EFFECTS OF ADIPOSE TISSUE EXTRACELLULAR MATRIX COMPONENTS ON BODY FAT DISTRIBUTION AND INSULIN SENSITIVITY IN BLACK AND WHITE SOUTH AFRICAN WOMEN

By

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"Perfection is lots of little things done well"

-Fernand Point

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DECLARATION

I, Liske Kotzé-Hörstmann do hereby declare that the experiments presented in this thesis were conceived and executed by myself except where otherwise indicated.

Neither the substance nor any part of this thesis has been submitted in the past, or is being, or is to be submitted for a degree in this university or any other university.

This thesis is presented in fulfilment of the requirements of the degree of PhD.

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SCIENTIFIC OUTPUTS FROM THIS THESIS

Peer reviewed publications related to this thesis

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ABBREVIATIONS

AIR_g	Acute insulin response to glucose
AT	Adipose tissue
ATBF	Adipose tissue blood flow
AU	Arbitrary units
BAPN	β-aminoproprionitrile
CCL2	Chemokine C-C motive ligand 2
CCR2	Chemokine C-C motive receptor 2
COL5A1	type V collagen alpha 1
COL6A1	type VI collagen alpha 1
CSF-1	*Colony stimulating factor-1*
CVD	Cardio vascular disease
DSAT	Deep subcutaneous adipose tissue
FFA	Free fatty acids
FGF1	*Fibroblast growth factor 1*
fm	fat mass
FSIGT	frequently sampled intravenous glucose tolerance test
HIF-1α	*Hypoxia inducible factor 1 alpha*
HOMA-IR	homeostatic model assessment of insulin resistance
HRE	hypoxia response element
HWE	Hardy-Weinberg equilibrium
IL-	Interleukin-
IRS	Insulin receptor substrate
LOX	*Lysyl oxidase*
MHO	Metabolically healthy obese
MIF	*Macrophage migration inhibitory factor*
MMP	Matrix metallopoteinases
MUNW	Metabolically unhealthy normal-weight
NCD	Non-communicable diseases
OLF	Ossification of the ligamentum flavum
OPLL	Ossification of the posterior longitudinal ligament of the spine
PDGF	*Platelet-derived growth factor*
PO_2	Oxygen partial pressure
PPARγ	*Peroxisome proliferator-activated receptor gamma*
PPIA	*Peptidylprolyl isomerase A*
PX-478	*S*-2-amino-3-[40-N,N,-bis (2-chloroethyl) amino]-phe-nyl propionic acid N-oxide dihydrochloride)
RPLP0	*60S acidic ribosomal protein P0*
RT-PCR	Reverse transcription polymerase chain reaction
SAT	Subcutaneous adipose tissue
S_I	Insulin sensitivity
SNP	Single nucleotide polymorphism
SSA	Sub-Saharan Africa

SSAT	Superficial subcutaneous adipose tissue
SVF	Stromal vascular fraction
T2DM	type 2 Diabetes Mellitus
TAG	Triacylglycerol or Triglyceride
TC	Total cholesterol
TGF-β1	*transforming growth factor β1,*
TIMPs	tissue inhibitors of MMPs
TNFα	*tumour necrosis factor* alpha
VAT	Visceral adipose tissue
VEGFα	*vascular endothelial growth factor alpha*
WHR	waist-hip ratio

GLOSSARY OF TERMS USED IN THIS THESIS

Black and white women: This thesis uses the term “black women” when referring to women of African ancestral origin as an ethnic group. With regards to the cohort of women referred in this study, the term black women refers to a cohort of Xhosa women requited in and around the Cape Town (South Africa) area for participation in this study. However, black women from other geographical areas are referred to in the context of their country of origin or in which the sampling was done, e.g. African American-, African Caribbean- or South African women. The term “white women” is used to describe women of European ancestral origin (also “Caucasian women”) as an ethnic comparator group.

Diabetes Mellitus: Most analyses do not distinguish type I from type II diabetes. However, as most cases (85-95 %) of diabetes in South African adults are classified as type II, the disease prevalence trends are therefore most likely due to cases of type II diabetes [3]. The term diabetes is used when no distinction between type I and type II is being made.

Glycemic metabolism: Insulin sensitivity (S_I) refers to the ability of endogenous insulin to promote glucose clearance into peripheral tissues and inhibit hepatic gluconeogenesis; Insulin resistance is defined as the decrease in insulin sensitivity. Hepatic insulin sensitivity relates to the ability of the liver to remove insulin from circulation and suppress gluconeogenesis (endogenous glucose production).

Insulin sensitivity and secretion measures: The hyperinsulinemic euglycemic clamp is widely considered to be the gold standard for measuring insulin sensitivity, and can differentiate between hepatic and systemic insulin sensitivity [4]. However, it is

expensive and technically challenging to undertake [5]. Due to the nature of the clamp procedure, no information is generated regarding pancreatic insulin response to a glucose load (AIR_g). In contrast, the frequently sampled intravenous glucose tolerance test (FSIGT) provides a reliable, cost effective alternative to the clamp method, including measures of peripheral insulin sensitivity, as well estimates insulin secretion (AIR_g) [6].

HOMA-IR is a less invasive alternative compared to both clamp and FSIGT. Although associated with the clamp technique, HOMA-IR does not provide information on postprandial glucose metabolism and skeletal / and adipose tissue insulin resistance and does not provide insight into beta-cell secretory capacity [7]

Body composition and body fat distribution determination:

Quantitative assessment of body fat mass and its distribution is important for evaluating the potential risk of development of obesity related pathologies [8] . Body fatness and composition in this thesis is quantified by using various direct measures and imaging technologies. BMI and WHR is used as a crude measure of obesity and central fat accumulation. In contrast, dual energy X-ray absorptiometry (DXA) is used as a sensitive measure to further characterize adipose tissue distribution across android- and gynoid regions of interest. Further, to characterize the intra-abdominal (central) adipose tissue depot, computerized tomography (CT) is used to estimate VAT, deep and superficial SAT accumulation and is also considered the gold standard for estimating these body composition measures [8].

Inflammatory products: The CC-chemokine family is secreted by macrophages and epithelial cells and include macrophage inflammatory protein 1α (MIP1α, also called CCL3) and monocyte chemoattractant protein 1 (MCP1, also known as CCL2).

Macrophage Markers include CD68, CD14 and CD163. Adipokines are proteins synthesized and secreted by adipocytes and include pro-inflammatory leptin and anti-inflammatory adiponectin. In addition, pro-inflammatory cytokines include interleukin (IL) -18, IL-6, tumour necrosis factor-α (TNF-α), colony stimulating factor-1(CSF-1) and macrophage migration inhibitory factor (MIF).

ABSTRACT

The global burden of non-communicable diseases (NCD's) is unacceptably high and disproportionately affects developing countries such as South Africa (SA). Black SA women have a higher prevalence of obesity and a greater associated risk for developing metabolic diseases (such as type 2 diabetes mellitus) than their white counterparts. An improved understanding of the ethnic-specific mechanisms underlying the increased risk of T2DM in black SA women is needed to inform future studies aimed at reducing the prevalence of this diseases.

One of the major determinants of insulin resistance is android/central body fat partitioning, with visceral adipose tissue (VAT) enlargement, in particular, being closely associated with increased risk. Conversely, lower-body fat accumulation is considered to be protective. However, these relationships between body fat distribution and its metabolic effects are altered by ethnicity. Black SA women have less abdominal and greater gluteal-femoral subcutaneous adipose tissue (SAT) but are more insulin resistant compared to BMI- and waist circumference-matched white SA women. A similar profile has been described in black African-American women. The reduced protective effect of peripheral fat distribution in black women remains to be understood.

The primary aim of this thesis was positioned within the context of adipose tissue expandability hypothesis, and aimed to examine the hypothesis that differences in SAT extracellular matrix (ECM)- and hypoxia-related gene expression and their ethnic-specific associations with body composition and insulin sensitivity may explain, in part, the higher rates of insulin resistance in black compared to white South African women.

Therefore, it was hypothesized that, as a consequence of increased adipose tissue hypertrophy in the gluteal depot of obese black compared to obese white women, gluteal SAT adipose tissue hypoxia and ECM component gene expression is higher in black compared to white women, and associates with their reduced insulin sensitivity (S_I) and higher insulin response. In order to address this hypothesis, four research studies were designed.

The first study (Chapter 3) in this thesis aimed to compare depot-specific (abdominal vs. gluteal) expression of hypoxia and ECM genes in normal-weight and obese black and white women, and to examine the ethnic-specific associations between these genes and body composition, measures of insulin sensitivity and secretion and inflammatory gene expression in black and white SA women by using a gene expression (Reverse transcription polymerase chain reaction (RT-PCR)) analysis. This thesis showed for the first time that hypoxia inducible factor 1 (*HIF-1α*), collagen type V α1 (*Col5A1*) and type VI α1 (*COL6A1*) gene expression were higher in the gluteal, but not the abdominal SAT depots, of black compared to white women, and associated with reduced insulin sensitivity in black women only. The expression of the hypoxia and ECM genes associated with inflammatory gene expression in both the gluteal and abdominal SAT depots of black women, whereas the expression of these genes associated with the inflammatory gene expression mostly in the abdominal SAT depots of white women.

The second study (Chapter 4) tests the hypothesis that higher hypoxia and ECM related gene expression would associate with higher central fat mass accumulation in black women and that the expression of these genes may be associated with changes in the measures of insulin sensitivity in black and white women. Thus, this longitudinal study aimed to determine whether changes in body composition and insulin sensitivity

variables over a 5 year follow-up period associated with variations in hypoxia and ECM related gene expression in the gluteal SAT of black and white women. Over the 5-year follow-up period, increased body fat mass in white women associated with increased *PPARγ* mRNA expression whereas increased body fat mass in black women associated with lower *COL5A1* expression. Furthermore, *HIF-1α*, and *COL6A1* expression correlated positively with the change in fasting insulin concentrations in black but not in white women.

It is not clear whether high circulating insulin may directly increase *HIF-1α* expression and contribute to the formation of excess ECM, or whether increased insulin may simply be a concomitant downstream effect of increased insulin resistance, as a consequence of increased fibrosis and the generation of inflammation. By using a cell culture based study, the third study in this thesis (chapter 5), investigated the effects of increasing insulin concentrations on the expression of hypoxia and ECM related genes under normoxic and hypoxic conditions in mature 3T3-L1 adipocytes. It was found that insulin and hypoxia treatment significantly elevated *HIF-1α* mRNA and protein levels but that the observed effects were not additive. Further, hypoxia, but not insulin treatment, increased the expression of *Col5a1* and *Col6a1* protein but not mRNA levels in mature 3T3-L1 adipocytes.

By using a genotyping analysis, the fourth study (Chapter 6) aimed to determine whether variants within two ECM component gene polymorphisms, collagen type 5α1 (*COL5A1*) rs12722 (C/T) and type 6α1 (*COL6A1*) rs35796750 (C/T) associates with body fat distribution and insulin resistance in black and white women. Allele and genotype distributions of the *COL5A1* rs12722 and *COL6A1* rs35796750 polymorphisms, as well as body fat distribution were significantly different between black and white women, the T- variant of the *COL5A1* rs12722 polymorphism was

associated with significantly less central fat mass, characterised by a smaller waist circumference and lower VAT, and this effect was independent of ethnicity. In addition, T- variant of the *COL5A1* rs12722 polymorphism was associated with lower fasted insulin concentrations and HOMA-IR in white but not in black women. In contrast, no genotype associations between *COL6A1* rs35796750 and any of the body fat mass, its distribution and insulin resistance measures in black or white women were reported.

This thesis used a hypothesis driven approach to provide preliminary evidence that the gluteal depot of obese black women has higher expression of hypoxia and ECM genes compared to that of obese white women and provides novel insight into the apparent paradox of reduced insulin sensitivity despite lower VAT and greater peripheral SAT accumulation in black compared to white women. An improved understanding of the ethnic-specific mechanisms underlying the increased risk of T2DM in black SA women will enable the development of cost-effective preventative care strategies within the South African demographic.

CHAPTER ONE

THEORETICAL FRAME

1.1 INTRODUCTION

Obesity is recognised as the major risk factor for multiple chronic diseases including diabetes, heart diseases, fatty liver syndrome and some forms of cancer [9]. The exact mechanisms that link these disorders to obesity are still unclear and may be specific for each disease [10]. Importantly, the most likely contributing factors are believed to be insulin resistance and hyperinsulinaemia, body fat distribution- including ectopic fat deposition in peripheral depots, and inflammatory pathways [10, 11]. Prior work in this area suggests that there are distinct ethnic differences in these obesity-related phenotypes that are evident form a young age and may help to explain the ethnic differences in non-communicable disease (NCD) prevalence and their outcomes [12-16]. Of particular relevance in South Africa is the high proportion of type 2 diabetes (T2DM) in the economically active (20-59 year old) age group [17]. Recent data suggests that the prevalence of T2DM is higher in black African women compared to their white counterparts [18-20] regardless of country in which the sampling took place. Currently, the reasons for the ethnic disparity in T2DM prevalence is still unclear. However, the prevalence of insulin resistance, a major risk factor for the development of T2DM, is also higher in black woman compared to age- and BMI-matched white women independent of variances in body fat distribution [21, 22].The mechanisms that contribute to the increased risk for insulin resistance in women of African ancestry remain largely unknown [23].

A better understanding of the relevant predisposing factors for the development of insulin resistance and T2DM in the South African context and their biological underpinnings is important for early disease detection and prevention. Therefore, the purpose of this thesis was to describe and investigate these differences in insulin resistance phenotypes and their biological underpinnings in two distinct ethnic groups in South Africa namely black African women and their white counterparts. However, the focus of this thesis falls mainly on South African women because of their markedly higher obesity prevalence compared to the South African men (69.3% in women vs 38.8% in men) [24-27]. Moreover, although the T2DM disease complications are not different by gender, women tend to have a 1.7 times higher T2DM-attributable mortality compared to men, with over half of these T2DM-related deaths in women occurring before the age of 40 years [17, 28, 29]. The higher risk of insulin resistance described for black compared to white women warrants further investigation. Investigating these two different insulin resistance phenotypes could identify putative pathways/ mechanisms underlying the different outcomes of obesity -related diseases in black and white women, and could highlight culturally appropriate intervention strategies.

1.2 AFRICAN PARADOX IN THE CONTEXT OF ADIPOSE TISSUE EXPANDABILITY

Obesity, characterised by the excess accumulation of adipose tissue, is strongly associated with insulin resistance, and is regarded as a major risk factor for the development of T2DM, although the causative molecular mechanisms that link obesity to the development of T2DM are not clearly understood. Epidemiological and clinical studies have suggested that 10-40% of individuals (depending on the criteria used, Table 1.1) are regarded as being obese but metabolically healthy (MHO) [30, 31]. MHO

Table 1.1 Criteria typically used to define MHO.

Factor	MHO definition
Metabolic syndrome (MetS) *	Absence of MetS or <3 MetS criteria
Insulin resistance	HOMA-IR <75th percentile, HOMA-IR <1.95
Blood lipid profile	Triglycerides ≤ 1.7mmol/L, TC ≤ 5.2 mmol/L HDL-C ≥ 1.3mmol/L LDL-C≤2.6 mmol/L
Inflammation	C-reactive protein levels ≥3.0 mg/L White blood-cell count <75th percentile
Obesity	BMI ≥ 30 kg/m^2 or body fat > 25% in men and 30% in women

Criteria used to define MHO are not universal but are typically characterised according to MetS criteria *(Based on NCEP ATP III definition), cut-points for insulin sensitivity (by HOMA-IR), blood-lipid profile, and recently also inflammation. For review see [1]

individuals are characterised as having high insulin sensitivity, absence of hypertension, and favourable lipid, inflammation, hormonal and liver enzyme profiles [32]. In contrast, recent analysis of the NHANES III cohort have estimated that 10 % of the US population who are normal-weight and have a BMI of below 25 kg/m^2, are metabolically unhealthy (MUNW) and display body fat related metabolic abnormalities [33] such as hyperinsulinaemia, insulin resistance, hypertriglyceridemia and arteriosclerosis [34]. Interestingly, based mainly around their better blood lipid profile (triglycerides ≤ 1.7mmol/L, TC ≤ 5.2 mmol/L HDL-C ≥ 1.3mmol/L and LDL-C≤2.6 mmol/L) and lower fasted glucose levels, earlier investigations into the adverse effects of obesity in black South African women reported that about 87% of these women could also be regarded as MHO and that their apparent “health” was likely due to their lower fat intake and high levels of physical activity [35, 36]. Black African and African American women have consistently been shown to have a more favourable lipid (higher HDL-C and lower TC) profile compared to BMI matched white women [37-39]. However, this high level of “health” described in the black women may be more dependent on the criteria used to characterise MHO because in spite of their favourable lipid profile, black women are more insulin resistant and maintain higher

levels of fasted insulin compared to white women [40]. These studies suggest that absolute fat mass is not the only determinant in the association between obesity and insulin resistance.

Rather, the regional distribution of adipose tissue has been shown to be an important determinant of metabolic risk [41-43] (for review see [44]). Different regional adipose tissue depots have different structural and functional characteristics, depending on their anatomical location within the body, and consequently differentially impact on the risk of development of metabolic disease such as T2DM. A major determinant of reduced insulin sensitivity is the centralisation of body fat (that is, upper body obesity) [45], and particularly visceral adipose tissue (VAT) [46] accumulation, while peripheral accumulation of body fat (that is lower body obesity) is considered protective [47-51], independent of BMI. Indeed, the MUNW individuals with centralised obesity (higher waist-to hip ratio) are more insulin resistant and have a higher mortality risk compared to either normal weight or obese individuals without central obesity (MHO) [52, 53]. However, this this notion has also been questioned [54] where metabolically healthy individuals (MHO) were found to still have some level of metabolic disorders.

