CLIENT SUPPORT SYSTEM

Have you ever had a loss in your life?	Yes	No			
If yes,	Who				
	When				
If the test is HIV positive, will you tell someone?	Yes	No	If Yes Who?		
Who else will you tell if you are HIV positive?	Family	Partner	Friend	Other (State)	
How will you tell this person you trust?					
Do you think you will get support from that person?	Yes	No	Would you like us to offer support?	Yes	No

PRE COUNSELLING SESSION

UNDERSTANDING HIV AND AIDS

COUNSELLOR TO USE CUE CARDS FOR COUNSELLING

Understanding of HIV/AIDS, client should be explaining mode of transmission and exchange of fluids.		Meaning of Window Period (What is it?)	

Benefits for HIV Testing		Importance of knowing ones HIV status (What does it mean?)	
Meaning of HIV testing		Meaning of HIV Negative Result	
Meaning of Confidentiality. (Counsellor to clarify confidentiality)		Meaning of HIV Positive Result	
		Perception of risk to HIV exposure. (Does the client think they are at risk to HIV infection?)	

HIV TESTING

Counsellor: Explain rapid testing processes. A rapid test for HIV will be done by the DPHRU lab. About a teaspoon full of blood will be collected (5ml to 10ml) and tested on specific HIV testing kits to check for HIV antibodies. Test results will be given to you in private when you check out today by a registered trained counsellor. If the report states negative it means that there are no antibodies to HIV. The window period will be explained. If the report states positive, it means that you are HIV positive and that there are antibodies to HIV. You will be given a letter to refer you to a clinic specialising in HIV treatment and you will be given a second test at the clinic to confirm this result. Sometimes we cannot clearly tell if the results are negative or positive. We will then repeat the test and if it is still indeterminate we will refer you to a clininc for a second testing that will help confirm the results

PATIENT CONSENT

I agree to have the HIV Rapid test. The procedure to be carried out has been explained to me. The possible discomforts, risks and benefits involved in taking part in the test have also been described to me. I understand that I can leave the study at any point. I also understand that if I have any questions concerning the test then the investigator will explain these to me.

Date: _____ Patient: _____

Contact details of researchers:

Associate Professor Lisa Micklesfield (PhD)
Principal Investigator
MRC/Wits Developmental Pathways for Health Research Unit
Chris Hani Baragwanath Hospital
Soweto Johannesburg
Tel: 021-650 3153(w) 083 9433172 (cell)
Email : lisa.micklesfield@wits.ac.za or
Associate Professor Julia Goedecke (PhD)
Principal Investigator
Honorary Associate Professor, School of Clinical Medicine, Wits University
Address: Room 230 RIND Building, South African Medical Research Council, Francie van Zijl Drive, Parow, 7725, Cape Town
Tel: 021-9380862(w) 0828255616 (cell)
Email : julia.goedecke@mrc.ac.za
Human Research Ethics Committee contact details:
Prof P Cleaton-Jones, Tel 011 717 2301, email peter.cleaton-jones1@wits.ac.za or Ms Zanele Ndlovu/ Mr Rhulani Mkansi/ Mr Lebo Moeng Administrative Officers 011 717

2700/2656/1234/1252 zanele.ndlovu@wits.ac.za; Rhulani.mkansi@wits.ac.za; and Lebo.moeng@wits.ac.za

POST TEST COUNSELING SESSION

NB. COUNSELOR: Identify Client with Name and ID number against HIV Test Results

HIV NEGATIVE TEST RESULT

We spoke earlier about what HIV positive and HIV negative results mean. Explain again. Your results are back and you are HIV negative; you do not have the HIV virus in your body

COUNSELORS KEY TASKS	CLIENTS NOTES	COMMENTS
Explain the implications of the negative test result		
Identify and prioritize the behaviours that correspond to the client's risk		
Motivate the client to develop a risk reduction plan		
Encourage clients to discuss their HIV status with current and future partners		

POST TEST COUNSELLING

HIV POSITIVE TEST RESULTS

We spoke earlier about what HIV positive and HIV negative results mean. Explain again. Your results are back and you are HIV positive; you do have the HIV virus in your body

COUNSELORS KEY TASKS	CLIENTS NOTES	COMMENTS
Inform client that the test results are available		
Provide results clearly and simply		
Review the meaning of the result		
Allow the client time to absorb the meaning of the result		
Explore the client's understanding of the result		
Assess how client is coping with the result		
Acknowledge the challenges of dealing with an initial positive result		

IDENTIFY SOURCES OF SUPPORT

COUNSELORS KEY TASKS	CLIENTS NOTES	COMMENTS

Identify current health care resources		
Address the need for health care providers to know client's test result		
Explore client's access to medical services		
Identify needed medical referrals		
Discuss situations in which the client may want to consider protecting his or her own confidentiality		
Discuss options of support groups (i.e. post test club)		
Provide appropriate referrals		

REFERRAL TO OTHER PROGRAMS

Refer the client with letter to Thandekile Essien and the clinic (ZAZI VCT service at Baragwanath Hospital); she will then ensure that the client has the appropriate support.