However, contrary to this prevailing hypothesis, black African and African American women have less VAT, and more SAT, but have consistently been found to be more insulin resistant than white women of comparable BMI and % body fat percentage (%fm) or waist circumference [22, 55-57]. This paradox suggests that the relationship between body composition and insulin resistance may be ethnic-specific. The bulk of the physiological investigations into the determinants of insulin resistance have been undertaken in predominantly white/Caucasian populations, with the mechanisms underlying the ethnic disparity in insulin resistance prevalence between black women and their BMI matched white counterparts mostly unknown.

Theoretical Frame

The metabolic effects of excess adipose tissue on individual disease risk are highly heterogenic in nature. Insulin resistance exists on a continuum within individuals at varying levels of body fat mass rather than a defined set point of adipose tissue mass that once reached defines the start of metabolic disease. Originally proposed by Vidal-Puig and colleges [58, 59] in an attempt to explain this phenomenon, *the adipose tissue expandability hypothesis* suggests that the link between obesity and metabolic complications is not dependent on total fat accumulation, but does depend on the remaining capacity for expansion and metabolic function of adipose tissue depots [58-60]. Once expansion capacity is reached, adipose tissue becomes dysfunctional and ceases to store excess nutrients effectively. SAT is the largest adipose tissue depot and also the preferred site to store excess nutrients from prolonged positive nutrient intake in the form of lipid [61]. A widely held view is that failure to store the excess nutrients in the SAT depot results in the storage of these products as lipid in various ectopic sites such as muscle, liver and pancreas [62]. Lipid spill-over from metabolically dysfuntional SAT results in lipotoxicity in these organs leading to central-obesity, dyslipidaemia, fatty liver, and beta-cell failure, complications which are typically associated with morbid obesity [62].

Studies in human and mouse models of obesity and insulin resistance have identified various environmental-, physiological- and genetic-predisposing factors that potentially contribute to a highly individualised expansion capacity of SAT [63, 64] (for review see [58, 59, 65]). However, mechanistically, it may be hypothesized that expanding adipose tissue causes oxygen deprivation in large adipocytes as their distance from the vasculature increases [66-68]. This local hypoxic environment (defined as a deficiency of O_2 in tissues) induces dysregulated adipokine secretion that contributes to the development of inflammation and the recruitment of immune cells into the adipose

tissue depot [69]. Hypoxia is also associated with the increased expression and deposition of extracellular matrix (ECM) components in adipose tissue, resulting in fibrosis [70]. It is proposed that increased adipose tissue fibrosis, through its mechanical constraints on adipocyte expansion, is one of the main triggers for the pro-inflammatory response and concomitant insulin resistance that characterizes adipose tissue of obese humans [71, 72].

Based on the increased prevalence of insulin resistance in obese black compared to obese white women despite the lower levels of VAT and more favourable lipid profile, it may be hypothesised that adipose tissue expansion in black women is associated with increased hypoxia and fibrosis and may account for the ethnic disparity insulin resistance between black and white South African women. Therefore, in this thesis I aimed to explore the black African paradox of lower VAT accumulation but greater insulin resistance compared to white women within the context of adipose tissue expansion. In the next section, a literature review provides a brief overview of the current research exploring the disproportionate prevalence of T2DM and insulin resistance in black African women when compared to their white/Caucasian counterparts (Section 2.1). Ethnic differences in body fat distribution and the associations with insulin sensitivity are explored (section 2.2). Possible mechanisms through which regional adipose tissue distribution (section 2.3) and limited SAT expansion (section 2.4) may contribute to increased local and systemic insulin resistance are discussed with a specific focus on hypoxia and extracellular matrix dysregulation in adipose tissue. Lastly, a potential model that assimilates the factors that distinguish between insulin sensitive and insulin resistant adipose tissue expansion is proposed (section 2.5).

CHAPTER TWO

LITERATURE REVIEW

2.1 INTRODUCTION

Globally, the burden of non-communicable diseases (NCD's) is steadily on the rise, with more than 38 million of the world's 56 million deaths in 2012 attributed to these diseases of lifestyle [73]. According to data from the World Health Organisation (WHO) it was reported that of this 38 million, 16 million deaths occurred from preventable causes, -an increase from 14.6 million in 2000 [73]. This burden is rising disproportionally in lower income countries in which over three-quarters of deaths in 2012 were attributed to preventable NCD's, and in which almost half of these deaths occurred before the age of 70 [73]. Together, the four main NCD's recognised by the WHO (cardiovascular diseases (CVD), cancers, diabetes and chronic respiratory disease) make up the leading causes of global mortality [73]. Although CVD, cancers and respiratory disease account for the highest NCD mortality rate, diabetes accounts for over 1.6 million deaths annually [73] and according to the International Diabetes Federations (IDF) represents a significant contributor to global disease burden, with particularly costly effects reported for low- and middle-income countries [29, 74].

Once described as a disease of affluence, diabetes now disproportionately affects the poorest of countries where the burden of diabetes, in terms of both prevalence and number of adults affected, has increased more rapidly compared to the higher-income countries [74, 75]. According to a pooled analyses of 751 population-based studies from across the world, low- and middle-income countries such as Indonesia, Pakistan,

Mexico and Egypt have, over the last three decades, replaced high-income countries such as Germany, Ukraine, Italy, and the UK on the list of top ten countries presenting with the highest adult diabetes prevalence * (1980-2014) [75]. For example, while there was little change in age-standardised diabetes prevalence in adult women in continental western Europe (less than 5% increase) over the same time period, diabetes prevalence increased rapidly in low- and middle-income countries such as Polynesia and Micronesia, at nearly 25%, followed by the Middle East and North Africa at 15% [75]. This disparity in diabetes prevalence and disease growth rate between the developed and developing countries is not fully understood. However, rapid urbanisation and increasing population age have been proposed as some of the major contributing factors to the increasing burden of diabetes in these lower-income countries [17, 76].

In accordance with the global trends, a steep increase in the diabetes prevalence has also been reported for sub-Saharan Africa (SSA) region where the IDF Atlas has estimated that 14.2 million people had diabetes in 2015 and that this number would rise to 34.2 million by 2040 - exceeding the predicted global increase of 55% [29]. The unavoidable consequence of this higher prevalence of T2DM will be a substantial rise in diabetes-related disability and mortality. Currently in the African region, age-standardised, high blood-glucose-related mortality rate (about 111.3 deaths per 100 000) was estimated to be almost double that of the European region (about 55.7 deaths per 100 000) [77].

* Importantly, this study does not distinguish type I from type II diabetes. Nevertheless, as most cases (85-95 %) of diabetes in adults are classified as type II, the disease prevalence trends are therefore most likely due to cases of type II diabetes

Notably, the prevalence of diabetes in Africa is not uniformly distributed. Nearly half of all adults living with diabetes in the African region live in some of Africa's most populous countries, including South Africa (2.3, [1.2-4.6] million), Nigeria (1.6, [1.2-3.8] million), and Ethiopia (1.3, [0.8-3.5] million) * [29]. In these countries, diabetes is posing an ever increasing threat to economic development not only because it is associated with increased mortality in the productive worker class category [29], but also associates with various psycho-social factors such as adverse life events [78] and a lower sense of coherence (SOC) [79], factors that may contribute to lower economic participation. For example, in South Africa a recent analysis of 1099 urban-dwelling black participants found that having T2DM was associated with a lower SOC in women and increased adverse life event score in women and men [76]. Therefore, to reduce the immense fiscal burden of these preventable diseases of lifestyle and their associated co-morbidities in these developing countries, an increased understanding of the socioeconomic and health-related risk factors are clearly warranted to identify specific, culture-appropriate interventions that would enable the development and implementation of effective preventative-care strategies [80, 81].

High levels of overweight and obesity, together with physical inactivity across all age groups are responsible for a substantial proportion of the global diabetes burden [82]. Results from the Global Burden of Disease (GBD) 2013 study, showed that the global prevalence of overweight and obesity, the major preventable risk factor for the development of T2DM, had risen by 27.5% for adults and 47.1% for children between 1980 and 2013 [25]. Moreover it was reported that by gender, the proportion of adults with a BMI of 25 kg/m^2 or greater increased from 28.8% (28.4-29.3) in 1980 to 36.9% (36.3-37.4) in 2013 for men, and from 29.8% (29.3-30.2) to 38.0% (37.5-38.5) for

* * Uncertainty range displayed in parenthesis.

women [25]. In SSA, the highest prevalence of obesity was found among South African women, where the prevalence of overweight and obesity was 69.3% compared to 52.6% in Botswana, 42.4% in Namibia and 41.9% in Zimbabwe [25].

Moreover, in an earlier population based survey, the South African Demographic and Health Survey (2003), the prevalence of obesity was found to be different between ethnic groups, with the highest estimates being 28.5% for black women, compared to 13.7% for white women; whereas 7.1 % of black men were affected compared to 23% of white men [27, 83]. More recent survey data demonstrated that compared to mixed-race (34.9%) and Asian/Indian (32.4%) populations, black women had the highest prevalence of obesity (39.9%) [25]. Notably, similar ethnic trends have been described for black women living in the United States of America (USA) and the United Kingdom (UK). According to NHANES (2014-2014), black women living in the USA have a higher age-adjusted obesity prevalence compared to non-Hispanic white- and Asian women (57.2% vs 38.2% and 12.4% respectively) [84] whereas survey data from the Health Survey for England (2014) also showed the prevalence of overweight and obesity was highest among black African women (70%) women residing in the UK compared to the general population (62%), whilst the lowest prevalence was found in Asian women (56%) [85].

As expected, the high prevalence of over-weight/obesity among ethnic-populations is also associated with a larger T2DM prevalence compared to their white counterparts. A recent meta-analysis of T2DM prevalence in ethnic-minority groups living in Europe revealed that South Asian, Sub-Saharan African and Middle Eastern and North African populations living in Europe were more than two-times more likely to be diabetic compared to the host populations [18] consisting mostly of white Europeans. Similarly, in the USA, the Nurse's Health Study, a large prospective cohort study ranging over a

20-year interval (1980-2000), reported that Asian, African and Hispanic-women living in the USA also had a higher risk of T2DM compared to their white counterparts [19]. Notably, this study showed that the high prevalence of T2DM could not wholly be attributed to the increased obesity prevalence of these ethnic groups, as adjusting for BMI only slightly attenuated the age-adjusted relative risk, whilst these ethnic differences still remained significant [19]. Indeed, previous studies have shown the risk of T2DM to be significantly higher among black African women than white women before and after accounting for differences in BMI [20, 86]. For example, in South Africa, young urban black women (30-44 years of age) have a 2-times higher T2DM prevalence (%) compared to their white counterparts [80]. Although the reasons for this disparity in T2DM prevalence are not clear, ethnic differences in tissue sensitivity to insulin (S_I) have been demonstrated, and may represent an ethnic-specific risk factor for the development of T2DM [22, 57].

Indeed, black women have been consistently shown to have lower S_I (measured by FSIGT) compared to white women and women from other ethnicities independent of adiposity [57, 87, 88, 89]. When comparing European American and African American women and girls, Chandler-Laney et al., found significantly lower S_I in African American females across all age groups, and that these difference remained significant when adjusting for differences in adiposity [90]. Similar results have been described for BMI-matched black and white South African women [57]. Notably, to compensate for their increased insulin resistance and to maintain normo-glycaemia, black Africans and African Americans (men and women) tend to hypersecrete and maintain higher basal levels of insulin [21, 91, 92], a compensation also evident during childhood [93]. This greater hyperinsulinaemic response has been clearly demonstrated when examining the acute insulin response to glucose (AIR_g) during a FSIGT in black

South African women compared to their white counterparts [57]. Similar results were also described in a large meta-analysis examining studies of ethnically homogenous cohorts in which S_I and AIR_g were measured [87] . In black women, this increased insulin response is partly due to increase β-cell response (first-phase secretion in response to intravenous glucose) and lower insulin clearance from the liver [21, 89, 94, 95]. These studies underscore the remarkable β-cell compensation ability in black Africans.

Whereas the exact scientific basis of increased obesity-induced insulin resistance and higher T2DM prevalence in black women compared to white women are still being defined, several mechanistic pathways have been identified as crucial, including the pattern of body fat distribution, rate of ectopic fat accumulation and dysregulated adipo/cytokine secretion. The next section investigates the contribution of these components to the ethnic disparity in T2DM risk in black and white women.

2.2 ETHNIC DIFFERENCES IN BODY FAT DISTRIBUTION AND IT'S ASSOCIATIONS WITH INSULIN RESISTANCE

2.2.1 Metabolic effects of abdominal/central vs peripheral fat mass accumulation in black and white women

One of the major determinants of insulin resistance is android/central body fat partitioning, with VAT enlargement, in particular, being closely associated with increased risk for obesity-mediated disease, including T2DM. Thus, it has been proposed that disparities in disease risk between black and white ethnic groups, may be explained, at least in part, by differences in whole body adiposity and/or body fat distribution [23]. Indeed, differences in body fat distribution have been described in black and white women. For the same level of adiposity, black South African and African American women have less central fat mass, specifically less VAT, and more

peripheral SAT (femoral and gluteal) compared to white women. Sumner [96] compared the waist-circumference and VAT mass relationship in black and white women and found a significantly higher increase in cross-sectional VAT area per unit increase in waist circumference of white women compared to black women. Similar results have also been described in smaller studies for black South African women [22, 37, 97] and African American [98] women when compared to their BMI-matched white counterparts. In addition, Conway et al. [98], showed that although waist circumference correlated significantly with VAT in both ethnicities, abdominal SAT levels were not different between these women, but black women had less VAT (cross-sectional area at level L2-L3) than white women.

A small study in South Africa showed that while VAT and abdominal SAT depots are similarly associated with insulin sensitivity in the white population, abdominal SAT, rather than VAT, was more closely associated with insulin sensitivity (S_I, as measured by FSIGT) in the black women [23]. These studies demonstrate that central fat mass may not be the most relevant factor differentiating MHO from NWMO in women of different ethnicities. Notably, abdominal SAT is anatomically divided by the stromal fascia into two distinct compartments namely, superficial subcutaneous adipose tissue (SSAT) and deep subcutaneous adipose tissue (DSAT) [99]. Ethnic differences in these abdominal SAT compartments have been reported, where obese black women have higher SSAT, but similar DSAT compared to white women [23, 40]. In black women, the relationship between S_I (as measures by FSIGT) and DSAT was stronger than for SSAT. It has been shown that DSAT has a positive association with insulin resistance with a similar magnitude as that described for VAT [40, 100] and therefore may be metabolically distinct to SSAT (Table 2.1).

Table 2.1 Studies investigating adipose tissue depot-specific associations with insulin resistance in black and white women

Study	Relationship with glucose metabolism	Additional comments
Body fat distribution		
Kelley et al [100]	[a]DSAT > VAT > SSAT á ↑ fasted glucose; VAT but not DSAT or SAT á ↑fasted insulin	SSAT is a weak determinant of S_I
Smith et al [99]	VAT + SSAT > DSAT á ↑fasting insulin in obese women	
Preis et al [45]	VAT > SAT á ↑HOMA-IR and ↑fasted insulin levels	VAT á with fasted insulin concentration stronger in obese compared to normal-weight (VAT × BMI interaction)
Lovejoy et al [104] & [40]	VAT+SAT á ↓S_I and ↑fasted insulin in AA whereas only VAT in WA; SAT > VAT á ↑fasted insulin in AA; SSAT > DSAT á ↑fasted insulin in AA but DSAT >SSAT á in WA	
Goedecke et al [23]	Abdominal SAT > VAT á ↓S_I in SA black women	Body fat distribution is differentially á with S_I in black and white women. VAT seems to be less important determinant of S_I in black
Goedecke et al [105]	Gluteal SAT á ↓S_I in black but not white women	Gluteal SAT not protective in obese black women
Keswell et al [88]	Trunk/fat mass ratio á ↑fasted insulin and ↑HOMA-IR whereas leg fat mass á ↓fasted insulin and ↓HOMA-IR in SA black and white women	Independent and opposite á of central and peripheral fat mass in ethnically diverse population
Chantler et al [106]	Baseline trunk:leg á ↓HOMA-IR in black SA (5.5 years follow-up); ↑trunk/leg á ↑fasted insulin and ↓HOMA-IR	Weight gain in black women associates with centralization % fm and predicted CVD

á , association; AA African American women, WA, white American women, HOMA-IR homeostatic model of insulin resistance; DSAT, deep subcutaneous adipose tissue; SSAT, superficial subcutaneous adipose tissue; VAT, visceral adipose tissue; % fm, a percentage of fat mass; S_I, Insulin Sensitivity; BMI, body mass index;

The association between increased central fat mass accumulation and the risk for developing T2DM have been known for more than 50 years since Vague [101] described two different patterns of fat accumulation, namely android (upper-body obesity) and gynoid (lower-body obesity) each with distinct associations with CVD and T2DM development. Subsequent research has shown that having a higher proportion of abdominal adipose tissue (crudely estimated by WHR) was predictive of metabolic abnormalities increasing the risk of T2DM and CVD [102, 103].

Paradoxically, however, less VAT in black women does not associate with a better insulin resistance profile as they are more insulin resistant compared to BMI matched white counterparts [40, 57, 107, 108]. This suggests that the relationship between body composition and insulin resistance may be ethnic-specific. Ethnic differences in the relationship between metabolic disease risk and body composition have been described [23, 44, 88, 109]. When matched for BMI, white women with upper-body obesity exhibited lower glucose tolerance, hyperinsulinaemia, and significant insulin resistance compared to white women with lower-body obesity [110]. However, in black women there were no differences in fasting glucose and insulin levels and/or peripheral insulin sensitivity measures between women with upper- body or lower-body obesity [110]. These findings suggest that the relationship of lower-body obesity and the development of insulin resistance appears to be attenuated in black women compared to white women.

Lower-body fat comprises of adipose tissue in the subcutaneous gluteal/femoral region and is considered to be protective against the development of metabolic disorders. In the AusDiab study, a large population based survey (n= 8400), Snijder et al., [49] reported that a larger hip circumference was associated with a lower prevalence of undiagnosed T2DM and dyslipidaemia, independent of age, BMI and waist circumference. Similarly, in the Hoorn study, where men and women (n=1357) were followed up after 6 years, it was found that larger hip and thigh circumferences were associated with a lower risk of T2DM, independently of BMI, age, and waist circumference, whereas a larger waist circumference was associated with a higher risk [47]. Although black women have been shown to have large peripheral fat depots, it is not clear whether these stores confer lower risk with increasing obesity in this population. This does not seem to be the case as having less central fat mass and to

a lesser extent, having more peripheral fat, was associated with lower fasting glucose levels in black women [88]. Using dual-energy X-ray absorptiometry (DXA), Keswell et al [88] showed that although a larger peripheral fat mass associated with lower fasted insulin levels and lower HOMA-IR in both black and white women, having a greater trunk fat mass explained significantly more variance in fasting glucose levels in the black compared to white women. Having lower central fat accumulation is thus vital for the prevention of metabolic disease in black women and it may be important to determine at which point storing fat in the peripheral depots no longer provides protection against higher metabolic disease risk. The attenuated protective effect of peripheral fat distribution in black women remains to be understood.

Ethnic differences in the relationship between abdominal adipose tissue distribution and insulin sensitivity may be explained by differences in chronic low-grade inflammation between the adipose tissue depots of white and black women. Adipose tissue inflammation plays a crucial role in the development of obesity- related insulin resistance with animal and human studies providing an increasing amount of correlative and causative evidence [111-113]. Inflammation leads to the development of insulin resistance by impairing insulin action at the post-receptor level by inhibiting the functions of the insulin receptor substrate (IRS) 1 and 2 [114-116]. Studies from our laboratory have shown that inflammation- and macrophage- specific gene expression are up-regulated in the gluteal SAT and DSAT depots of black women independent of age, total adiposity and amount of VAT [55]. However, in black women, the SAT inflammatory gene expression only accounted for 20.9% of the variance in insulin sensitivity, compared to over 56% in the white women. These findings were mirrored by circulating levels of leptin, adiponectin and hpCRP correlating significantly with S_I in white women but not in black women. The relationship between adipose tissue

inflammation and S_I, being stronger in white women compared to black suggests that there are other factors that may also be contributing to reduced S_I in the black population.

2.2.2 Ectopic fat and insulin sensitivity in black and white women

Increased ectopic fat, the spill over of triglycerides into peripheral organs such as liver, pancreas and muscle, represents another factor linking obesity to greater metabolic disease risk. Studies have shown that ectopic fat deposition in liver and skeletal muscles may differ by ethnicity [117, 118] (Table 2.2).

The Dallas Heart study, the largest study measuring hepatic fat accumulation showed that black women had lower prevalence of hepatic fat accumulation and a favourable lipid profile but still were more insulin resistant (lower S_I) compared to white (and Hispoanic) women [119] Additionally, in African American women, having greater hepatic fat content, associated with 49 % lower S_I vs African American women with a low hepatic fat content whereas in Hispanic women, having higher hepatic fat content associated with 24% lower S_I vs those women with a low hepatic fat content [12].. Importantly, these observations may be explained, in part, by the different physiological implications of insulin action as measured at different sites in the body, that is, peripheral vs hepatic insulin action (see also [7]). For example, hepatic insulin sensitivity in black women is more closely associated with their reduced VAT mass than with SAT mass, whereas peripheral (skeletal muscle and adipose tissue depots) S_I is more closely associated with SAT than VAT [57, 120]. In a cohort of black and white women who were matched for body fatness and fat distribution (BMI, fat free mass and VAT mass) and S_I, black women had higher hepatic insulin sensitivity, expressed as the % suppression of endogenous glucose production, as measured by low-dose euglycemic, hyperinsulinemic clamp [120]. In this study, liver fat was more strongly

Table 2.2 Studies investigating adipose tissue depot-specific associations with insulin resistance in black and white women

Study	Relationship with glucose metabolism	Additional comments
Ectopic fat distribution		
Browning et al [119]	AA have ↓hepatic steatosis compared to WA and Hispanics, independent of BMI and insulin sensitivity [119].	Despite lower peripheral insulin sensitivity, AA have higher hepatic insulin sensitivity. Ethnic differences in hepatic steatosis may be explained by the smaller VAT in AA compared to WA. Inadequate SAT depot may lead to ectopic fat storage with its associated metabolic sequelae [121]
Alderete et al [12]	AA LF < Hisp LF. LF á ↑VAT in AA and Hispanics. AA with ↑LF had 49% lower S_I compared to AA with ↓LF whereas Hisp with ↑LF had 24% lower S_I compared to Hisp with ↓LF	AA stronger inverse relationship between LF and S_I when compared with Hisp. LF in AA especially detrimental to DI (AIR_g compensation for ↓S_I) and may underlie β-cell failure
Taksali et al [121]	AA have ↓VAT vs Hisp & white obese. When stratified by VAT, the highest VAT tertile á ↑ LF and á ↓ BMI and ↓ BF%. Highest VAT tertile á ↑ fasted insulin and ↑ HOMA-IR	Ability to retain and expand SAT(SSAT) á ↓VAT and ↓LF and more favourable metabolic profile
Goedecke et al [120]	↓LF fat in BSA vs WSA women. ↑LF á ↓ S_I in BSA only. EMCL & IMCL not different in BSA and WSA	The paradox of ↑hepatic S_I but similar basal EGP in black vs white SA women may be explained by lower hepatic insulin clearance in obese BSA
Lawrence et al [56]	↑EMCL in AA. IMCL á ↓ S_I in EA only (ethnic × IMCL interaction)	↑EMCL in AA vs EA but was not á ↓ S_I. Ethnic difference may exist in DAG, ceramide or other FFA metabolites.

á, association; LF, liver fat; AA, African American women; WA, white American women; Hisp, Hispanic women; BSA, black South African women; WSA, white South African women; HOMA-IR, homeostatic model of insulin resistance; SSAT, superficial subcutaneous adipose tissue; VAT, visceral adipose tissue; BF%, a percentage of fat mass; S_I, Insulin sensitivity; BMI, body mass index. IMCL, intra-myocellular lipid; EMCL, extra-myocellular lipid; EGP, endogenous glucose production; ↑, higher; ↓ lower.

associated with S_I in black compared to white women. These results underscore the significant role that the liver plays in maintaining normal glycaemia in black women.

In sum, ethnic differences in the relationship between VAT/liver fat content and insulin resistance have been investigated by several studies. Black African and African American women have consistently been found to have higher serum insulin levels and reduced S_I compared to women of other ethnicities despite their lower VAT and liver fat.

2.2.3 Genetic control of fat mass and body composition

As discussed, not all obese individuals have the same risk of developing insulin resistance, hyperlipidaemia, CVD and T2DM. For example, individuals with peripheral

obesity have a reduced risk of obesity associated metabolic abnormalities compared to individuals with central obesity who are more prone to these complications. While considerable research have been done in the physiological and metabolic determinants of appetite and energy expenditure that underlie obesity development, much less is known about the genetic factors that determine adipocyte number, body fat distribution or their associations with metabolic disease.

2.2.3.1 Genetic regulation of BMI and body fat distribution

Studies undertaken in monozygotic twins have consistently demonstrated a significant genetic influence on the development of obesity and body fat distribution [122-124]. For example, a study in free-living middle-aged female monozygotic twins found that up to 65% of total variance in fat mass (%) could be attributed to genetic variation [125] while another monozygotic twin study (males) found a significant genotype effect to the responsiveness to an overfeeding stimulus [126]. Furthermore, a study comparing the interclass associations for all body fat mass (%) parameters among monozygotic and dizygotic twins found a significant genetic (heritability, h^2) component for total- and regional fat mass distribution (lower-body fat mass % and trunk/lower-body fat mass %) in both young and elderly twin pairs [123]. These studies highlight that body fat mass and its distribution are under extensive genetic control.

In support, large genome-wide association studies (GWAS) have also confirmed the profound role of genetic regulation on BMI and suggested that common genetic variation may account for 40-70% variation in obesity-related phenotypes [127, 128]. Based on findings from these studies, several different metabolic pathways implicated in higher obesity-related pathophysiology risk have been proposed, and may include a role for the central nervous system in obesity susceptibility, along with other novel

pathways related to insulin signalling, energy metabolism, lipid biology and adipogenesis [128]. Notably, these GWAS studies have identified over 100 different loci associated with BMI [128] [129-131] and other obesity-related traits such as MetS [131].

In a meta-analysis of GWAS, Heid et al., [132] demonstrated that genetic regulation of body fat distribution involved loci and regulatory pathways that are distinct from those that influence BMI and risk of obesity. In support, another meta-analysis of GWAS conducted by Lindgren et al., [133] investigated the genetic regulation of waist circumference (WC) and WHR, and found that variation near the *lysophospholipase-like protein 1* gene (which is thought to act as a triglyceride lipase and is up-regulated in SAT of obese individuals) was associated with variation in WHR (BMI-adjusted) in women, although further investigations are still warranted.

Importantly, the heritable effect on fat mass and its distribution is likely a result of genetic variation at several different regulatory sites that may either be dependent or independent of extrinsic influence. For example, Gesta et al., [134] showed expression differences of multiple embryonic-developmental genes (*HoxA5*, *Gpc* and *Tbx15*) that were independent of interstitial factors such as innervation, vascularization, oxygenation or other factors between adipocytes sourced from VAT and SAT in mouse models prone to, or protected against obesity-related pathology. This study also showed similar inter-depot differences in developmental gene expression in lean humans with comparable WHR. These differences in gene expression were shown to be intrinsic, and persisted during *in vitro* culture and differentiation, suggesting that they are cell-autonomous and are independent of the microenvironment [134]. These results suggest that genetically programmed developmental differences in adipocyte biology may play important roles in body fat distribution that may be independent of obesity and therefore, by extension, associated with higher T2DM risk. Notably,

although these cell-autonomous characteristics may be independent of adipose tissue depot-interstitial microenvironment, a subset of factors may also be influenced by extrinsic (environment) factors such as glycaemia. It therefore follows that cell-autonomous factors may exaggerate this risk for T2DM by augmenting adipocyte biology in a depot-specific manner leading to differences in the pattern of body fat distribution.

2.2.3.2 Effects of ethnicity

Genetic explanations for the phenotypic differences in body composition and the disparity in metabolic disease risk between African and European/white populations remain to be elucidated. Few studies have demonstrated ethnic differences in allelic frequencies for multiple genes affecting a single biological mechanism in a way that might produce a biologically significant effect that is able to explain a significant proportion of the variation in disease risk [135].

Previous GWAS studies have mostly been conducted in European cohorts. However, recent studies aiming to replicate these findings in different ethnic populations have not only confirmed some significant relationships between risk loci and body composition measures, but have also identified others [130] [132, 136, 137]. Due to ethnic differences in allele frequencies and haplotype structures, conducting GWAS across ethnically diverse populations may highlight important disease risk loci not readily detected in European populations [130] [131]. For example, a recent GWAS found ethnic-specific associations were involved in the genetic determination of BMI, highlighting the importance of ethnic-specific approaches in discovery of genetic associations with obesity-related phenotypes. This may have implications for the development of gene-based therapies [130]. Moreover, another GWAS conducted in African Americans

identified credible candidate loci associated with fasted insulin levels and insulin resistance with successful replication within another independent sample of West Africans, but failed to do so in a European population [138].

Taken together, these studies show that BMI and body fat distribution are under considerable genetic regulation and that the increased risk for T2DM could be exacerbated by obesity-independent genetic risk factors. Currently, it remains to be investigated whether genetic variation could potentially explain the ethnic-specific risk factors contributing to increased insulin resistance and higher T2DM risk in women of African ancestry, providing a strong rational for studies investigating genetic and extrinsic factors regulating adipose tissue depot development.

2.3 ADIPOSE TISSUE DEPOT SPECIFIC DETERMINANTS OF INSULIN RESISTANCE

Unlike other organs, adipose tissue exists throughout the body in distinct depots. VAT surrounds the inner organs in the abdominal cavity and comprises both the greater- and lesser omentum and mesenteric adipose depots while SAT lies directly under the skin in the upper and lower body and connects with the fasciae surrounding the muscles. Functionally, while the combination of these adipose tissue depots serve as energy repositories to maintain normal systemic equilibrium during energy intake and expenditure, each depot adopts certain constitutive morphologic and physiological characteristics that enables them to fulfil distinctive primary metabolic functions in energy regulation. These functions are maintained through integrated neural and hormonal regulation of lipid metabolism, cellular proliferation and differentiation, adipo/cytokine signalling and vascularization, and are discussed next.

2.3.1 Depot specific differences in lipid metabolism and associations with insulin resistance

After a meal, non-esterified free fatty acids (FFA) are transported into the cell through lipoprotein lipase (LPL) breakdown of circulating triacylglycerol (TAG) attached to chylomicrons from the gut and very low-density lipoprotein lipase (VLDL) from the liver, but may also be directly taken up from the circulation [139, 140]. A proportion of these FFAs released by LPL activity remain in the circulation as non-esterified fatty acids (NEFA). On the other hand, when energy demand increases during energy expenditure, for example during bouts of exercise, TAG stored in the adipocyte are then again hydrolysed into FFA and glycerol through adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL), and are exported from the adipocyte to be used as an energy substrate for β-oxidation in mitochondria of other tissues, such as muscle [141]. In response to insulin, glucose is also transported into the cells and utilised in the production of TAG for storage through *de novo* lipogenesis [142]. The various characteristics within VAT compared to SAT with regards to their differences in lipid storage and metabolism are shown in Table 2.3. In brief, human studies have demonstrated that compared to SAT, VAT has a higher turnover of triglyceride/FFA, at least in part due to a combination of its high lipolytic and lipogenic rates, along with a lower sensitivity to the anti-lipolytic effects of insulin [143, 144]. VAT therefore serves as short term lipid buffer, protecting other organs from the lipotoxic effects of high circulating levels of lipid in the post-prandial state. In accordance with this function, VAT displays small, insulin sensitive adipocytes with a high capillary density. In contrast, the SAT depots have been shown to have larger cells which are more sensitive to the anti-lipolytic effects of insulin and therefore are considered to be metabolically more inert, in accordance with their long term energy storage function

Table 2.3 The Morphologic and physiologic characteristics of VAT compared to SAT: Lipid Metabolism

Lipid metabolism characteristic		Depot differences in primary metabolic function	Contribution to IR
Lipogenesis			
VAT > SAT	Basal rate of glucose uptake [145, 146] Insulin stimulated glucose uptake [145, 147] FFA and TAG uptake [148, 149] LPL protein levels Lipid synthesis[150]	VAT has a high lipid turnover rate that constitute its major functional role in post-prandial lipid buffering	Increased lipogenesis in VAT contributes to enlarged, insulin resistant adipocytes.
VAT < SAT	Insulin stimulated LPL activity in women[151] Adipocyte size	Larger adipocyte size in SAT may allow for larger storage capacity in this depot.	Increased VAT hypertrophy á with increases splanchnic FFA delivery and increases hepatic IR.
gSAT < VAT	Insulin-regulated FFA uptake [152]	GSAT is favoured for FFA sequestering excess and safe storage.	VAT is more resistant to insulin's antilipolytic effects than leg and non-splanchnic upper body fat
Lipolysis			
VAT > SAT	ATGL HSL content[153] Catecholamine-induced lipolysis[149, 154, 155] β-Adrenoreceptor lipolysis[156]	Inhibition of lipolysis is more pronounced in SAT than VAT adipocytes. Decreased insulin receptor affinity and signal transduction in VAT. SAT protects against lipotoxicity and ectopic fat deposition	Dysfunctional SAT during obesity leads to elevated circulating FFA's and results in lipotoxicity and ectopic fat deposition, which leads to peripheral and hepatic IR
VAT < SAT	Basal rate of lipolysis[150, 153, 154, 157] HSL activity[153, 157] α-Adrenoreceptor-dependent anti-lipolysis[158, 159] Anti-lipolytic effect of insulin[149, 160]		
abSAT > gSAT	β-Adrenoreceptors mRNA [161]	Enhanced catecholamine induced lipolysis in abSAT compared to gSAT [161]	

Á, associates; LPL, lipoprotein lipase enzyme; FFA, free fatty acid, VAT, visceral adipose tissue, SAT subcutaneous adipose tissue; IR, insulin resistance, ATGL, adipose triglyceride lipase; HSL, hormone-sensitive lipase; TAG, triglyceride

[143], [144]. Thus, during normal physiological conditions, VAT and SAT function synergistically to facilitate the flux of excess dietary energy for storage in the peripheral SAT depots until mobilisation from these depots during times of metabolic demand.

Metabolic dysregulation during the pathological expansion of adipose tissue associates with the development of central obesity, a process characterised by the increased storage of fat in the central depots (VAT and abdominal SAT) rather than peripheral depots (leg or gluteal/femoral SAT), and correlates closely with metabolic abnormalities underlying increased risk for T2DM and CVD [100, 162-165]. Excess VAT accumulation has been shown to be a critical characteristic that distinguishes MHO

from obese people with metabolic abnormalities and from those characterised as MUNW (for review, see [166]). In a large cohort of Caucasian women (n=1452) participating in the Framingham Heart Study, it was shown that although both SAT and VAT were correlated with metabolic risk factors, VAT remained more strongly correlated with an adverse metabolic risk profile, even after accounting for standard anthropometric indexes such as BMI and total body fat % [167]. One hypothesis aimed at explaining the added risk of VAT over SAT is the portal/visceral hypothesis. Originally proposed by Björntorp [144], this hypothesis proposes that the direct release of FFA as products of the increased lipolytic rate of VAT into the portal vein reduces hepatic insulin sensitivity, impairs peripheral glucose utilisation and increases hepatic glucose overproduction [168-170] and thereby results in glycaemic abnormalities that may predispose individuals to developing T2DM. Chronic exposure of the liver to elevated FFAs influence several important hepatic functions, including gluconeogenesis, VLDL secretion, and insulin clearance [171] and thereby can contribute to both the development and exacerbation insulin resistance [152, 172]. FFAs release from VAT into the portal vein increases with increasing VAT mass, although its relative contribution of total circulating FFAs varies greatly at a given visceral adiposity [170]. Additionally, Fabbrini et al., [173] demonstrated that intrahepatic triglyceride content has a stronger association with the development of the metabolic disturbances associated with obesity than does VAT volume per se.