COUNSELLORS NOTES:

_____

_____

_____

_____

_____

_____

_____

Counsellor's Signature:……

Print Name of Researcher_____

Signature of Researcher _____

Date _____

Copy provided to participant _____ (initialed by researcher)

WITNESS:
Printed name:_____ **Signature/Mark or Thumbprint**

Date and Time: _____

PARTICIPANT INFORMATION SHEET 4

Type 2 diabetes mellitus (T2D) risk in middle-aged black South African (SA) women: dissecting the role of menopause, HIV infection and adipose tissue biology

Hello, my name is Maphoko Adelade Masemola and I am a PhD student at the MRC/Wits Developmental Pathways for Health Research Unit (DPHRU) from the University of the Witwatersrand.

Thank you for completing the research testing for the study entitled "Determinants of type 2 diabetes mellitus (T2D) risk in middle-aged black South African men and women: dissecting the role of sex hormones, inflammation and glucocorticoids". This study provided valuable information on the changes in diabetes risk, body composition and lifestyle factors in middle-aged men and women. We are now interested in gaining a more detailed understanding about the **reasons** for the differences in body fat distribution between pre-menopausal and post-menopausal black South African women who are either HIV positive or HIV negative.

I would like to invite you to participate in the follow-up testing of the research study. Before, agreeing to participate, it is important that you understand the purpose of the study, the study procedures, benefits and risks as well as your right to withdraw from the study at any time. If you have any questions, do not hesitate to ask me. You should not agree to take part unless you are satisfied with all the procedures involved. If you decide to take part in this study, you will be asked to sign this document to confirm that you understand the study. You will also be given a copy to keep.

Why is the study being done?

Studies have shown that body fat distribution changes as one enters menopause, and is characterised by changing from a 'pear shape' (greater leg and buttock fat) to an 'apple shape' (greater tummy or abdominal fat). This change in body fat distribution with menopause is associated with an increased risk for type 2 diabetes (sugar disease). However, most of these studies were undertaken in white women. It is important to study this in black African women as their body fat distribution differs to that of white women, with black women having more of a 'pear shape and white women having more of an 'apple shape'. Further, it is not known how body fat distribution and the risk of diabetes is affected by HIV infection, which is known to alter body fat and its distribution. This study will examine the **reasons** for the differences in body fat distribution between pre-menopausal and post-menopausal black South African women who are either HIV positive or HIV negative by examining factors (proteins and hormones) produced within the fat cells of the tummy (abdomen) and the buttocks. We will then examine how these factors associate with the risk of type 2 diabetes in these women. The factors that we are interested in examining include oestrogen receptors (involved in female hormone functioning), inflammatory markers (involved in immunity), glucocorticoids (involved in the stress response) and factors involved in the formation of fat cells.

Who can participate?

A sub-sample of women (n=100) will be selected from the Birth-to-Twenty Caregivers who participated in Part 1 of the study. Only women who fall within these four groups and meet the inclusion and exclusion criteria (below) will be selected to participate in this study:

1) 25 pre-menopausal HIV-negative women;
2) 25 pre-menopausal HIV-positive women;
3) 25 post-menopausal HIV-negative;
4) 25 post-menopausal HIV-positive women.

Women will be selected based on the following criteria:

Inclusion criteria:

I. Pre-menopausal women within the age range 35-45 years;
II. Post-menopausal women within the age range 55-65 years;
III. All women: body mass index (BMI = weight in kg divided by height in metres squared) = 25-40 kg/m^2

Exclusion criteria:

I. Cardiovascular event, diabetes, thyroid dysfunction, inflammatory, hepatic and renal diseases;
II. Use of hormone replacement therapy; hormonal contraceptives, oral cortisone, anti-inflammatory drugs;
III. Peri-menopausal (based on blood results obtained from Part 1 of the study);
IV. Currently pregnant or lactating;
V. Tobacco use.

All pre-menopausal women will be tested during the follicular phase of their menstrual cycle (between day 1 and 10 from the start of their menstrual period).

What will happen if you decide to take part in the study?

If you meet all the criteria listed above and decide to take part in the study, you will be required to complete two testing sessions described below, at DPHRU at Chris Hani Baragwaneth Hospital in Soweto.

You are under no obligation to take part in the study and are not required to give a reason if you do not wish to participate. If you decide to take part in the study, you are free to withdraw at any time and without giving a reason and without prejudice. If you decide to withdraw from the study, we will discuss with you what will happen to any information or samples that you have provided. If the incomplete samples and information can usefully contribute to the study, we will ask your permission to store them and use them in our analysis. Alternatively, on your request all your information and samples will be destroyed.

Testing Session 1: Testing in the early morning and will take 5 hours of your time.

You will be requested to come to DPHRU in the morning (±8 am) after an overnight fast. In other words, you must not eat or drink anything, except water, from 10pm the night before (at least 10 hours). You cannot take part in any exercise training for 72 hours (3 days) before this test.

Insulin test – a measure of insulin secretion and insulin sensitivity:

A small plastic tube will be placed into a vein in each arm. You will then be required to undergo a test that will measure how much insulin your body produces and how sensitive your body is to insulin (insulin is the hormone that helps to transport glucose from your blood into your tissues, such as your muscle). We will inject a concentrated glucose solution (~ 30-60 ml, depending on your weight) into one vein over a 1-minute period. Small amounts of blood (1 teaspoon) will be withdrawn from the other arm at regular intervals (1-2 minutes) for 20 min. After 20 min, insulin will be infused into your arm, which will assist your body to take up the glucose into the cells. Further blood samples (1 teaspoon each) will be drawn from your other arm for a further 3.5 hrs. During this test, a maximum of 200 ml of blood will be drawn (less than 1/2 of the amount drawn when you donate blood). A medical doctor will perform the procedure. During the tests, you will be required to sit or lie quietly and DVDs will be provided for entertainment.