Obesity-induced pathophysiology of the liver may also be a result of the inability of SAT to effectively store excess energy. Nielsen et al., demonstrated that SAT may account for up to 90% of circulating FFAs, with almost 70% of these FFAs coming from the abdominal SAT depot [170, 174]. In contrast, lower-body/peripheral fat, which comprises the hip and gluteal-femoral SAT areas, is considered to act as a metabolic

"sink" to store excess FFA when there is an energy surplus [50, 175]. The protective effect of peripheral fat mass is believed to result from increased FFA sequestration from circulation for storage in this depot, and thereby protect from ectopic fat distribution into organs less adapted for this purpose [47]. Votruba and Jensen [176] reported that women preferentially store post-prandial FFA in the gluteal femoral adipose tissue depot, demonstrating its important role in excess FFA disposal into the lower-body SAT. Thus, it may be hypothesised that the ability of peripheral SAT to effectively sequester excess FFAs from circulation for storage within this depot rather than in VAT may be protective against the lipotoxic effects of ectopic fat deposition. In support of this notion, higher TAG sequestration was demonstrated to be a determinant of insulin sensitivity in insulin sensitive compared to insulin resistant BMI-matched individuals [177]. On the other hand, when the ability to safely store FFA in SAT is inhibited, these FFAs may be deposited in VAT and/or other ectopic sites less adapted for this purpose where they may contribute to the deterioration of systemic and hepatic insulin sensitivity.

2.3.2 Adipo/cytokine production

Another consequence of expanding adipose tissue, described as the endocrine hypothesis, relates to increased VAT mass affecting insulin sensitivity through the increased secretion of products other than FFA, such as adipo/cytokines (Table 2.4). VAT and its associated macrophages have been shown to secrete higher levels of pro-inflammatory adipokines than SAT [107]. Measures of visceral adiposity have been shown to correlate with increased serum levels of pro-inflammatory cytokines (eg. IL6, IL8) [178, 179], and VAT adipocytes secrete these cytokines in larger quantities than SAT adipocytes [180, 181]. Serum levels of both IL-6 and IL-8 have been show to negatively influence insulin resistance, and that IL-6 has predictive value in the risk of T2DM

Table 2.4 The Morphologic and physiologic characteristics of VAT compared to SAT: Adipo/cytokines production and secretion

Depot adipo/cytokine secretion characteristics		Primary metabolic function	Contribution to IR
VAT > SAT	Adiponectin secretion Angiotensinogen Inflammatory cytokines (IL-6, IL-8, PAI-1)	Adiponectin and leptin function in the regulation fat metabolism throughout different depots.	Endocrine hypothesis
VAT < SAT	Leptin secretion [154, 184, 185]	Leptin expression from abdominal SAT relays nutritional status to the energy regulating centres of the brain [186, 187]	Resistance to leptin may promote ectopic fat deposition and further exacerbate insulin resistance. Additionally, defects in leptin signal transduction may lead to insulin resistance [188]
VAT > SAT	Glucocorticoid receptor (GR-α) [189] 11β-hydroxysteroiddehydrogenase 1 (11β-HSD-1)[190]	Conversion of inactive cortisone to active cortisol by 11β-HSD-1.	Elevated 11β-HSD-1 activity in VAT associates with increased VAT accumulation and insulin resistance [191]

development [182]. Importantly, DSAT and SSAT of obese individuals are not homogenous in terms of inflammatory profile. DSAT has been reported to have higher inflammatory gene expression compared to SSAT [183], and suggest a distinct role for this depot in obesity-associated metabolic complications.

Other common adipo/cytokines associated with insulin resistance is adiponectin, leptin and angiotensin. Notably, leptin was first identified as the product of the *obese* (*ob*) gene in mice in which the expression of a non-functional protein product results in profound obesity and T2DM [187], a phenotype considered synonymous to morbid obesity in humans. Leptin is an adipocyte-derived factor that communicates the level of fat stores to the energy regulating centres of the brain (e.g. hypothalamus) [187]. Leptin levels have been measured to be approximately 4 times higher in the peripheral circulation in obese compared to normal-weight individuals [192]. However, during obesity, although high amounts of leptin are produced from the abdominal SAT depots, adipocytes become resistant to its actions, termed leptin resistance [193]. Notably,

increased IL-6 and lower leptin levels have been demonstrated in the portal vein compared to the peripheral artery *in vivo,* and demonstrates the close association between increased VAT and reduced insulin sensitivity [172, 194].

2.3.3 Structural component differences between adipose tissue depots and associations with insulin resistance

In addition to the metabolic/secretory differences described between VAT and SAT depots and their respective effects on insulin sensitivity, depot-differences in cellular and structural components have also been reported to differentially impact insulin sensitivity, and are discussed next.

2.3.3.1 Cellularity

During adipose tissue development, depot enlargement can occur either by hyperplasia, the recruitment of progenitors/pre-adipocytes to form new lipid laden adipocytes, or by hypertrophy, the development and enlargement of existing adipocytes. These two process are tightly regulated in each adipose tissue depot and the result is that adipose tissue compartments such as VAT and abdominal SAT differ in both cell number and cell size, the ratio of which is often referred to as the cellularity of a depot [195]. Apart from the mature lipid filled adipocytes that make up the bulk of adipose tissue volume, the cellularity of the depot also refers to the non-adipocyte component present in adipose tissue, which is known as the stromal vascular fraction (SVF). These include pluripotent mesenchymal stem cells (MSC's), fibroblasts, vascular endothelial cells of blood and lymphatic vessels, macrophages, mast and dendritic cells [196]. Additionally, pre-adipocytes are generated during adipogenesis from fibroblast-like MSCs cells. In this process, MSC's that reside in perivascular locations [197-200] are recruited into adipose tissue to form new adipocytes.

Table 2.5 The Morphologic and physiologic characteristics of VAT compared to SAT: Structural characteristics: Cellularity

Structural characteristic: Cellularity		Primary metabolic function	Contribution to IR
Adipocytes			
VAT > SAT	Mesentric adipocyte size[150, 184, 202] Apoptosis susceptibility /senescence[203, 204]	Omental adipocytes are 20-30% smaller than abdominal adipocytes over large BMI range. However, lower-body adipocytes can develop rapidly into mature adipocytes to buffer incoming lipid.	Mean adipocyte size from all adipose depots increase with obesity. During weight gain, having a lower differentiation capacity in lower body SAT depot results in enlarged abdominal (VAT and SAT) adipocytes. These large adipocytes are more insulin resistant.
VAT < SAT	Omental adipocytes[150, 184, 202] Higher proliferation and differentiation capacity in vitro[154, 205, 206] PPARγ production[207, 208] Increased cell size[150, 184, 202]		
Cells of the SVF			
VAT > SAT	Macrophage infiltration Endothelial cells [206] SVF cells per gram tissue [206]		

Differentiation of these MSC's into mature lipid-laden adipocytes is a two-step developmental process. Firstly, the commitment stage involves a reduction of the developmental potential of the stem cell that ultimately renders it incapable of differentiating into other cell types. This determination step is an irreversible commitment of the pluripotent MSC to a pathway of differentiation to the adipocyte lineage [199]. In the second step, the terminally differentiated pre-adipocyte, now incapable of further mitotic division then acquire the features of mature adipocytes and undergo lipogenesis to acquire its characteristic lipid droplet. Therefore, the formation of all "new" mature adipocytes during depot expansion is a result of the proliferation and differentiation of pre-adipocytes and their more primitive from, MSC's, in response to the metabolic demand. It is estimated that the half-life of a mature adipocyte is about 8 years and that approximately 10% of adipocyte are renewed annually [201].

Experiments using the murine 3T3-L1 and 3T3-F442A pre-adipocyte cell lines have become the "gold standard" for investigating the *in vitro* transcriptional cascade that promotes adipocyte differentiation [209, 210]. CCAAT-enhancer-binding proteins

(C/EBPs), basic leucine-zipper class of transcription factors and peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear receptor subfamily, function together as pleiotropic transcriptional activators of a large group of genes that produce the adipocyte phenotype [210-212]. The sterol regulatory element-binding proteins (SREBP's), basic helix-loop-helix-leucine zipper proteins, regulate gene expression involved in cholesterol and lipid metabolism. SREBP1c mRNA expression is activated downstream of C/EBPα and PPARγ-induced induction of differentiation. Under insulin regulation, SREBP1c is cleaved to its activated form after which it is translocated to the nucleus. Here it initiates the transcription of lipogenic enzymes which induce lipid biosynthesis and produce the mature adipocyte characteristics [213].

2.3.3.1.1 Adipocyte hypertrophy versus hyperplasia and metabolic health

The ratio of adipocyte hyperplasia to hypertrophy during adipose tissue expansion ultimately determines the cellularity of each adipose tissue depot and as such, its relationship with energy metabolism. In contrast to the recruitment of new progenitor cells and their differentiation into adipocytes, mature/differentiated adipocytes increase their cell size/volume predominantly by the taking up FFA from circulation and increasing TAG storage. It is accepted that the lager adipocyte cell-size is associated with increased BMI, and is directly related to the development of metabolic dysregulation [214]. However, the relationship between cell size and metabolic abnormalities is now increasingly being shown to be depot- and gender-dependent.

The dimorphic nature of hypertrophic versus hyperplastic depot expansion of VAT and SAT depots is most clearly demonstrated murine models. Studies in mice have shown that the epididymal fat pad increases in cell number up to a certain age, and then mainly expands by hypertrophy [215]. However, in the subcutaneous depot and retroperitoneal depots of rodents, prolonged fat feeding results in depot growth mainly

by hyperplasia [215, 216]. VAT tissue growth occurs mostly through hyperplasia in both mice [217, 218] and humans [219],while SAT does not seem to show significant hyperplasia in response to high fat feeding [217, 218] during which depot enlargement mainly occurs through increased cell size. In contrast, in response to bariatric surgery-induced weight loss, abdominal SAT adipocyte cell size is significantly reduced with no significant reduction in adipocyte number noted [201]. The differences in adipocyte growth rate and/or differentiation and proliferation responses between the different adipose tissue depots have been proposed to be dependent on both intrinsic and extrinsic factors [220], and are discussed next. These studies indicate that depot-specific mechanisms are important in the regulation of adipose tissue growth during obesity [220] and determines its relationship with insulin sensitivity and metabolic disease.

2.3.3.2 The microenvironment

2.3.3.2.1 The structure of the microenvironment

Human adipose tissue consists primarily of large spherical cells in which a large lipid droplet fills almost the entire volume. Adipocytes vary widely in size and can range from 30-200 µm in diameter [221, 222]. Since only a lipid monolayer forms the boundary between the large fat droplet and the cytosol, mechanical stress could easily lead to the disruption of the organelle [223, 224]. To structurally support the cell, a strong external connective tissue framework is needed.

Napolitano, using a murine model, was the first to demonstrate adipose tissue ECM as fibrillar collagen structures that surround adipocytes [225]. This extracellular framework is formed by the pericellular basement membrane, situated around each adipocyte, and the interconnecting interstitial fibres. The ECM is made up of structural proteins (collagens I-VI) and various adhesion molecules including fibronectin,

laminins, elastins and proteoglycans (decorin and lumican) and matricellular proteins such as MMP's, secreted proteins acidic in nature (SPARC) and thrombospondins (THBS) [226]. Table 2.6 lists the key matrix adhesion and structural collagen components and their functional relevance in adipose tissue. In short, Triple-helical type I collagen molecules form interwoven bundles and provide the major ECM network necessary to sustain the structure and function of all mesenchymal tissues [227] , while type IV collagen forms the pericellular basement membrane [228]. The basement membrane provides the structural support to the mature adipocyte and also interacts with cell surface integrins for cell ECM signalling. Collagen IV and laminins make up the major components of the basement membrane. These molecules are assembled in the extracellular space and form a basket-like ultrastructure of the basement membranes that surrounds each adipocyte. Nidogen and perlican bridge the laminin and type IV collagen network to stabilize and maintain basement membrane integrity [229] (Figure 2.1).

Table 2.6 Adhesion molecules and collagen components of the ECM in adipose tissue

Component	Composition	Matrix structure	Function	Refs
Adhesion proteins				
Fibronectin	Dimers, non-collagenous 250 kDa multidomain subunits	Fibrillar meshwork, Stromal ECM	Modulates cell shape, Inhibits adipogenesis. Communication between cell and ECM. Organization of other ECM components	191
Laminins	Triple-helix of α, β and γ chains	Integrates with Col IV to form basement membrane	Major component of basement membranes. Signalling ECM-cell interactions	230
Elastic fibres	Hierarchical assembly and cross-linking of multiple tropoelastin (60kD) monomers that accumulate on a micro-fibrillar skeleton.	Asymmetric coil, contains cell binding domains. Pericellular distribution around adipocytes and are associated with blood vessels	Provides flexibility and extendability in tissue enabling long-range deformability and passive recoil without energy input	231-233
SPARC	Matricellular glycoprotein momomer 35kD	Bound to collagen network	Mediator of collagen deposition and promotes fibrosis. Inhibits adipocyte differentiation and adipogenesis, and adipose tissue hyperplasia	234
MMP's	Ca^{2+}-dependent Zn-containing endopeptidases. Consists of N-terminal pro-domain, catalytic domain, hinge region, and C-terminal hemopexin-like domain	Secreted by fibroblasts, osteoblasts, endothelial cells, macrophages, neutrophils, and lymphocytes.	Responsible for tissue remodelling and degradation of the ECM, including collagens, elastins, gelatin, matrix glycoproteins, and proteoglycans	235
THBS	Matricellular glycol-protein momomer includes THBS 1 and 2	Resides in pericellular space	THBS 1 regulates TGF-β activity and correlates with inflammation. THBS2 clears MMP 2 from pericellular space	234
Structural components				
Collagen Type I (ColI)	Triple-helix of $[\alpha1(I)]_2$ α2(I) heterotrimers	Interwoven with each other to form thick collagen bundles	ECM framework for adipocyte support. Tensile strength and tissue rigidity	227, 236
Collagen Type IV (ColIV)	Triple-helix of $[\alpha1(I)]_2$ α2(I) heterotrimers	Forms major component of basement membranes around adipocytes	Structural support to mature adipocytes. Mechano-signal transduction between adipocyte and interstitial matrix	72
Collagen Type V (ColV)	minor-fibrillar α 1(V) α 2(V) heterotrimers	Pericellular ECM. Co-assembles with type I collagen to form heterotypic fibril	Controls collagen fibril assembly in several tissues	237
Collagen Type VI (ColVI)	Non-fibrillar α 1(VI) α 2(VI) α 3(VI) heterotrimers	Micro-fibrils in the interspace between basement membranes and thick ColI bundles	Regulates adipocyte hypertrophy. Links basement membrane to interstitial space	238

Matricellular proteins are non-structural, extracellular regulators of cell function and facilitate ECM cell interactions [234]. The matricellular protein SPARC also contributes to the basement membrane integrity [234]. Type VI collagen, a non fibrillar collagen binds various other ECM components in the basement membrane and interstitial fibres and

links the adipocyte to the ECM through its interaction with cell surface integrins [238]. Integrins are heterodimeric transmembrane receptors that bind externally to the ECM and internally to the adipocyte cytoskeleton [239]. Integrins transduce mechanical information from the ECM to regulate gene expression and function. In doing so, integrins may provide the adipocyte with a means to sense its own size [240, 241].

Type V collagen molecules also form part of the basement membrane assembly [242, 243] where they exist as micro-fibers that are closely associated with type I collagen bundles, and have been demonstrated to regulate fibril assembly and thickness [237, 244]. Early ECM development has mostly been investigated *in vitro* in differentiating 3T3-L1 adipocytes. Studies on the differentiating adipocyte secretome indicate that the induction of ECM expression is one of the earliest events induced during differentiation when the collagen/fibronectin-rich matrix surrounding the pre-adipocyte is replaced by type IV collagen, laminin, and heparin sulphate proteoglycans that form the basement membrane which is more characteristic of the mature adipocyte. This conversion is crucial for the fibroblast-like cell to accomplish its new spherical form [209].

ECM synthesis has also been shown to be a prerequisite for pre-adipocyte differentiation and TAG accumulation. Mouse 10T1/2 pre-adipocytes treated with a collagen synthesis inhibitor, ethyl-3,4-dihydroxybenzoate (EDHB), fail to develop into mature, lipid laden adipocytes [245]. Similarly, upon EDHB treatment, bovine intramuscular pre-adipocytes (BIP) cells do not accumulate collagens (types I-VI) or triglyceride, which are generally associated with mature adipocytes. However, when exogenous type V and type VI collagens were supplied to the culture dishes, these cells were able to differentiate and restore the mature adipocyte phenotype [246], highlighting the importance of these matrix components, as well as other matrix remodelling enzymes (Table 2.6) to adipocyte development .

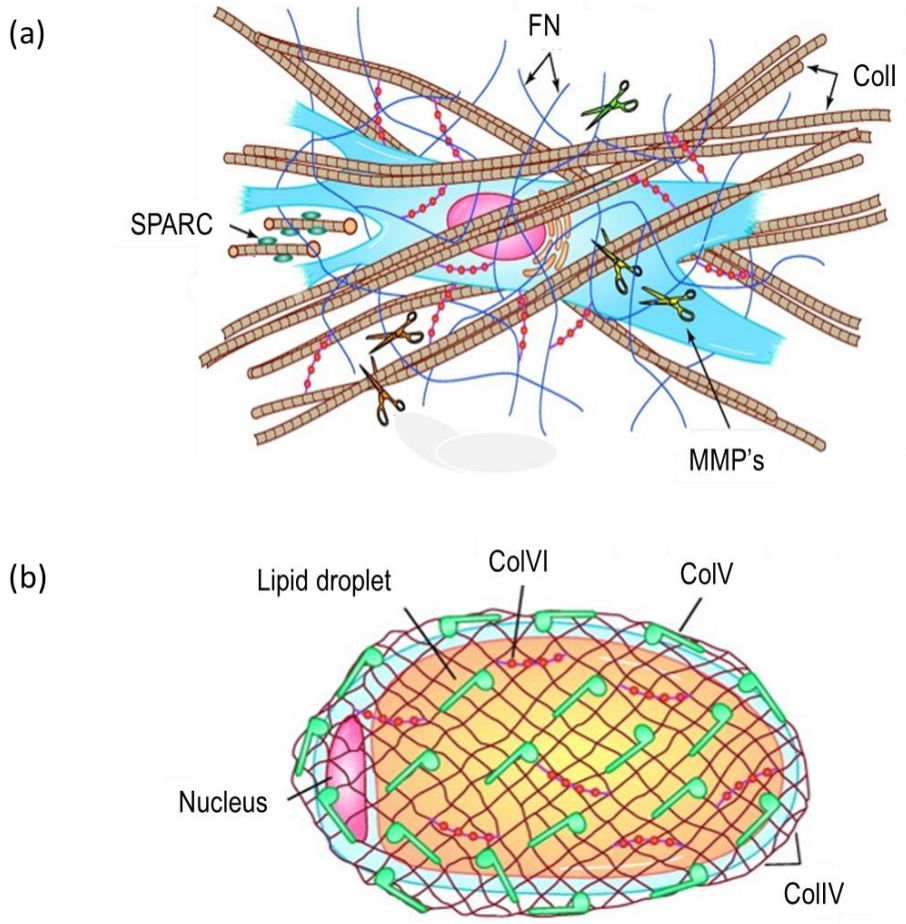


Figure 2.1 Structural organization of the ECM and basement membrane components around the adipocyte. a.) Fibroblastic pre-adipocyte associates with ECM comprising of large Collagen type 1 (Col1) fibers (cross-banded tan lines), fibronectin (FN) fibers (thin blue lines) and Collagen type VI (ColVI) red beaded lines. The matrix metalloproteinases (MMP's) are depicted by the scissors and b) mature adipocyte and ECM and basement membrane components. Pre-adipocyte differentiation –Adapted from Haung and Greenspan 2012 [2]

2.3.3.2.2 Regulation of ECM expression in soft connective tissue

Adipose tissue has a unique plasticity demonstrated by its ability to quickly expand during times of nutrient excess and to retract during nutrient scarcity. To accommodate the ever-changing adipose tissue organ, the ultra-structure and the composition of the ECM needs to be regularly remodelled. The factors regulating ECM remodelling in adipose tissue are not fully understood. However, as a soft connective tissue, the

mechanisms that regulate adipose tissue ECM turnover may reflect those that regulate ECM remodelling within other connective tissues such as tendons, ligaments and bone [247].

The expression of structural proteins in soft connective tissues are regulated by signalling cascades in response to stimuli such as repetitive mechanical loading. This process results in adaptive ECM reorganization which allows for tissue repair and healing [248]. Macrophages respond to tissue injury with the ultimate goal of wound healing and phagocytosis of contaminating components. Activated macrophages secrete pro-inflammatory factors (e.g. MCP-1, -2, -3; MIP-1α, -1β; osteopontin) that exacerbate inflammatory cell infiltration into the sites of tissue injury [249]. They are considered the master regulators of the ECM turnover [249] through their production of pro-fibrotic factors such as transforming growth factor β (TGF-β1) and platelet-derived growth factor (PDGF) [250, 251]. Macrophages are also the main source of several types of matrix metallopoteinases (MMPs), the major enzymes involved in ECM degradation [252], and their endogenous suppressors, tissue inhibitors of MMPs (TIMPs). The inflammatory mediated tissue repair process consists of two stages. Normal tissue repair, prompted by an acute inflammatory state, involves an orderly remodelling of ECM components and removal of dead or damaged cells. However, failure to resolve the inflammatory state results in maladaptive ECM remodelling and the formation of fibrosis.

Hypertrophic adipocytes may experience increased mechanical stress due to increasing static strain of the enlarging lipid droplet. Indeed, cell stiffness (resistance to deformation) has been shown to increase during adipogenesis as preadipocytes mature and acquire their characteristic lipid droplet [253]. Increased mechanical loading has also been reported to influence stochastic events in the adipocyte life cycle (e.g.

cell proliferation, commitment and differentiation) [254]. Shoham et al., [254] demonstrated that stretching in 3T3-L1 adipocytes promoted differentiation but did not have effects on mitosis. Shear stress and cyclic strain altered the structure and cytoskeleton of the cells in a dose-dependent manner and are also subject to threshold levels [255]. Additionally, Chien et al., [256] showed that cultured human aortic endothelial cells responded to sustained shearing but that the resulting mRNA and protein expression was dependent of the direction of the stress (stress with a forward component vs stress without a significant forward component). These studies show that adipocyte function is regulated by cytoarchitecture and suggests that chronic exposure to adipocyte cell deformation e.g. increased weight-bearing fat tissue mass, may contribute to alterations in ECM in adipose tissue which may have exacerbating effects on inflammation and insulin resistance.

Intrinsic factors

Excessive mechanical loading is often reported as a risk factor for soft tissue injuries [257]. Carpal tunnel syndrome in the hands [258], Achilles tendinopathy in the heel [259], anterior cruciate ligament (ACL) injuries in the knee [260] and rotator cuff tendinopathy in the shoulder [248, 261] are commonly found in sporting activities that involve sudden deceleration or change in direction [262]. Although the mechanisms of these overuse and acute exercise-associated soft-tissue injuries are poorly understood, the expression of collagens, proteoglycans and/or glycoprotein content have been demonstrated to be altered in disease states such as tendinopathy and ligament injuries [248]. These injuries are considered multifactorial disorders since several extrinsic and intrinsic risk factors for developing these disorders have been identified [259]. With genetic and familial factors being important intrinsic risk factors [248],

susceptibility to these injuries are determined by exposure to extrinsic risk factors such as repetitive mechanical loading.

Rare Mendelian connective-tissue disorders that are independent of environmental exposures such as classic Ehlers-Danlos syndrome (cEDS) [263], Bethlem myopathy [264-266] and Ulrich congenital Muscular dystrophy [264-266] have highlighted the importance of genes encoding type V and type VI members of the collagen protein family in the normal structure and function of connective tissues. Although the phenotypes resulting from these mutations occur without any input from environmental factors, similar mutations resulting in less severe/extreme phenotypes may require sufficient environmental exposures interacting on a genetic background to result in the multifactorial phenotypes such as those observed in soft tissue pathology.

One such polymorphism, *COL5A1* rs 12722 (C/T) in the type V collagen gene occurs within the 3'untranslated region (URT), and has previously been shown to be associated with soft tissue injuries and connective tissue pathology [267-269],and shows an association with body weight and BMI [270, 271]. The *COL5A1* BstU1 rs12722 has been suggested to be functional. Specifically, the T-allele is thought to increase mRNA stability and increase α1(V) chain synthesis [272], which may have an impact on fibrillogenesis and influence tissue elasticity. Further, the TT-genotype has been associated with increased risk for developing achilles tendinopathy, whereas the CC-genotype was over-represented in the apparently healthy control group [267].

COL6A1 rs 35796750 (C/T) in the type VI collagen gene has been associated with a number of multi-factorial conditions such as ossification of the posterior longitudinal ligament of the spine (OPLL) [273] and ossification of the ligamentum flavum (OLF) [274]. Epidemiological studies have identified a variety of risk factors associated with the development of OPLL [274] including diabetes, high BMI, hormonal imbalance, dietary

habits, trauma, gender and ethnicity [275]. Specifically, the *COL6A1* rs 35796750 (T/C) was significantly associated with both OPLL and OLF in two independent Japanese populations [274] where a high BMI after age 20 years and T2DM were independent risk factors for developing these disease states [276].The *COL6A1* rs 35796750 SNP has also been proposed to be functional. Although a splice variant has not yet been identified, the T→C substitution at intron 32 [-29] occurs near the branch site of the intron and consequently may affect the lariat-shaped structure resulting in aberrant protein splicing [277].

2.3.3.3 Vascularisation and adipose tissue blood flow (ATBF)

Adipose tissue comprises a dense capillary network that delivers oxygen and energy substrates to the adipocytes. Moreover, the vascularization provides a transport route for hormones and cytokines and energy substrates to and from the adipocytes [278]. Dietary lipids from the small intestinal enterocytes are transported within the circulation and delivered to the adipocytes for storage. In addition to its role in FFA delivery, the microvasculature of adipose tissue serves as a transport route for immune and adipocyte progenitor cells [279].

Gluteal SAT is considered to be less metabolically active compared to abdominal SAT based mainly on its reduced β-adrenergic lipolytic-response and its increased sensitivity to the anti-lipolytic effects of insulin stimulation (summarised above in Table 2.3). Further, post-absorptive ATBF in lean individuals has been shown to be higher in the abdominal SAT compared to the gluteal-femoral depot, perhaps reflecting its special metabolic role for meal FFA uptake [280]. These depot differences are best illustrated through the markedly different depot response to catecholamine-induced lipolysis in abdominal- compared to the gluteal SAT depots [281].

Depot differences in ATBF between VAT and SAT depots in the obese state are less clear. Villaret et al [282] found increased capillary density in VAT compared to SAT in paired samples from *29* obese patients undergoing bariatric surgery[282]. In contrast Gealekman et al [283] demonstrated that SAT from obese individuals had higher capillary density per adipocyte *in vivo* and higher capacity for angiogenic growth *ex-vivo* compared to VAT. Further, Lemoine et al [284] investigated vessel density in *29* obese humans undergoing bariatric surgery and found no differences in vessel number per adipocyte in VAT compared to SAT depot. These authors also reported a significant positive association between waist circumference and capillary density in VAT, but not in the SAT depot.

Notably, vascular density not only differs between adipose tissue depots, but also within each depot. For example, in rodents, Cho et al [285] demonstrated that the tip portion of epididymal adipose tissue depot contained a higher density of vasculature compared to the rest of the depot. These findings suggests that different gradients of oxygen and nutrient supply may exist not only between different adipose tissue depots, but also within a given adipose tissue depot. Therefore, it is conceivable that the degree of vascularization throughout each adipose depot may have different implications for hypoxia and inflammatory signalling.

2.4 REDUCED ADIPOSE TISSUE EXPANSION AND INSULIN RESISTANCE

Adipose tissue dysfunction represents one of the earliest abnormalities in the development of obesity, and constitutes an important contributor to individual risk to develop metabolic and CVD diseases. Adipose dysfunction may develop under conditions of excess calorie intake in patients with an impaired expandability in the SAT depot. Fat storage deficiency, as a major determinant of insulin resistance and dyslipidaemia, has been demonstrated in genetic forms of lipodystrophies in human

and murine models and are characterised by the complete or partial loss of adipose tissue, severe insulin resistance and ectopic fat deposition (see Medina-Gomez., [286] for review). In contrast, a transgenic mouse model of morbid obesity that displayed increased amounts of body fat mass, but limited VAT accumulation and lower fat accumulation in the liver and muscles, had a better metabolic profile compared to its wild type litter mates [287]. Results from a seminal study utilising adipose tissue transplantation into a lipoatrophic (low fat mass) mouse model showed that the addition of normal SAT reversed insulin resistance, hepatic steatosis and hypoleptinemia [288]. These results suggest that the lack of adipose tissue was causative in the metabolic abnormalities displayed in these lipodystrophic phenotypes [288]. The mechanisms leading to these improvements are still unknown but may involve the enhanced FFA uptake by adipocytes and concomitant improvement of inflammatory profile.

2.4.1 Insufficient adipogenic capacity in SAT insulin resistance

PPARγ is considered to be the master regulator of adipogenesis and lipogenesis in adipose tissue. Activation of *PPARγ* promotes adipocyte differentiation through FFA storage whilst it simultaneously represses lipolysis genes and the release of FFA from the adipocyte [289]. Reduced expression of this factor results in dysregulation of lipid storage and mobilization. Adipose tissue hypertrophy (due to increased cell diameter) has been shown to be a major determinant of obesity development [201] and correlates with parameters of insulin sensitivity and lipid metabolism [290]. Obese patients with larger cell size in their SAT are more hyperinsulinaemic and glucose intolerant whereas similarly obese patients with a larger proportion of smaller adipocytes are more insulin sensitive [291]. Extremely filled adipocytes are insulin resistant and therefore less effective metabolic buffers. Adipocyte hypertrophy is a feature of non-

obese T2DM patients [292]. Adipocyte hypertrophy reflects both reduced pre-adipocyte recruitment along with impaired differentiation capacity in the setting of increased TAG demand.

2.4.1.1 Hypoxia

Atmospheric oxygen tension at sea level is about 160 mmHg (21%). However, physiological oxygen levels vary dramatically between different tissues, and can range from about 150 mmHg in the upper respiratory tract to 1- 10 mmHg in the retina [66, 293]. In adipose tissue, oxygen tension can vary dramatically depending on species studied, adipose tissue depot and obesity level. For instance, by using O_2-electrodes, it was shown that obese rodents had lower *in vivo* oxygen partial pressure than normal-weight rodents (15mm Hg vs 48 mm Hg respectively) [294]. Similarly, obese humans had lower adipose tissue partial pressure (46 mmHg) compared to lean controls (55 mmHg) [295].

Generally, hypoxia occurs when oxygen demand from cells or tissue exceeds the supply, resulting in reduced oxygen tension. Specifically, in adipose tissue, hypoxia may result from inadequate blood flow to the adipose tissue depot, increased cell size and/or enhanced oxygen consumption by the tissue [296]. It is postulated that the increased cell size of hypertrophic adipocytes associated with obesity increases the distance that oxygen has to diffuse from the microvasculature into the cells [221]. As a result, oxygen partial pressure reduces [297], causing a local hypoxic environment [294, 298-301]. Hypoxia has been measured in adipose tissue of obese humans [70, 301] and rodents [294, 302] (Table 2.7). Cellular adaptation to hypoxia is accomplished through the activation of an array of oxygen-sensing transcription factors, including hypoxia inducible factor-1 (*HIF-1*α) [303, 304]. *HIF-1*α is a heterodimer that consists of an oxygen-

Table 2.7 Direct measurement of oxygen partial pressure (PO_2) in human and mouse adipose tissue studies

Study	Population	Measurement method	PO_2
Human			
Kabon et al [300]	Men and women under-going surgery (n= 46)	Micro Clark type electrode, inserted 1cm into SAT (arm and abdomen)	Lean vs obese: 57 vs 36 mmHg
Fleishmann et al [299]	Men and women undergoing surgery (n= 35)	Micro Clark type electrode, inserted 1cm into SAT (upper arm)	Lean vs obese: 57 vs 41 mmHg
Pascarica et al [70]	Men and women (n= 21)	Micro Clark type electrode, inserted 1 cm into SAT (abdominal area)	Lean vs obese: 55 vs 47 mmHg
Goossens et al [309]	Men (n=20)	Opto-chemical measurement system using microdialysis (Abdominal SAT area)	Lean vs obese: 48 vs 67 mmHg
Murine			
Yin et al [310]	Mice (♂), (aged 6 -12 weeks) (n= 3)	Optic-fiber oxygen micro sensor (epididymal fat pad)	Wild-type vs *ob/ob:* 6 weeks 57 vs 35 mmHg 12 weeks 57 vs 20 mmHg
Ye et al [294]	Mice (♂) (n= 3)	Optic-fiber oxygen micro sensor (epididymal- and retroperitoneal fat pad,	Wild-type vs *ob/ob:* Epididymal: 48 vs 15.2 mmHg Retroperitoneal: 48 vs 21 mmHg
Rausch et al [311]	C57BL/6J Mice (♂) (n= 4)	OxyLite fluorometric micro oxygen sensor.(perigonadal fat pad,	C57BL/6J lean vs DIO + *ob/ob:* 35-40 vs 20-25 mmHg

SAT: Superficial adipose tissue, DIO: diet induced obesity, ♂:male

regulated α subunit and a constitutively expressed β subunit [304, 305]. Under normoxic conditions, *HIF-1*α is targeted for proteasomal destruction by proline hydroxylation by an E3 ubiquitin ligase complex containing the von Hippel-Lindau tumor suppressor protein (pVHL) [306]. During hypoxic conditions, the lack of molecular oxygen, an absolute requirement for *HIF-1*α hydroxylation, results in increased *HIF-1*α protein accumulation. Upon stabilization, *HIF-1*α translocates to the nucleus where it heterodimerizes with *HIF-1*β and other co-activators in order to bind to the hypoxia response element (HRE), through which it activates its target genes [307, 308].

*HIF-1*α mediates the adaptation and survival to hypoxia by inducing the expression of pro-angiogenic proteins such as vascular endothelial growth factor (*VEGF*α) and pyruvate dehydrogenase kinase-1 (PDK1) [312], and by inducing the switch to anaerobic-glycolytic metabolism. In tumour biology, *VEGF*α expression in tumour cells [313] allows for vascular development and tumour expansion. However, in hypertrophic obese adipose tissue, even though *HIF-1*α protein increases, *VEGF*α does not increase [70, 294, 301, 307] and is accompanied by reduced capillary density [116, 294]. This unresponsiveness of *VEGF*α transcription to elevated *HIF-1*α levels was proposed to be related to hyperglycaemia in *ob*/*ob* mice, as *VEGF*α expression was previously shown to be inhibited by high glucose [294, 314].

Hypoxia, via the induction of *HIF-1*α expression, has also been shown to directly reduce insulin signalling. Regazzetti and colleagues [315] showed that hypoxia treatment of 3T3-L1 cells decreased the ability of insulin to bind to its receptor and reduced the tyrosine phosphorylation of IRS without affecting their respective protein concentration levels [315]. This reduction of insulin signalling was rapid and robust, with almost a complete inhibition of insulin-stimulated glucose transport, and was restored after re-oxygenation. In addition, insulin signalling may be further reduced by hypoxia through the inhibition of adiponectin expression [302].