Session 2: Testing in the early afternoon and will take 1 hour of your time.

You will be requested to come to DPHRU in the early afternoon (± 2pm) after fasting for FOUR hours before the test. In other words, you must not eat or drink anything, except water for 4 hours before your visit. You may not take part in any exercise training for 72 hours (3 days) before this test.

Fat biopsies:
You will be requested to undergo a fat biopsy from your buttocks (bum) and your abdominal area (tummy). The samples will be used to analyse the factors within your fat tissue that may be associated with your body fat distribution and diabetes risk. A medical doctor will perform the biopsies. We will use local anaesthetic to numb the skin and fat underneath, which may cause a momentary sting. **Please inform us if you have had any previous reactions to any other anaesthetics, for example at the dentist.** The biopsy would then be taken by inserting a needle into the fat tissue and applying suction through the needle. Approximately 1 gram of tissue is taken out (about the size of a couple of grains of rice). After this procedure, a waterproof sterile dressing will be applied.
Refreshments will be provided after each testing session.
What are the risks and discomforts of this study?

Insulin test – a measure of insulin secretion and insulin sensitivity:
There are no appreciable risks for this test, other than those associated with routine blood sampling, including discomfort, bruising, swelling and local infection. The risks associated with the infusion of dextrose (sugar) include fluid leaking out of the vein into the surrounding tissue, which may cause pain, swelling and redness. However, this is a very rare occurrence. The infusion of insulin (hormone that controls sugar levels in the blood) may result in low blood sugar levels. However, blood glucose is monitored throughout the trial. In the unlikely event that your blood sugar drops to very low levels and/or you experience symptoms of low blood sugar (sweating, confusion, heart beating fast), a concentrated sugar solution will be infused immediately to increase your blood sugar levels, and the test will be immediately terminated. Very occasionally participants may 'faint'. This is a stress response to a trigger (e.g. the sight of blood) and has no long-term effects. All procedures will be supervised and carried out by a medical doctor and appropriately trained medical personnel using sterile techniques to minimise any risks of infection. These tests are used routinely in research to accurately determine insulin secretion and insulin sensitivity. A maximum of 200 ml of blood will be drawn during the entire study, which is less than half that drawn during standard blood donation.

Fat biopsies:
You may feel some local stinging for a few seconds after the local anaesthetic is given. You will experience some discomfort during the biopsies, and after the biopsies you may experience some bruising, which will generally feel better within 2-3 days. We have performed many fat biopsies and have had very few adverse events. There is a very small risk of infection and haematoma (large bruise). Very occasionally participants may 'faint'. This is a stress response to a trigger (e.g. the sight of blood) and has no long-term effects. Rarely there may be an allergic reaction to the local anaesthetic or to the preservative in it, methylparaben, which could cause itching and if severe, wheezing or low blood pressure that are symptoms of anaphylactic shock. Severe reactions will be treated with adrenaline, which will be available during the procedure.

Are there any benefits to you for being in the study?

There are no personal benefits to taking part in this study. However, you will contribute to our understanding of why changes in body fat distribution that occur during menopause may change the risk for developing diabetes. In addition, we would like to see how this might differ in women with and without HIV. This information can be used to tell us which are the best strategies to test in future research in order to help prevent, treat and manage diabetes risk in middle-aged black South African women.

What will happen when the study is over?

Detailed analysis of the tissue samples will take some time, but once these analyses have been completed, the final results of the study will be shared with you. In addition, the results of the study will be published in scientific journals, as well as in the local media. Your name will not be used in any publication of the results.

Will you receive reimbursement for transport, time and inconvenience?
You will receive R200 to cover your transport costs to DPHRU for each of the two testing sessions. The transport money will be paid to you at the end of each session.

Who will see the information that is collected about you during the study?

Strict confidentiality of results will be maintained. Your name will be removed from all data, and you will be assigned a number, which will be used to identify data relating to you. All records will be kept in a locked room and in a secure computer database in the research unit. Your name will not be used in any publication of the results.

Who do I contact if I have any questions about the study?