The expansion of adipose tissue during obesity is characterised by a local chronic inflammatory state, in addition to increased circulating inflammatory markers. Inflammation is considered causal to the development of T2DM and links obesity to the metabolic syndrome [114, 316]. Adipocytes respond to hypoxia though inflammatory signalling, possibly to attract macrophages into the adipose tissue to remove necrotic cells and tissue debris resulting from increased apoptosis [317]. Yin et al., [310] reported that 3T3- L1 adipocytes under hypoxic (1% O_2) conditions had increased adipocyte

death, as measured by triglyceride release into culture medium and flow cytometry. However, the cause of adipocyte death during hypoxic conditions is still unknown [310].

Hypoxia through the stabilisation of *HIF-1α* has been shown to induce inflammatory gene expression not only from adipocytes but also from the resident macrophages and other cells of the SVF, which are also exposed to reduced oxygen partial pressure. *In vitro* exposure of human VAT SVF to hypoxia results in increased expression of *tumour necrosis factor α* (*TNFα*), *Interleukin* (*IL*)*-6*, *IL-10* and *Chemokine (C-C motif) ligand 2* (*CCL-2*) [318]. It is proposed that the increased inflammatory signalling from the resident macrophages and endothelial cells which are stimulated by the lower adipose tissue PO_2 from the expanding adipocytes, may be necessary to induce angiogenesis and subsequent vasculature formation in adipose tissue to alleviate effects of hypoxia [319].

2.4.2 Aberrant vascularization:

One of the earliest events during adipose tissue depot growth is the formation of capillaries [285]. Concomitant with increased adipogenesis, increased expression of potent pro-angiogenic factors such as *fibroblast growth factors* (FGFs) and VEGF, facilitate the migration of endothelial cells and pre-adipocytes into the adipose tissue and induce the formation of new vasculature that is required for adipose tissue growth [320]. However, during obesity development, vascular development does not always match the level of hypertrophy and results in the development of a local hypoxic state. Pasarica et al., [301] showed reduced capillary density and reduced *VEGF* mRNA in adipose tissue of obese humans compared to lean suggesting adipose tissue rarefaction (capillary drop out). Thus, it is suspected that oxygen supply in obese adipose tissue may be restricted. One reason for which increasing obesity might correlate with lower capillary density in adipose tissue of obese individuals is insulin

resistance. Indeed, Gealekman et al., [283] demonstrated that HOMA-IR associated negatively with SAT angiogenic capacity in a group of obese patients. It is hypothesised that angiogenic capacity increases during the development of adipose tissue but it is unable to continue in the correct proportion to adipocyte size and/or number during the progression towards obesity [283]. This lower capillary density would be unable to supply adequate O_2 to the expanding adipocytes which leads to the development of a relative hypoxic sate and associates with increased insulin resistance.

2.4.3 Dysregulated ECM remodelling

ECM dynamics need to be altered around the expanding adipocyte during tissue expansion. As adipose tissue develops, the ECM and the connecting interstitial-network is remodelled to accommodate the increasing adipocyte diameter and higher tissue mass. This process involves the enzymatic processing of the structural components within the tissue by synthesising new structures and degrading redundant ones. Previous studies have shown that during the pathological expansion of SAT, the expression of ECM components and their associated remodeling enzymes become dysregulated [72, 321, 322]. This process results in increased adipose tissue fibrosis [323] and is considered a hallmark of pathological tissue alterations leading to the development of insulin resistance [324]. Studies summarising the contribution of collagen and ECM accumulation in adipose tissue on the development insulin resistance is shown in Table 2.8.

This phenomenon was highlighted in C57BL/6 mice by Strissel et al [325] who showed that during the progression of obesity, induced by overfeeding, extensive adipose tissue ECM remodelling occurred that was associated with enhanced collagen deposition and increased inflammatory markers [325]. Moreover, Heneger et al., [326]

demonstrated that red picrosirius staining collagens grouped in bundles in SAT of obese humans and that ECM, adipocyte metabolism and inflammatory markers associated with the degree of obesity [326].

Increased mRNA and protein expression of type I, IV and VI collagen has consistently been shown to be higher in obese compared to lean subjects [295, 323, 327]. Divoux et al., [327] observed higher quantities of type VI collagen around adipocytes in obese than lean omental human adipose tissue. Further, Pasarica et al. [295], demonstrated that the expression of type VI collagen is up-regulated after overfeeding in humans, with a concomitant increase in inflammatory gene expression. Fibrosis and collagen accumulation in obese adipose tissue is closely correlated with BMI and inversely correlated with insulin sensitivity [295, 328]. Because obese patients with smaller adipocyte cell size display a more favourable lipid profile compared to those who exhibit hypertrophy [290], the increased fibrosis generation around the expanding adipocytes has been considered as an adaptive response to restrain excessive adipose tissue hypertrophy in obesity [323]. Fibrosis may thus act as an adaptive mechanism that contributes to the slowing down of the negative effects of adipocyte hypertrophy such as hypoxia, inflammation and insulin resistance [327].

Conversely, other studies have shown that reduced fibrosis and/or collagen deposition is associated with reduced insulin resistance. In a murine model, a reduction in collagen protein content in adipose tissue has been shown to alleviate the negative metabolic effects of excess adipocyte hypertrophy. Using type VI collagen-null mice, Khan et al [323], demonstrated that the lack of type VI collagen improved several metabolic parameters, including insulin resistance and inflammation. They showed that collagen VI-null *ob*/*ob* mice have less weight gain on a high fat diet compared to mice with only the *ob*/*ob* mutation. The type VI collagen-null mice also showed

Table 2.8 The Morphologic and physiologic characteristics of VAT compared to SAT: Structural

Structural characteristic: Microenvironment		Primary metabolic function	Contribution to IR
Human			
VAT > SAT	Tot Collagen (%)[329]	ECM intrinsic determinant of differentiation capacity [330]	PC collagen (%) in VAT á with ↑ fasted Insulin and ↑HOMA- IR [329]
VAT<SAT	Age á ↑Collagen (%) (Tot and PC) in SAT but not VAT [329] Tot Collagen (%)á ↑Inflammation ↑Col6a3 mRNA [321]	Strong tot collagen ECM framework supports adipose tissue mass.	Dysregulated genes in SAT of IR vs IS women included ↑inflammation genes (TNF), insulin pathway genes (IRS), and angiogenesis (VEGF) [331]
VAT=SAT	PC collagen (%) ↑ Adipocyte diameter á ↑ PC collagen (%) [329]; ↑ PC collagen % á ↑Inflammation[329]	Fibrosis hypothesised to limit adipocyte hypertrophy in order to limit effects of large cell-size [332]	↑Collagen expression in both depots á ↑inflammation that may increase IR Dysregulated genes in VAT and SAT á ↑IR. Common pathgways include integrin cell-surface signalling, focal adhesion and ECM expression (*COL5A1*) [331]
Obese > lean	PC collagen (%)[329] ↑ColVI in VAT	BMI and body weight á ↑ PC collagen (%)	↑Areas of fibrosis in obese á ↓ S_I and ↑ macrophage number
Murine			
VAT<SAT	*Col1a1*, *Col3a1*, and *Col5a1* mRNA in rat in vivo.	High SAT ECM gene expression in rats age 3-12 weeks. ECM intrinsic determinant of differentiation capacity [330]	
VAT=SAT	*COL6a1* mRNA in rat		AT SVF secretes ECM that directly modulates terminal differentiation in depot-specific manner [330].

á, associates with; Tot, total; PC, pericellular (%)

improved carbohydrate and lipid metabolism, despite having an increased adipocyte cell size.

As a structural tissue, adipose tissue protects organs and muscles from mechanical- and sheer stresses that are generated through movement. Similar to other connective tissues such as ligaments, tendons, fascia and bone, repeated mechanical loading may similarly result in pathological alterations to the cellular components and composition of the ECM in the affected adipose tissue depot itself [257]. Increasing adipose tissue mass during the development of obesity is indeed characterised by altered ECM expression and increased M1-activated macrophage infiltration.

Another transcriptional target of *HIF-1*α is lysyle oxidase (*LOX*) [333]. *LOX* facilitates collagen cross-linking and stabilisation, specifically type I and III collagen, and thereby, increases tensile strength of the ECM components [334] and increases ECM deposition in adipose tissue. *HIF-1*α over-expressing mice show elevated levels of *LOX* protein, increased adipose tissue fibrosis, laid down in adipose tissue as fibrotic streaks, and reduced glucose tolerance [307]. Conversely, in *HIF-1*α over-expressing mice, inhibition of *LOX* activity by β-aminoproprionitrile (BAPN) treatment reduced fibrotic areas in adipose tissue, reduced inflammation and improved glucose tolerance [307]. *LOX* is up-regulated by *HIF-1*α in a dose dependant manner in transgenic mice. However, *LOX* is down-regulated by *PPAR*γ treatment with a resulting improvement of metabolic phenotype.

2.5 INSULIN SENSITIVE VS INSULIN RESISTANT ADIPOSE TISSUE EXPANSION

The relationship between body composition and insulin resistance has been shown to be ethnic-specific. Black African and African American women have less VAT, and more SAT, but have consistently been found to be more insulin resistant than white women of comparable BMI and body fat percentage (%fm) or waist circumference (section 2.2) [22, 55-57]. The notion of AT expandability has not been used to investigate the ethnic-specific associations of body composition, its distribution and measures of insulin sensitivity and secretion in black and white women (Section 2.2). Following this notion, the major factors that can influence the capacity of adipose tissue to expand are:

(1) reduced adipogenic capacity (section 2.4.1),

(2) insufficient and inappropriate vascularisation (section 2.4.2) and

(3) aberrant ECM remodelling (Section 2.4.3).

Accordingly, it could be hypothesised that the increased systemic insulin resistance in obese black women may be a consequence of dysregulated gluteal SAT expansion following obesity development, resulting in the inability of this depot to effectively store excess TAG with a concomitant redistribution of fat to the central depots. In this context, the insulin sensitive vs insulin resistant model of adipose tissue expansion is proposed (Figure 2.2) with the specific aim of investigating whether these factors may differ by ethnicity and explain the ethnic-specific associations between body composition and insulin sensitivity measures in black and white South-African women.

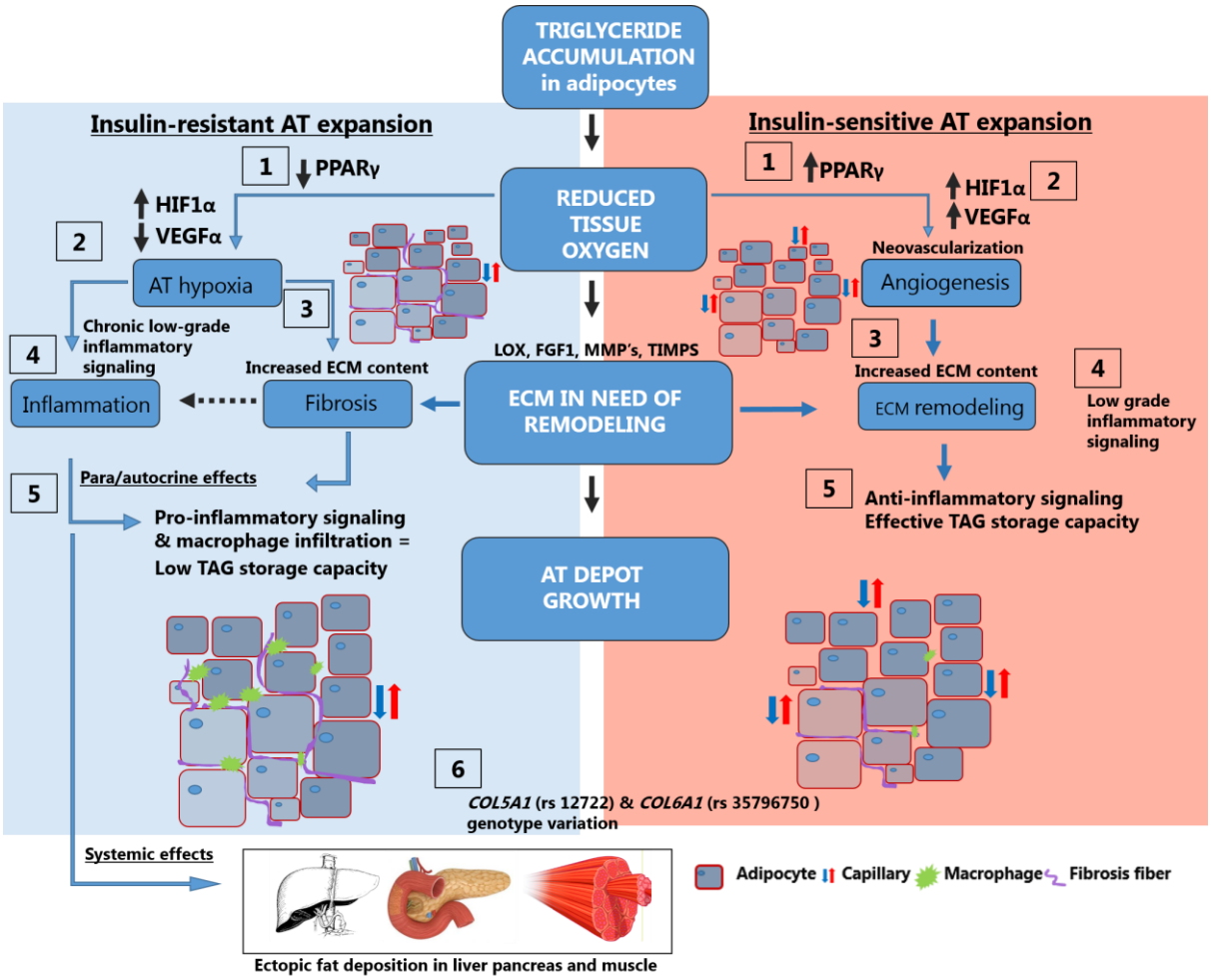


Figure 2.2 Model of insulin sensitive vs. insulin resistant adipose tissue expansion **Insulin resistant adipose tissue expansion (Blue block to the left)**: Pathologic adipose tissue expansion results in local and systemic insulin resistance. Increased adipocyte cell size results in local hypoxic state in adipose tissue. Increased HIF-1α stabilization does not induce angiogenic response, but rather invokes an inflammatory state with a high degree of macrophage infiltration, limited vasculature development and fibrotic response. Such inflammatory activation ultimately results in systemic insulin resistance. **Insulin sensitive adipose tissue expansion** (**Red block to the right)**: Healthy, insulin sensitive adipose tissue expansion encompasses the enhanced recruitment of adipocyte progenitors that may differentiate into small insulin sensitive adipocytes, appropriate increased vascularization, minimal induction of ECM and minimal inflammation

1) During adipose tissue expansion, adipocytes take up excess calories to store as TAG, resulting in either a larger cell diameter (hypertrophy) and/or by recruiting and differentiating new adipocytes from the precursor cells in the SVF (hyperplasia). Adipocyte hypertrophy and hyperplasia are regulated by genetic and depot-extrinsic factors. *PPARγ* induction activates gene transcription that promotes lipid storage.

Increased *PPARγ* expression during obesity development is associated with insulin sensitive adipose tissue expansion (right panel). In contrast, adipose tissue expansion characterised by low expression of *PPARγ* is associated with reduced insulin sensitivity (left panel) and hypertrophic obesity.

2) This increased adipocyte cell-diameter results in a local hypoxic state due to the greater distance that oxygen has to diffuse from the microvasculature to the cells [68, 335]. Cellular adaptation to hypoxia involves the activation of the master regulator of the hypoxia response, *HIF-1α* [336]. *HIF-1α* aims to induce angiogenesis through the expression of pro-angiogenic factors, such as *VEGFα,* in order to induce vasculature formation in the adipose depot to satisfy the oxygen requirement of the expanding adipocytes (right panel). In contrast, during pathological adipose tissue expansion, increased *HIF-1α* expression does not result in adequate *VEGFα* expression [70, 294, 301, 307], ultimately resulting in limited angiogenesis and the perpetuation of the hypoxic state (left panel).

3) ECM is remodelled through matrix remodelling enzymes such as *LOX* and *FGF1*. However, during obesity, increased ECM production and aberrant remodelling leads to increased fibrosis which makes the tissue stiffer and less accommodating to adipocyte expansion and capillary development (left panel) with concomitant increased inflammatory signalling.

4) Hypoxic adipocytes increase pro-inflammatory expression that associates with the influx of M1-activated macrophages. Adequate vascularisation and appropriate ECM remodelling resolves the hypoxic state which in turn reduces inflammation (right panel). In contrast, chronic inflammation can lead to further, more excessive ECM accumulation resulting in increased fibrosis around the adipocytes and in the adipose tissue depot (left panel).

5) A chronic higher inflammatory state, and resultant increased fibrosis, limits the TAG storage capacity of the adipose tissue and associates with increased local and systemic insulin resistance with resulting redistribution of adipose to the central depots (left panel). In contrast ECM remodelling that allows for appropriate adipocyte expansion, capillary development and TAG storage would provide protection against ectopic fat deposition in liver, muscles and pancreas, preserving the insulin sensitive state (right panel).

6) A genetic contribution to the profibrotic state within adipose tissue has not yet been investigated. The T- variant of the *COL5A1* rs 12722 polymorphism associates with connective tissue pathology [267-269], proposedly through its role in type I collagen fibrillogenesis and resultant tissue stiffness [272]. However, having at least one copy of C- allele attenuates its impact on soft tissue pathology risk [267]. It could be hypothesised that the T- allele of *COL5A1* rs12722 polymorphism that associates with increased tensile strength of the collagen network in tendons and ligaments, may represent a mechanical limitation to adipose tissue expandability with a commensurate effect on body fat distribution. The *COL6A1* rs 35796750 (T→C) SNP is strongly associated with ectopic bone formation in paravertebral ligaments, for which ethnicity, a high BMI after age 20 years, dietary habits and T2DM were independent risk factors for developing these disease states [274] [273, 276]. It remains to be elucidated whether genetic variation that associates with altered ECM component expression could potentially explain the ethnic-specific risk for insulin resistance and T2DM in black and white South African women.

Therefore the primary hypothesis of this thesis posits that during the development of obesity, the excess generation and deposition of ECM components within adipose tissue may represent a mechanical limitation to adipose tissue expandability, and

result in adipose tissue depot-specific associations with ethnicity. This model of insulin sensitive vs insulin resistant adipose tissue expansion may be potentially implemented to explain the different associations between body composition and insulin sensitivity measures in black and white South African women.

2.6 RESEARCH OBJECTIVES

The primary aim of this thesis is positioned within the context of adipose tissue expandability hypothesis, and aims to examine the hypothesis that differences in SAT ECM- and hypoxia-related gene expression and their ethnic-specific associations with body composition and insulin sensitivity may explain, in part, the higher rates of insulin resistance in black compared to white South African women. This thesis was divided into four separate research studies to investigate different aspects of this central hypothesis as described below.

Chapter Three

In the first study, three hypotheses are investigated: a) gluteal SAT of black women has greater expression of ECM and remodelling components and genes relating to the cellular hypoxia than white women and b) that the expression of these genes differ between gluteal and abdominal SAT; c) the expression of these genes in SAT of black and white women are differentially associated with insulin sensitivity and inflammatory gene expression. Therefore, the aims of this study are to i) compare depot-specific expression of ECM and matrix remodelling components and genes relating to the cellular hypoxia response in normal-weight and obese black and white women; ii) to examine the ethnic-specific associations between these genes and body composition, measures of insulin sensitivity and secretion and inflammatory gene expression in black and white SA women.

Chapter Four

The second study tests the hypothesis that higher hypoxia and ECM related gene expression would associate with higher central fat mass accumulation in black women and that the expression of these genes may be more closely associated with changes in the measures of insulin sensitivity in black women compared to white women. Therefore the aims of this study are to i) measure the expression of ECM and matrix remodelling components and genes relating to the cellular hypoxia response over a 5 year free-living follow-up period in black and white women; ii) to examine the associations between the change in the expression of these genes and changes in the measures of insulin sensitivity and body composition in black and white women over 5-year free-living period.

Chapter Five

The third study investigates the hypothesis that the expression of *HIF-1*α, *COL5A1* and *COL6A1* will increase in a dose-dependent manner in insulin-treated 3T3-L1 adipocytes under hypoxic compared to normoxic conditions. Therefore, the aim of this study is to determine the effects of increasing insulin concentrations on the mRNA and protein levels of *HIF-1*α, Col5a1 and Col6a1 under normoxic and hypoxic conditions in vitro in mature 3T3-L1 adipocytes.

Chapter Six

The fourth study investigates the hypothesis that differences in body fat distribution, and consequently insulin resistance, is associated with ethnic differences in the distribution of *COL5A1* (rs12722) and *COL6A1* (rs35796750) polymorphisms in black and white women. Thus, the aims of this study are to i) examine differences in the frequencies of the rs12722 polymorphism within the *COL5A1* gene and the

rs35796750 polymorphism within the *COL6A1* gene between black and white women, and to ii) examine the ethnic-specific associations with body fat, its distribution and insulin resistance.

Chapter Seven

In this final chapter, a summary of the main findings from the four research studies in this thesis are presented in the context of the main research question. Lastly, the strengths and limitations of the research design, are discussed.

CHAPTER THREE

HYPOXIA AND EXTRA-CELLULAR MATRIX GENE EXPRESSION IN ADIPOSE TISSUE ASSOCIATES WITH REDUCED INSULIN SENSITIVITY IN BLACK SOUTH AFRICAN WOMEN

Data from this chapter have been published in part in Kotzé-Hörstmann L M, Keswell D, Adams K, Dlamini T and Goedecke J H. Hypoxia and Extra-Cellular Matrix gene expression in adipose tissue associates with reduced Insulin Sensitivity in black South African women. 2017 *Endocrine* (55) p144-152

3.1 INTRODUCTION

Insulin resistance, a major risk factor for T2DM [337], is closely associated with central obesity, in particular visceral adiposity [162]. In contrast, accumulating fat in the lower body (peripheral region) is considered to be protective against insulin resistance [50]. Paradoxically, black South African and African American women accumulate more fat in the femoral-gluteal region and have less VAT than white women, but are more insulin resistant [23, 55, 338]. This suggests that the relationship between fat deposition and insulin resistance differs by ethnicity [23].

To gain a better understanding of the ethnic differences in body fat distribution and its relationship with insulin sensitivity, previous investigations in our laboratory explored the adipogenic and lipogenic capacity of abdominal and gluteal SAT of black and white women [105]. It was reported that black women had reduced expression of adipogenic genes such as *PPARγ* and *PPARγ*-responsive genes in the gluteal SAT, which correlated with reduced insulin sensitivity in black women, but not white women [105] . Further, the reduced adipogenic capacity in the gluteal depot of black women was

associated with adipocyte hypertrophy (Keswell et al., unpublished data, 2013) and correlated with a higher inflammatory profile [105] [339] and insulin resistance.

Adipocyte hypertrophy has been proposed to be the underlying cause of local cellular hypoxia due to the greater distance that oxygen has to diffuse from the microvasculature to the cells [68, 335]. The prevailing hypothesis around the pathological expansion of adipose tissue during obesity predicts that the increasing cell diameter and subsequent hypoxia results in increased cellular inflammatory signalling and dysregulated ECM remodelling, which leads to aberrant glucose metabolism (Reviewed in [67, 71] and section 2.4.3). Specifically, cellular adaptation to hypoxia involves the activation of the master regulator of the hypoxia response, *HIF-1α* [336], which aims to induce angiogenesis through the expression of pro-angiogenic proteins such as *VEGFα* and *FGF1* [340] . However, in obese adipose tissue, higher *HIF-1α* protein expression does not increase the expression of *VEGFα* [70, 294, 301, 307], but is rather associated with reduced adipose tissue vascularisation [70, 294, 301, 307] and the formation of ECM components such as collagen type V [341] and VI [295, 323, 327, 342] and ECM remodelling enzymes, *LOX* [307] and *FGF1* [343] and higher levels of inflammatory signalling molecules/proteins.

Given the reduced adipogenic capacity and greater adipocyte size observed in the gluteal SAT depot of black compared to white women, it is hypothesised that the gluteal SAT of black women has greater expression of ECM- and hypoxia-related genes than white women. Further, it is proposed that the expression of these genes differs between gluteal and abdominal SAT and are differentially associated with insulin sensitivity and inflammatory gene expression in black and white women. Therefore, the aims of the study are to i) compare depot-specific expression of ECM and matrix remodelling components and genes relating to the cellular hypoxia

response in normal-weight and obese black and white women; ii) to examine the ethnic-specific associations between these genes and body composition, measures of insulin sensitivity and secretion and inflammatory gene expression.

3.2 MATERIALS AND METHODS

3.2.1 Participants

This cross-sectional study included 14 normal-weight [body mass index (BMI) < 25kg/m^2] white; 12 obese [BMI > 30 kg/m^2] white; 15 normal-weight black and 15 obese black pre-menopausal women. These women were recruited from church groups, community centres and universities. Inclusion criteria were: aged between 18 and 45 years of age, no previous diagnosis of, or not taking chronic medication for T2DM, hypertension, HIV, or other metabolic diseases; and not currently pregnant or lactating. Ethics approval was obtained from the Human Research Ethics Committee of the Faculty of Health Science at the University of Cape Town and written informed consent was obtained from all participants prior to testing.

3.2.2 Testing procedures

3.2.2.1 Body composition assessment

Basic body composition, including weight, height, hip (largest gluteal area) and waist (at level of umbilicus) circumferences were recorded. Total body fat mass (kg and %) and fat mass distribution were measured by dual-energy X-ray absorptiometry, (DXA) (Discovery- W, Software version 12.7.3.7, Hologic Inc., Bedford, MA,USA). Body measurements were divided into regions of interest corresponding to arms, legs, and trunk [344, 345] , according to cut-off lines positioned at anatomical markers (Figure 3.1) Trunk fat region/central fat compartment was determined by measuring fat content from below the chin to the waist (line above the iliac crest) with vertical borders lateral

to the ribs positioned to achieve separation from the upper arm and trunk at the glenoid fossa. The arm region was demarcated by placing a line from the crease of the axilla through the glenohumeral joint. The leg region (kg) or lower body segment was isolated from the trunk region by vertical lines extending downward from the waist cut-line and diagonal cut lines (passing through the femoral neck) that intersect the central vertical line between the legs [344, 345]. The android region (kg) was measured between the ribs and the pelvis and is totally enclosed by the trunk region with upper demarcation a 1/5th of the distance from neck to waist cut line and the lower demarcation at the top of the pelvis. The gynoid region (kg) overlaps the trunk and leg region and includes the upper thigh and hip area. The height of the gynoid region is twice the height the android region with the upper demarcation below the top of the iliac crest at a distance of 1.5 times the android height [346]. The percentage of fat mass of each these regions of interest was calculated by dividing the body fat distribution variable (kg) by the total body fat mass (kg) and then multiplying by 100 (%fm). As a measure of body fat distribution, the central-to-leg fat mass ratio was calculated by dividing trunk fat mass (kg) by the leg fat mass (kg). Abdominal visceral adipose tissue (VAT), deep subcutaneous adipose tissue (DSAT) and superficial subcutaneous adipose tissue (SSAT), using the fascia superficialis to discriminate between DSAT and SSAT [99], were measured by computerized tomography (CT) at the level of L4-L5 lumbar vertebrae.

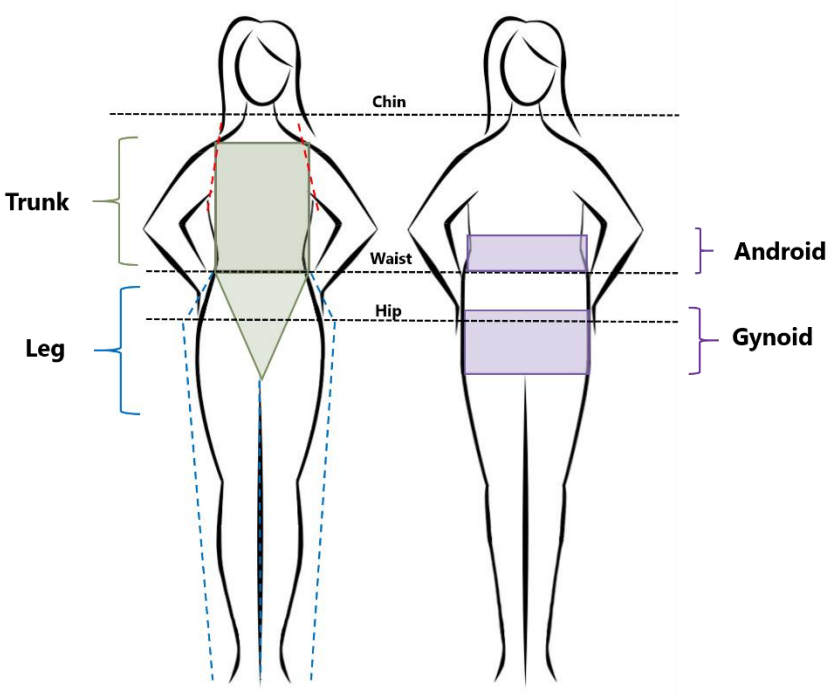


Figure 3.1 Body composition analysis by DXA region of interest.
Body measurements were divided into regions of interest corresponding to arms (red lines), legs (blue lines), and trunk (green boxes), according to cut-off lines positioned at anatomical markers. Purple boxes demarcate android and gynoid regions of interest

3.2.2.2 Insulin sensitivity measures (S_I)

Overnight fasted (10-12 hrs) participants underwent an insulin-modified frequently sampled intravenous glucose tolerance test (FSIGT). Baseline blood samples were collected at -15, -5 and -1 min prior to glucose infusion. Glucose (50% dextrose, 11.4 g/m^2 body surface area) was infused over 1 min. After 20 min, human insulin (0.02 units/kg; Actrapid, Novo Nordisk) was infused at a constant rate for 5 min. A total of 32 samples 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 following the infusion of glucose and was used to measure insulin and glucose concentrations. Insulin sensitivity (S_I) and the acute insulin response to glucose (AIR_g) were calculated based on Bergman's minimal model of glucose kinetics [347].

3.2.2.3 Biochemical analyses

Fasting plasma glucose concentrations were calculated using a glucose oxidase method (YSI 2300 STAT PLUS, YSI Life Sciences Yellow Springs, OH). Serum insulin concentrations were determined using a Microparticle Enzyme immunoassay (AxSym Insulin kit Abbot, IL, USA).

3.2.2.4 Adipose tissue biopsies

Under ultrasound guidance, abdominal SAT biopsies were collected from above (SSAT) and below (DSAT) the fascia superficialis by mini-liposuction method. In short, a small incision was made directly above the umbilicus after which 200 ml saline containing 0.1% adrenalin (Intramed, Port Elizabeth, South Africa) and 0.75% Lidocaine (Intramed) was infused using an infiltration cannula (Lamis 14 ga x 15 cm, Byron Medical Inc., Tucson, AZ). After infusion, an aspiration cannula (Coleman, 12 ga x 15 cm, Byron Medical Inc.) attached to a 10 ml syringe was used to collect approximately 2 ml of fat from each adipose tissue depot and washed 3 times in normal saline until no blood was visible. Gluteal samples were collected from the upper right quadrant of the gluteal depot using a similar procedure. Biopsy samples were immediately frozen in liquid nitrogen and stored at -80˚C until analysis.

3.2.3 Gene selection

Candidate genes were selected for their potential involvement in metabolic abnormalities related to obesity and insulin resistance as described in Chapter 2. Specifically, *HIF-1α* and *VEGFα* were chosen for their role in hypoxia signalling during obesity [116, 294, 348] and *PPARγ* for its role during adipogenesis. The *COL5A1* gene encodes an α1 (V) chain found in all type V collagen isoforms and plays an important regulatory role in fibrillogenesis [259], whereas type VI collagen-null mice, produced by

type VI α1 collagen chain disruption [323], display increased adipocyte cell size and improved glucose tolerance compared to wild type *ob*/*ob* littermates [323]. *LOX* [333], a transcriptional target of *HIF-1α*, was chosen as it facilitates collagen cross-linking and stabilization, and increases insoluble ECM deposition in adipose tissue. Finally, *FGF1* has been reported to be essential for ECM remodelling during adipose tissue expansion [343] and has recently been demonstrated as a therapeutic treatment for insulin resistance in obese mice and results in insulin dependent glucose lowering and whole body insulin sensitization [349]. In addition, a previously generated RT-PCR data set from a panel of inflammatory genes was also included in this study [55]. These genes included, chemokines (Chemokine (C-C motif) ligand 2 (*CCL2*) and its receptor *CCR2*), macrophage markers (*CD14*, *CD68* and *CD163*) and colony stimulating factor 1 (*CSF-1*), macrophage migration inhibitory factor (*MIF*), and cytokines (*TNF-α*, Interleukin (*IL*)-*6*, *IL-10* and *IL-18*).

3.2.4 RNA extraction and reverse transcription

Total RNA was extracted using the TriPure Isolation Reagent (Roche Diagnostics Ltd, Burgess Hill, UK) and isolated using QIAGEN RNeasy Lipid Tissue Mini Kit, Qiagen GmBH (Hilden, Germany) according to manufacturer's instructions, and purified with Ambion Turbo DNase treatment kit (Ambion Ink, Austian, USA). RNA was evaluated for purity and concentration using a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Lafayette, CO). Total RNA was reverse transcribed to cDNA with High Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA manufacturer's protocol). A duplicate set of reactions were prepared without RT enzyme (RT-) and was used to calculate the amount of genomic DNA contamination.

3.2.5 Quantitative Real-time PCR for gene expression analyses

RT-PCR was carried out on a StepOnePlus™ Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), with inventoried gene specific TaqMan™ primer/probes gene expression assays for *PPARγ* (Hs01115513_m1), *HIF-1α* (Hs00153153_m1), *VEGFα* (Hs00900055_m1), *LOX* (Hs00942480_m1), *COL5A1* (Hs00609088_m1), *COL6A1* (Hs00242448_m1) and *FGF1* (Hs00265254_m1), using the StepOnePlus™ Real-Time PCR software Version 2. Inflammatory genes investigated were similarly amplified by using TaqMan™ inventoried primer/probes gene expression assays for *CD14* (Hs00169122_g1), *CD68* (Hs00154355_m1), *CD163* (Hs01016657_m1), *CCL2* (Hs00234140_m1), *CCR2* (Hs00356601_m1), *CSF-1* (Hs00174164_m1), *MIF* (Hs00236988_g1), *TNF-α* (Hs00174128_m1), *IL-6* (Hs00985639_m1), *IL-10* (Hs00174086_m1), *IL-18* (Hs99999040_m1). Transcript levels are presented as a ratio of abundance of the gene of interest to the mean of the expression of endogenous control genes, 60S acidic ribosomal protein P0 (*RPLP0*) (Hs99999902_m1) and *18S* (Hs99999901_s1), and peptidylprolyl isomerase A (*PPIA*) (Hs999999904_m1) for the inflammatory genes, as determined by Normfinder [350].

3.2.6 Statistical Analysis

Results are presented as means ± standard deviation (SD) with significance being defined as $p<0.05$. All data sets were tested for normality according to the Shapiro-Wilk test and non-normally distributed variables were logarithmically transformed for parametric analysis. Differences between body composition and gene expression in normal-weight and obese, black and white women in each SAT depot were determined using two-way ANCOVA, adjusting for age, with Fisher least significant difference (LSD) *post hoc* analysis. Pearson's correlations (r) were used to explore the relationships between gene expression and body composition in black and white

women and partial correlations, adjusted for age and body fat mass (kg) to explore associations with insulin sensitivity measures, were used. Data were analysed using STATISTICA version 12 (Statsoft Inc., Tulsa OK, USA).