If you have any questions or you experience any problems during or after the tests, please contact Maphoko Adelade Masemola or Associate Professor Julia Goedecke or a representative of the Human Research Ethics Committee:

Maphoko Adelade Masemola
PhD student
MRC/Wits Developmental Pathways for Health Research Unit
Chris Hani Baragwanath Hospital
Tel: 0832417729 (cell)
Email : maphokomasemola@gmail.com
or
Associate Professor Julia Goedecke (PhD)
Principal Investigator
Honorary Associate Professor, School of Clinical Medicine, Wits University
Address: Room 230 RIND Building, South African Medical Research Council, Francie van Zijl Drive, Parow, 7725, Cape Town
Tel: 021-9380862(w) 0828255616 (cell)
Email : julia.goedecke@mrc.ac.za

Human Research Ethics Committee contact details:

Prof P Cleaton-Jones, Tel 011 717 2301, email peter.cleaton-jones1@wits.ac.za or Ms Zanele Ndlovu/ Mr Rhulani Mkansi/ Mr Lebo Moeng Administrative Officers 011 717 2700/2656/1234/1252 zanele.ndlovu@wits.ac.za; Rhulani.mkansi@wits.ac.za; and Lebo.moeng@wits.ac.za

INFORMED CONSENT 4

Type 2 diabetes mellitus (T2D) risk in middle-aged black South African (SA) women: dissecting the role of menopause, HIV infection and adipose tissue biology

Consent to participate in the study:

"I, _____, hereby give consent to participate in this research trial to be conducted by DPHRU, within the Department of Paediatrics at the University of Witwatersrand.

I understand that I will undergo two testing sessions at DPHRU at the Chris Hani Baragwanath Hospital in Soweto. I understand that I will undergo a test to measure insulin secretion and sensitivity, which will take approximately 5 hours of my time. I also understand that fat samples (±0.3-1 gram) will be taken from the fat stores in my abdominal (belly) and buttocks, which will take approximately 1 hour. I understand that my fat samples will be sent to the University of Oxford for analysis.

I have read and have had explained to me the procedures described. I have had an opportunity to ask questions and my questions have been answered in a satisfactory way. I understand the nature of the trial and the risks and benefits associated with my participation and that I am free to withdraw from this study at any time.

I understand that all the information collected during the study will be treated with the strictest confidentiality and will only be used for scientific research purposes. All samples will be kept in a freezer in a secure facility with access limited to research personnel. All records will be kept in a locked room and in a secure computer database in the research unit. My name will not be used in any publication of the results. I understand that for data verification and quality control purposes regulatory authorities and/or members of the Wits Human Research Ethics Committee (Medical) may be allowed access to my personal data under conditions of strict confidentiality.

I have read the information, or it has been read to me. I have had the opportunity to ask questions about it and my questions have been answered to my satisfaction. I consent voluntarily and understand that I have the right to withdraw my consent without this affecting the current research study or my medical care.

Print Name of Participant _____

Signature of Participant _____

Date _____

I have fully and carefully explained the study to the person named above and confirm that to the best of my knowledge, they clearly understand the nature, risks and benefits of taking part in the study. I confirm that I have given them an opportunity to ask questions and answered their questions to the best of my ability.

Print Name of Researcher_____

Signature of Researcher _____

Date _____

Copy provided to participant _____ (initialed by researcher)

WITNESS:
Printed name:_____ Signature/Mark or Thumbprint

Date and Time: _____

PARTICIPANT INFORMATION SHEET 5

Type 2 diabetes mellitus (T2D) risk in middle-aged black South African (SA) women: dissecting the role of menopause, HIV infection and adipose tissue biology

Information sheet

Will any of your blood samples be stored and used for research in the future?

The researchers will ask your permission to store your blood and fat samples for future research indefinitely. All samples will be kept in a freezer in a secure facility with access limited to research personnel only; all records will be kept in a locked room and in a secure computer database in the research unit. Your name will not be used in any publication of the results. For data verification and quality control purposes regulatory authorities and/or members of the University of the Witwatersrand Human Research Ethics Committee may be allowed access to your personal data under conditions of strict confidentiality.

Future research analyses on the stored blood and fat samples will be based on new research that we are at present not aware of, but may be important in our understanding of the risk for disease. Any research done in the future will only be executed once it has been approved by the Human Research Ethics Committee (Medical) of the University of Witwatersrand that is set up to determine that the research is done according to accepted standards. You will not be penalized in any way for not allowing the use of your blood or tissue for future research. If you decide not to donate blood or fat for future research, it will be destroyed on completion of this study.

When entering into the study, you will receive a unique code that will be used for sample and data analysis, which serves to maintain your confidentiality. When storing samples, you may choose that we keep the unique code on the sample so that we can link any new results to your existing data. If any clinically relevant information relating to this sample is found, we will inform you of the results. Alternatively, you can remove the identifying number, so that your information will not be linked to the sample and you will not be informed of any clinical results relating to the new analyses.

Maphoko Adelade Masemola
PhD student
MRC/Wits Developmental Pathways for Health Research Unit
Chris Hani Baragwanath Hospital
Tel: 0832417729 (cell)
Email : maphokomasemola@gmail.com
or
Julia Goedecke (PhD)
Principal Investigator
Honorary Associate Professor, School of Clinical Medicine, Wits University
Address: Room 230 RIND Building, South African Medical Research Council, Francie van Zijl Drive, Parow, 7725, Cape Town
Tel: 021-9380862(w) 0828255616 (cell)
Email : julia.goedecke@mrc.ac.za

Human Research Ethics Committee contact details:
Prof P Cleaton-Jones, Tel 011 717 2301, email peter.cleaton-jones1@wits.ac.za or Ms Zanele Ndlovu/ Mr Rhulani Mkansi/ Mr Lebo Moeng Administrative Officers 011 717

2700/2656/1234/1252 zanele.ndlovu@wits.ac.za; Rhulani.mkansi@wits.ac.za; and Lebo.moeng@wits.ac.za

INFORMED CONSENT 5

Type 2 diabetes mellitus (T2D) risk in middle-aged black South African (SA) women: dissecting the role of menopause, HIV infection and adipose tissue biology

Certificate of Consent:

1. If any of the **BLOOD** that I have provided for this research project is unused or leftover when the project is completed

☐ I give permission for my **blood** sample to be stored indefinitely

AND if my **blood** sample is to be stored:
 I give my permission for my **blood** sample to be stored and used in future research, which has been properly approved

AND if some research on my **blood** sample cannot easily be done in South Africa:
 I give my permission for a portion of my **blood** sample to be sent out of the country for analysis if appropriately approved

2. If any of the **FAT** that I have provided for this research project is unused or leftover when the project is completed

☐ I give permission for my **FAT** sample to be stored indefinitely

AND if my **FAT** sample is to be stored:
 I give my permission for my **FAT** sample to be stored and used in future research, which has been properly approved

 I give my permission for my **fat** samples to be sent to the University of Oxford for analysis,
AND if additional research on my **fat** samples cannot easily be done in South Africa:
 I give my permission for a portion of my **fat** samples to be sent out of the country for analysis if appropriately approved.

I have read the information, or it has been read to me. I have had the opportunity to ask questions about it and my questions have been answered to my satisfaction. I consent voluntarily and understand that I have the right to withdraw my consent without this affecting the current research study or my medical care.

Print Name of Participant _____

Signature of Participant _____

Date _____

I have accurately read or witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print Name of Researcher_____

Signature of Researcher _____

Date _____

Copy provided to participant _____ (initialed by researcher)
WITNESS:
Printed name:_____ Signature/Mark or Thumbprint

Date and Time: _____

Appendix D: Demographic Questionnaire

IDENTIFICA

SUBJECT CODE:

Date _____

Name _____

ID number:
_____

Date of Birth : _____ Age: _____

Physical Address:
_____
_____

_____
_____

Postal Address:
_____
_____

_____
_____

E-mail:
_____

Tel No's: _____ (h) _____(w) _____ (Cell) _____

Contact details of a relative or a friend who will <u>always</u> know where you live:

Alternative contact Person: _____
Relationship:_____

IDENTIFICATION AND CONTACT DETAILS	
Tel No's: _____ (h) _____(w) _____ (Cell) _____ **TO BE KEPT SEPARATE FROM QUESTIONNAIRE DATA**	

Determinants of type 2 diabetes mellitus risk in middle-aged black South African (SA) men and women: dissecting the role of sex hormones, inflammation and glucocorticoids – Part 1

PARTICIPANT QUESTIONNAIRE

DATE: Day | | | **Month** | | | **Year** | | | | |

BTT ID NUMBER: | | | | | | | |

DXA (SCAN) ID NUMBER: | |

Components	Tick & DAY (1 or 2)
Study information sheet	
Consent form	
ID COPY	
Photograph of medications	
Demographic & SE Details	
General Health	
Lifestyle	
HIV Test	
STRAW + 10	
HORNE-OSTBERG Questionnaire	
Food Frequency Questionnaire	
Anthropometric Measurements (Height, Weight, WC, HC)	
Blood pressure	
DXA scan	
Fasting bloods samples	
OGTT	
Accelerometer (ActiGraph)	
ActivPAL	
GPAQ	
Grip strength test	
PITTSBURGH Sleep Quality Questionnaire	
THE EPWORTH SLEEPINESS Scale	
K10	
STRESSFUL EVENT Scale	
Payment	
Feedback (comment)	
Date : _____	
Signature: _____	

SECTION A: DEMOGRAPHIC AND SOCIO-ECONOMIC DETAILS

1. CURRENT MARITAL STATUS (tick option that applies)

Single	
Married/cohabiting	
Widowed	
Separated/Divorced	

2. HIGHEST LEVEL OF EDUCATION ATTENDED (tick options that applies)

No education	
Grade 1-2	
Std (1-3) Grade (3-5)	
Std (4-5) Grade (6-7)	
Std (6-7) Grade (8-9)	
Std (8) Grade (10)	
Std (9) Grade (11)	
Matric Grade (12)	
Post Matric qualification	
Diploma	
Tertiary education (university / technikon)	

3. CAREGIVER'S CONFIRMATION DETAILS

Question	**Answer**
Where were you born? (City/Town & Province SA) (Country & Rural/Urban)	
Where did you spend most of your school years, which includes primary and high school?	

How many years have you been living in Gauteng?	

4. GENERAL HOUSEHOLD INFORMATION

Questions	Answers
4.1 How many people live in your house including you?	
4.2 How many rooms are in your house (including kitchen, dining room, bedrooms, excluding bathrooms)	
4.3 How many bathrooms are in your house?	
4.4 How many rooms are there for sleeping?	

5. WHICH OF THE FOLLOWING DO YOU HAVE IN YOUR HOUSEHOLD (Tick for YES and X for NO)?

Electricity		Television		Radio	
Motor vehicle		Fridge		Washing machine	
Telephone/Cell phone		Microwave		Bicycle	
Tablet/ Laptop/Personal Desktop		DSTV/Satellite		MNet	

6. HOW WOULD YOU DESCRIBE YOUR HOME (tick the one that best describes it)?

House		Flat/Cottage/Townhouse		Residence/hostel	
Shack/Zozo		Government housing (e.g. municipal/RDP housing)		Room in backyard of house (or shared house)	

7. WHAT ARE THE WALLS OF YOUR HOUSE MADE OF? (tick appropriate box)

Brick/concrete		Mud/ cement		Plastic/cardboard	
Clay/Mud		Corrugated iron/zinc		Other	

Prefab		Plaster/finished			

8. WHAT IS THE **ROOF** OF YOUR HOUSE MADE OF? (tick appropriate box)

Straw/Thatch		Galvanised iron		Other (specify)	
Earth/sand/Mud		Wood/planks			
Concrete		Tiles/slates			

9. WHAT IS THE **FLOOR** OF YOUR HOUSE MADE OF (tick one box only)

Earth/sand/mud		Stone/Brick		Cement	
Wood		Vinyl/linoleum		Other	
Carpet		Ceramic tiles			

10. WHAT IS THE MAIN SOURCE OF DRINKING WATER IN THE HOUSE? (Tick one box only)

Bottled water		Protected dug out well		Public tap/standpipe	
Running water (tap water)		Unprotected dug well		Tanker truck/cart with small tank	
Piped water into yard/plot		Protected spring		Piped water into dwelling	
Surface water		Rain water		Other	

11. WHAT IS THE TYPE OF TOILET FACILITY IN THE HOUSE (tick one box only)

Flush to piped sewer system		Protected dug out well		Bucket system	
Flush to septic tank		Ventilated improved pit (VIP) latrine		Other	
Traditional pit toilet		No facility or bush or field			

12. HOUSEHOLD FOOD INSECURITY ACCESS SCALE

1. In the past four weeks, did you worry that your household would not have enough food?			
1. No	2. Rarely	3. Sometimes	4. Often
2. In the past four weeks, were you or any household member not able to eat <u>the kinds of foods you preferred</u> (i.e. VEGETABLES, FRUIT, MEAT/CHICKEN <u>NOT</u> "luxury" food such as pizza, burgers or fried chicken) because of a lack of resources?			

1. No	2. Rarely	3. Sometimes	4. Often
3. In the past four weeks, did you or any household member have to eat a <u>limited variety of foods</u> (e.g. Pap with <u>NO meat</u> OR pap with sweetened water) due to a lack of resources?			
1. No	2. Rarely	3. Sometimes	4. Often
4. In the past four weeks, did you or any household member have to eat some foods that you really did not want to eat because of a lack of resources to obtain other types of food?			
1. No	2. Rarely	3. Sometimes	4. Often
5. In the past four weeks, did you or any household member have to eat a smaller meal than you felt you needed because there was not enough food?			
1. No	2. Rarely	3. Sometimes	4. Often
6. In the past four weeks, did you or any other household member have to eat fewer meals in a day because there was not enough food?			
1. No	2. Rarely	3. Sometimes	4. Often
7. In the past four weeks, was there ever no food to eat of any kind in your household because of lack of resources to get food?			
1. No	2. Rarely	3. Sometimes	4. Often
8. In the past four weeks, did you or any household member go to sleep at night hungry because there was not enough food?			
1. No	2. Rarely	3. Sometimes	4. Often
9. In the past four weeks, did you or any household member go a whole day and night without eating anything because there was not enough food?			
1. No	2. Rarely	3. Sometimes	4. Often

13. EMPLOYMENT AND INCOME

13. 1 Are you currently employed? (tick)	YES NO
13.2 If YES TO 13.1, what type of employment?	FORMAL INFORMAL
13.3 If YES to 13.1, which best describes the work that you are employed to do? (tick relevant option)	

Skilled manual work (i.e. sewing, beadwork, arts and craft, administrative assistants)	
Unskilled manual work (i.e. hotel maids, cleaner, sweepers or farm worker, domestic work)	
Clerical support, service or sales (i.e hairdresser, taxi service)	
Managerial/professional	
Own business	
Other (specify)	

15.4 Other household incomes (tick appropriate box):

Grants(PLUS number of people receiving grant)			
Child support grant		Support from a partner	
Disability grant		Support from family	
Care dependency Grant		Other (specify)	
Grants for older persons			
Foster care grant			

Income is a sensitive question to many people. However, it is very important for the study to have an idea of your monthly income.

13. 5 Monthly Household Income (including all sources of income e.g. grant, spousal support or family support) (Tick appropriate range):

R 1 to R800		R51 201 +	
R801 to R3 200		No income	
R 3 201 to R12 800		Not willing to disclose	
R12 801 to R51 200			

13.6 How many people do you support with this income? _____Adults _____Children

SECTION B: GENERAL HEALTH

FOR WOMEN ONLY:

SECTION A: MENSTRUAL HISTORY

This section will tell us more about your menstrual cycle.

1. Are you currently pregnant? **Y** **N**

2. Have you had a hysterectomy? **Y** **N**

   2.1 IF YES, what date was it? _____

   2.2 IF YES, do you know whether you had a partial hysterectomy or a full hysterectomy? **Partial** **Full**

3. Do you have regular periods? **Y** **N**

   3.1 IF YES, when did you have your last period? (month and year)_____

   3.2 IF NO, when was your last period?

3 months ago	6 months ago	1 year ago	more than one year

   3.3 For how long have you not had even a drop of blood?

12 months ago	24 months ago (12 months after FMP+1 year=2 years)	2-6 years ago after FMP	more than 6 after FMP

4. Are you on a contraceptive? **Y** **N**

   IF YES which one?

a) Oral contraceptive ☐

b) Sterilisation ☐

c) IUD ☐

d) Injectables ☐

5. Are you on hormone replacement therapy (hormone therapy) | Y | N |

5.1 IF YES, what type? _____

6. Have you been sterilized? | Y | N |

7. Have you ever had a pap smear? | Y | N |

7.1 IF YES, when did you have it? _____

7.2 IF YES, did you get the results? | Y | N |

8. Do you understand what menopause (change of life) means?

SECTION B: MENOPAUSE RATING-SCALE (MRS II)

The aim of this questionnaire is to see whether you have certain symptoms associated with the menopause transition and to understand how severe or mild they are compared with other women worldwide.

Which of the following complaints do you experience at this time or recently?

We will mark the following symptoms with a tick in the box which best describes the severity of your symptom. If you are not experiencing that symptom the interviewer will tick the box saying "none"

	None	slight	medium	strong	very strong
	0	1	2	3	4
1. Body Temperature Disturbances (hot flushes, sweats and night sweats)	☐	☐	☐	☐	☐
2. Heart Complaints (irregular heartbeat, palpitations, chest pains,)	☐	☐	☐	☐	☐
3. Sleep disturbances (interrupted sleep, trouble in sleeping through the night, waking too early)	☐	☐	☐	☐	☐
4. Mood Disorders (listlessness, sadness, tearfulness, lack of energy mood swings)	☐	☐	☐	☐	☐
5. Irritability	☐	☐	☐	☐	☐

(nervousness, tension, aggressiveness)

6. Fearfulness……. ☐ ☐ ☐ ☐ ☐
(panic attacks and anxiety)

7. Physical and mental exhaustion……. ☐ ☐ ☐ ☐ ☐
(lack of energy, forgetfulness, not concentrating well, confused about time or place)

8. Sexual Problems……. ☐ ☐ ☐ ☐ ☐
(change in sexual desire, change in sexual activity and sexual satisfaction)

9. Urinary Problems……. ☐ ☐ ☐ ☐ ☐
(difficulty in urinating, urinating more often, increased urgency, leaking, stress incontinence – urinating during physical activity, such as coughing, sneezing, laughing, or exercise.)

10. Vaginal dryness…….. ☐ ☐ ☐ ☐ ☐
(feeling of dryness or burning, pain during sexual intercourse)

11. Joint and muscle complaints…….. ☐ ☐ ☐ ☐ ☐
(pain in the vicinity of the joints, arthritic complaints)

SECTION C: MENOPAUSE TRANSITION (MT)

Circle the appropriate classification following the aforementioned symptoms

Pre-menopause ☐

Peri-menopause ☐

Post-menopause ☐

Cannot be stage ☐

If cannot be staged;
Reasons(s) Hysterectomy☐ , Contraception☐ or Hormone replacement therapy☐

13. CLINICAL CONDITIONS

Y	N

NO.	QUESTIONS AND FILTERS	CODING CATEGORIES
13.1	Would you say your health is poor, average, good, or very good/excellent?	POOR ..... AVERAGE ..... GOOD..... VERY GOOD/EXCELLENT .....

NO.	QUESTIONS AND FILTERS	CODING CATEGORIES
13.2	Do you personally think that you are underweight, normal weight or overweight?	UNDERWEIGHT..... NORMAL WEIGHT..... OVERWEIGHT. ..... OBESE..... DON'T KNOW.....
	Has a **doctor** or **nurse** or **health worker** at a **clinic** or at **hospital told you** that you had or have any of the following conditions:	
13.3	High Blood Pressure?	YES ..... NO ..... DON'T KNOW.....
13.4	Heart attack or angina (chest pains)?	YES ..... NO ..... DON'T KNOW.....
13.5	Stroke?	YES ..... NO ..... DON'T KNOW.....
13.6	High blood cholesterol or fats in the blood?	YES ..... NO ..... DON'T KNOW.....
13.7	Diabetes or Blood Sugar?	YES ..... NO ..... DON'T KNOW.....
13.8	Emphysema/Bronchitis?	YES ..... NO ..... DON'T KNOW.....
13.9	Asthma?	YES ..... NO ..... DON'T KNOW.....
13.10	Sore joints, e.g. Arthritis, gout?	YES ..... NO ..... DON'T KNOW.....
13.11	Osteoporosis?	YES ..... NO ..... DON'T KNOW.....
13.12	Epilepsy / fits?	YES ..... NO ..... DON'T KNOW.....
13.13	TB?	YES ..... NO ..... DON'T KNOW.....
13.15	Cancer?	YES ..... NO ..... DON'T KNOW..... If yes, what? ……..

14 MEDICATION AND SUPPLEMENT USE

14.1	Do you use any medicine regularly or daily that a doctor or nurse has prescribed?	YES ..... NO .....
14.2	Please provide the following information of your medication (s): **Name of medication(s)?** **What are they used for?** **What long have you been using the medication?** **Examples of medical conditions that you could be using the medication (s) for:** high blood pressure, heart attack or angina (chest pains), stroke, high cholesterol (fats in the blood), diabetes, emphysema, bronchitis, asthma.	
14.3	Do you use corticosteroids currently or have you used them in the last month? This includes inhaled or tablets.	YES ..... NO .....
14.4	Do you use nutritional or other supplements?	YES ..... NO .....
14.5	If YES to 8.3, Name the supplement, what is it used for, dosage, frequency and duration of use.	
14.7	Do you use any herbal medicine?	YES ..... NO .....
14.8	Name of herbal medicine, what you are using for, state the dosage, frequency and duration of use.	
14.9	Have you been sick in the past week?	YES ..... NO .....
15.0	If YES, what sickness?	YES .....

	8.9.1 Did you take medicine? (yes/no) What medication(s) did the nurse or doctor prescribe you? State the dosage, frequency and duration of use.	NO .....

15. FAMILY AND MEDICAL HISTORY

NO.	QUESTIONS AND FILTERS	CODING CATEGORIES
	Now I would like to ask you about your family. Do you have a **close blood relative** (father, mother, brother, sister or child) who has ever had any of the following conditions:	
15. 1	High Blood Pressure?	YES..... NO..... DON'T KNOW..... If yes, who? …… ……
15.2	Heart attack or angina or chest pain when exerting himself/herself?	YES..... NO..... DON'T KNOW..... If yes, who…….. …… ……
15.3	Was this relative younger or older than 50 years old when they first had a heart attack, angina or chest pain?	YOUNGER THAN 50 YEARS..... …… …… OLDER THAN 50 YEARS..... …… …… DON'T KNOW..... …… ……
15.4	Stroke?	YES..... NO..... DON'T KNOW..... If yes, who? …… …… ……....
15.5	Diabetes?	YES..... NO..... DON'T KNOW..... If yes, who? …… ……

NO.	QUESTIONS AND FILTERS	CODING CATEGORIES
		Adult/ child onset? …… ……
15.6	oBESITY **Obesity (were they abnormally large? Or have difficulty moving?)**	YES..... NO..... DON'T KNOW..... If yes, who? …… ……

SECTION C: LIFESTYE

	TOBACCO USE (WHO STEPwise Questionnaire)	
1.1	Do you **currently smoke** any tobacco products, such as cigarettes, cigars, or pipes?	YES ..... NO .....
1.2	Do you currently smoke tobacco products **daily**?	YES ..... NO .....
1.3	**How old** were you when you first **started** smoking daily?	YEARS OLD _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ IF "YOU DON'T REMEMBER", ...
1.4	If you do not remember how old you were, do you remember how long ago it was?	WEEKS AGO_ _ _ _ _ _ _ _ _ _ _ _ _ _ _ —
		MONTHS AGO _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _
		YEARS AGO_ _ _ _ _ _ _ _ _ _ _ _ _ _ _
1.5	On average, how many of the following items do you smoke each day?	MANUFACTURED CIGARETTES _ _ _ _ _
	(CHECK EACH ITEM, IF NOT SMOKING AN ITEM, CODE 00)	HAND-ROLLED CIGARETTES _ _ _ _ _
		PIPES FULL OF TOBACCO _ _ _ _ _ _ _ _ _ _ _ _
		CIGARS/CHEROOTS/CIGARILLOS _ _ _ _ _ _ _ _
		OTHER_ _
1.6	**In the past,** did you ever smoke daily?	YES ..... NO .....
1.7	**How long ago did you stop** smoking daily?	WEEKS AGO_ _ _ _ _ _ _ _ _ _ _ _ _ _ _ MONTHS AGO _ _ _ _ _ _ _ _ _ _ _ _ _ _ _

		YEARS AGO_ _ _ _ _ _ _ _ _ _ _ _ _ _

2. Do you use snuff?

2.1 IF YES, how often do you use snuff?

Once a day	
Twice a day	
Three times a day	
More than three times a day	
Other: Specify _____	

3. Do you use e-cigarettes (electronic cigarettes?)

3.1 IF YES, how often do you use e-cigarettes?

Once a day	
Twice a day	
Three times a day	
More than three times a day	
Other: Specify _____	

4. Do you use chewing tobacco?

2.1 IF YES, how often do you use chewing tobacco?

Once a day	
Twice a day	
Three times a day	
More than three times a day	
Other: Specify _____	

5. ALCOHOL INTAKE

1 standard drink is equal to 10 g of pure alcohol:

- 200 ml of beer
- 1 glass of wine

- 1 tot (25 ml) spirits
- 1 small glass (50ml) of sherry/port

5.1 How often do you have a drink containing alcohol?

Never		2-3 times per week	
Monthly or less		4 or more times per week	
2-4 times per month			

5.2 On a typical WEEK day, how many drinks containing alcohol do you drink? (how many standard drinks)

1 or 2		7, 8 or 9	
3 or 4		10 or more	
5 or 6		0	

5.3 On a typical WEEKEND day, how many drinks containing alcohol do you drink? (how many standard drinks)

1 or 2		7, 8 or 9	
3 or 4		10 or more	
5 or 6		0	

Appendix E: Turn-it-in report

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by Maphoko Masemola

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