3.3 RESULTS

3.3.1 Participant characteristics

The participant characteristics are shown in Table 3.1. Obese women were significantly older than normal-weight women ($p<0.01$) and consequently all subsequent analyses were adjusted for age. Black women were shorter than white women, but were similar in terms of BMI, DXA-derived measures of body fat (kg and %) and waist and hip circumferences. Normal-weight black women had higher gynoid fat (%fm) than obese black women, whilst this did not differ between normal-weight and obese white women. Android fat (%fm) was higher in obese compared to normal-weight women, independent of ethnicity. Although VAT and abdominal SAT did not differ between normal-weight black and white women, obese black women had less VAT, but more SSAT than obese white women, while DSAT did not differ by ethnicity.

Fasting glucose concentrations were not different by ethnicity, but were higher in obese compared to normal-weight women. However, fasting insulin levels were higher in black compared to white women, and were higher in obese compared to normal-weight women. S_I was lower, and AIR_g was higher in black compared to white women.

Table 3.1 Body Composition and insulin sensitivity in normal-weight and obese white and black SA women

	White women		Black women	
	Normal-weight	**Obese**	**Normal-weight**	**Obese**
n	14	12	15	15
Age (years)	25 ± 2[a]	32 ± 2[b]	24 ± 2[a]	28 ± 2[b]
Body Composition				
BMI (kg/m^2)	22.4 ± 1.5[a]	35.9 ± 5.8[b]	22.7 ± 1.2[a]	37.7 ± 3.7[b]
Waist (cm)	80.0 ± 5.7[a]	107.9 ± 16.1[b]	76.2 ± 4.1[a]	113 ± 12.1[b]
Body fm (%)	29.7 ± 6.5[a]	45.5 ± 3.7[b]	30.5 ± 5.6[a]	46.9 ± 3.1[b]
Central fm (%fm)	44.9 ± 4.4[a]	49.0 ± 4.2[b]	42.7 ± 5.0[a]	51.5 ± 3.5[b]
Leg fm (%fm)	44 ± 1.35[a]	40 ± 1.46[b]	47 ± 1.30[a]	37 ± 1.30[b]
Cent/leg fat ratio	1.1 ± 0.2[a]	1.2 ± 0.3[b]	1.0 ± 0.2[a]	1.4 ± 0.3[c]
Gynoid fm (%fm)	9.1 ± 1.2[a]	8.7 ± 0.8[a]	10.3 ± 1.4[b]	8.6 ± 1.6[a]
Android fm (%fm)	5.1 ± 1.1[a]	7.8 ± 1.7[b]	5.3 ± 1.2[a]	9.1 ± 1.3[b]
VAT (cm^2)	55.5 ± 20.5[a]	144.2 ± 69.5[b]	60.5 ± 16.9[a]	101.5 ± 37.7[c]
Total SAT (cm^2)	178.4 ± 63.2[a]	510.0 ± 104.9[b]	169.3 ± 50.8[a]	591.2 ± 92.2[c]
SSAT (cm^2)	95.2 ± 14.1[a]	242.7 ± 16.0[b]	99.4 ± 13.5[a]	321.4 ± 14.1[c]
DSAT (cm^2)	83.3 ± 14.0[a]	267.3 ± 16.0[b]	70.1 ± 13.5[a]	269.9 ± 14.0[b]
Insulin Sensitivity				
Glucose (mmol/l)	4.3 ± 0.1[a]	4.6 ± 0.1[b]	4.5 ± 0.1[ab]	4.6 ± 0.1[b]
Insulin (mU/l)	4.2 ± 1.3[a]	8.4 ± 1.4[b]	8.7 ± 1.3[b]	15.1 ± 1.2[c]
S_I (x10^{-4} min^{-1}/[pmol/l])	6.1 ± 0.5[a]	4.0 ± 0.5[b]	2.4 ± 0.5[c]	1.8 ± 0.5[c]
AIR_g (µU/ml)	49.5 ± 52.2[a]	68.2 ± 54.6[a]	217.72 ± 46.7[b]	316.7 ± 46.7[b]

Values represent unadjusted means ± SD. Means with different letters are significantly different as determined by *Post hoc* analysis ($p<0.05$) and adjusted for age. BMI, body mass index; DSAT deep subcutaneous adipose tissue; SSAT, superficial subcutaneous adipose tissue; VAT, visceral adipose tissue; % fm, a percentage of fat mass; S_I, Insulin Sensitivity; AIR_g, Acute Insulin Response to Glucose

3.3.2 Hypoxia and ECM related gene expression in gluteal and abdominal SAT of black and white SA women

Transcript levels of ECM- and hypoxia related genes in gluteal and abdominal SAT (DSAT and SSAT) depots are presented in Figure 3.2. When examining the ethnic differences in gene expression within each of the adipose tissue depots, it was found that the gluteal depot of black women had higher *HIF-1α* and *COL5A1* expression,

and lower *VEGFα* expression compared to the gluteal depot of white women (ethnic effect, p<0.05).

In the DSAT depot, *COL5A1* expression was higher, but *FGF1* expression was lower in black women compared to white women (ethnic effect, p<0.05). In contrast, black women had lower *HIF-1α* and *COL5A1* expression in the SSAT depot compared to white women (ethnic effect, p<0.05).

When examining the between-depot differences in ECM- and hypoxia related gene expression in black and white women, it was found that in both black and white women, expression levels of *PPARγ*, *VEGFα*, and *LOX* were higher in the SSAT depot compared to the gluteal and DSAT depots whilst *COL6A1* and *FGF1* expression levels were higher in the gluteal depot compared to the abdominal SAT depots (depot effect, P<0.05). Although *HIF-1α* expression did not differ between the gluteal and abdominal SAT depots of the black women, *HIF-1α* expression was higher in both of the abdominal SAT depots (DSAT and SSAT) compared to the gluteal depot of white women (depot × ethnicity interaction, p=0.01). In contrast, the expression of *COL5A1* did not differ between the gluteal and abdominal SAT depots of the white women, but *COL5A1* expression was higher in the gluteal and DSAT depots compared to the SSAT depot in the black women (depot × ethnicity interaction, p=0.01).

It was then determined whether the expression of these genes were different in normal-weight and obese black and white women within each of the adipose tissue depots (BMI effect). In the gluteal depot of the obese white women, *LOX* and *FGF1* expression level were higher, while *PPARγ*, *VEGFα*, *HIF-1α*, *COL5A1* and *COL6A1* expression did not differ between the normal-weight and obese white women. In contrast, *COL5A1* and *LOX* expression did not differ between normal-weight and obese black women, but *PPARγ* and *VEGFα* expression were lower, and *HIF-1α*,

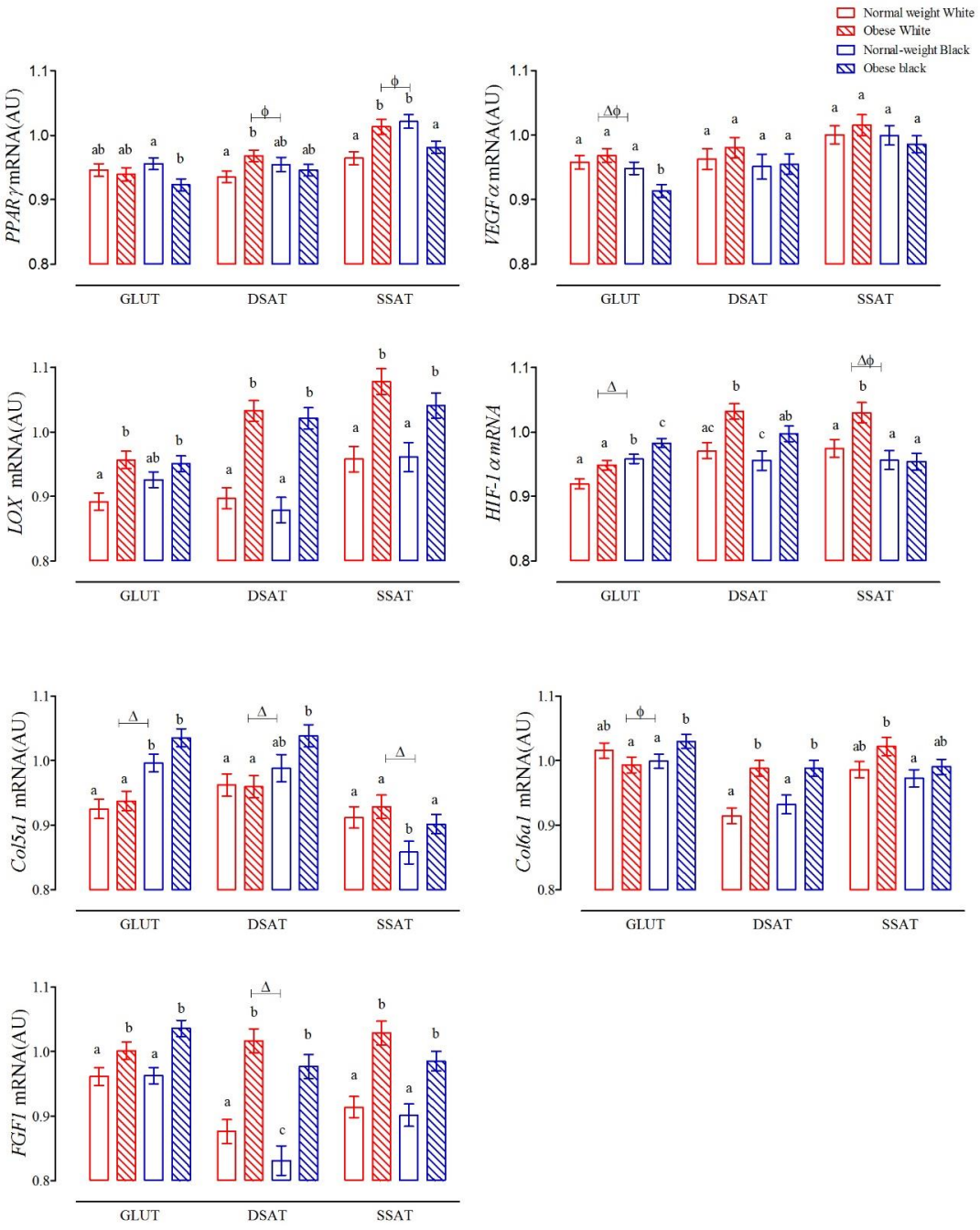


Figure 3.2 Gene expression in gluteal SAT, abdominal DSAT and SSAT depots of normal-weight and obese white and black women.

Bars represent unadjusted means ± SD. Similar groups, as determined by post-hoc analysis, are denoted with the same letter. Δ, ethnic effect (white vs. black) $p < 0.05$; φ, BMI x Ethnicity interaction (white BMI effect vs. black BMI effect) $p<0.05$. mRNA expression relative to internal control genes and expressed in arbitrary units (AU). GLUT, gluteal adipose tissue; DSAT, deep subcutaneous adipose tissue; SSAT, superficial subcutaneous adipose tissue

COL6A1 and *FGF1* expression were higher in obese compared to normal-weight black women. Moreover, the lower *VEGFα* expression and the higher *COL6A1* expression in the obese compared to the normal-weight black women was significantly different

from the gene expression in the obese and normal-weight white women (BMI × ethnicity interaction, p<0.05 for both).

In the DSAT depot of both ethnicities, *HIF-1α*, *LOX*, *COL6A1* and *FGF1* expression were higher in obese compared to normal-weight women, while *VEGFα* and *COL5A1* did not differ by BMI group. In contrast, obese white women had higher *PPARγ* expression compared to normal-weight women, whereas *PPARγ* expression did not differ between normal-weight and obese black women (BMI × ethnicity interaction, p=0.04).

In the SSAT depot of both black and white women, *LOX* and *FGF1* expression were higher in obese compared to the normal-weight women, while *COL6A1* did not differ by BMI. Obese black women had lower *PPARγ* expression compared to normal-weight black women, while obese white women had higher *PPARγ* expression compared to normal-weight white women (BMI × ethnicity interaction, p<0.01). Further, *HIF-1α* expression was significantly higher in obese white compared to normal-weight white women, but were similar in normal-weight and obese black women (BMI × ethnicity interaction, p=0.04).

3.3.3 Associations between the expression of hypoxia and ECM related genes, in gluteal and abdominal SAT depots, and body fat and its distribution, in black and white women

Correlations (r) between SAT gene expression and body fat mass (kg) and central-to-leg fat ratio (cent/leg fat ratio) are shown in Table 3.2. In black women, body fat (kg) correlated positively with *HIF-1α*, *COL5A1, COL6A1* and *FGF1* and reduced *VEGFα* expression in the gluteal depot, and with *LOX* and *FGF1* expression in both the abdominal SAT depots (both DSAT and SSAT). Central obesity, as described by

cent/leg fat ratio, associated positively with *COL6A1* and *FGF1* expression, and negatively with *PPARγ* and *VEGFα* levels in the gluteal depot. In both the abdominal SAT depots, cent/leg fat ratio associated positively with *LOX*, *COL6A1* and *FGF1* expression.

In white women, body fat mass correlated positively with *LOX* expression in the gluteal depot. In both the abdominal SAT depots, body fat mass correlated positively with *HIF-1α*, *LOX*, and *FGF1* expression and in the DSAT depot with *COL6A1* expression. Cent/leg fat ratio associated positively with *HIF-1α* and *FGF1* and reduced *VEGFα* expression in the gluteal SAT depot. In both abdominal SAT depots cent/leg fat ratio associated positively and with *LOX* and *FGF1*expression and in the SSAT depot with increased *HIF-1α* expression.

3.3.4 Associations between the expression of hypoxia and ECM related genes in abdominal and gluteal SAT and insulin sensitivity and secretion in black and white women

The partial correlation coefficients (r), adjusted for age and body fat %, between adipose tissue gene expression in the gluteal and abdominal SAT depots and measures of insulin sensitivity and secretion are shown in Table 3.2. In the gluteal depot of black women, S_I correlated with reduced *HIF-1α*, *COL5A1*, *COL6A1*, and *FGF1* expression, whereas AIR_g correlated with increased *HIF-1α* and *COL5A1* expression. In the SSAT depot of white women, fasting insulin concentrations correlated with increased expression of *HIF-1α*, *COL5A1*, *COL6A1* and *FGF1*. In both the abdominal SAT depots, S_I correlated with lower *LOX* and *FGF1* expression. Further, AIR_g correlated with increased *COL5A1* expression in the DSAT depot.

Table 3.2 Associations between the expression of hypoxia and ECM related genes in abdominal and gluteal SAT and body composition and insulin sensitivity and secretion in black and white women

	Body Composition				Insulin Sensitivity					
	Body fat mass		Cent/leg fat ratio		Fasting Insulin		S_I		AIR_g	
	White	Black	White	Black	White	Black	White	Black	White	Black
Gluteal SAT										
PPARγ	-0.28	-0.35	-0.12	-0.43[a]	-0.46	-0.27	0.12	0.01	-0.11	0.23
HIF-1α	0.37	0.53[b]	0.53[a]	0.35	-0.28	0.14	0.07	-0.55[b]	0.04	0.41[a]
VEGFα	-0.16	-0.59[b]	-0.43[a]	-0.54[b]	-0.30	-0.11	-0.08	0.04	0.30	0.12
LOX	0.57[b]	0.32	0.30	0.26	-0.22	-0.09	-0.04	-0.13	0.24	0.09
COL5A1	0.23	0.40[a]	0.25	0.16	-0.23	-0.13	0.30	-0.41[a]	0.03	0.47[a]
COL6A1	-0.08	0.51[b]	-0.09	0.44[a]	-0.15	0.17	-0.26	-0.47[a]	0.12	0.26
FGF1	0.41	0.66[b]	0.45[a]	0.62[b]	-0.17	0.14	-0.32	-0.45[a]	0.11	0.05
Deep SAT										
PPARγ	0.28	-0.02	0.07	-0.43	-0.31	0.31	0.32	-0.28	0.16	0.13
HIF-1α	0.51[a]	0.50	0.27	0.47	0.08	0.12	-0.15	0.14	0.35	-0.14
VEGFα	-0.07	0.00	0.01	-0.36	-0.03	0.23	-0.20	-0.27	0.36	0.10
LOX	0.81[b]	0.86[b]	0.49[a]	0.82[b]	0.22	-0.23	-0.53[a]	0.01	0.07	0.19
COL5A1	0.17	0.59[a]	-0.21	0.21	-0.12	-0.38	0.17	-0.47	0.56[a]	0.36
COL6A1	0.50[a]	0.74[b]	0.17	0.77[b]	0.07	0.22	-0.15	0.09	0.32	0.03
FGF1	0.68[b]	0.79[b]	0.47[a]	0.74[b]	0.23	0.38	-0.61[a]	-0.16	0.05	0.17
Superficial SAT										
PPARγ	0.35	-0.52[a]	0.19	-0.40	-0.31	-0.12	-0.24	-0.11	0.07	0.28
HIF-1α	0.57[a]	-0.17	0.56[a]	0.37	0.32	0.54[a]	-0.35	-0.09	-0.01	-0.04
VEGFα	0.02	-0.16	-0.04	-0.03	-0.20	0.26	0.00	-0.21	-0.21	0.58
LOX	0.63[b]	0.49[a]	0.54[a]	0.70[b]	0.29	0.58	-0.69[b]	-0.19	0.16	0.14
COL5A1	0.21	0.44[a]	0.09	0.45[a]	0.24	0.56[a]	-0.35	-0.12	0.40	0.35
COL6A1	0.40	0.16	0.24	0.60[b]	0.35	0.64[b]	-0.19	-0.31	0.12	0.34
FGF1	0.78[b]	0.62[b]	0.55[a]	0.78[b]	0.71[b]	0.63[b]	-0.76[b]	-0.27	0.06	0.19

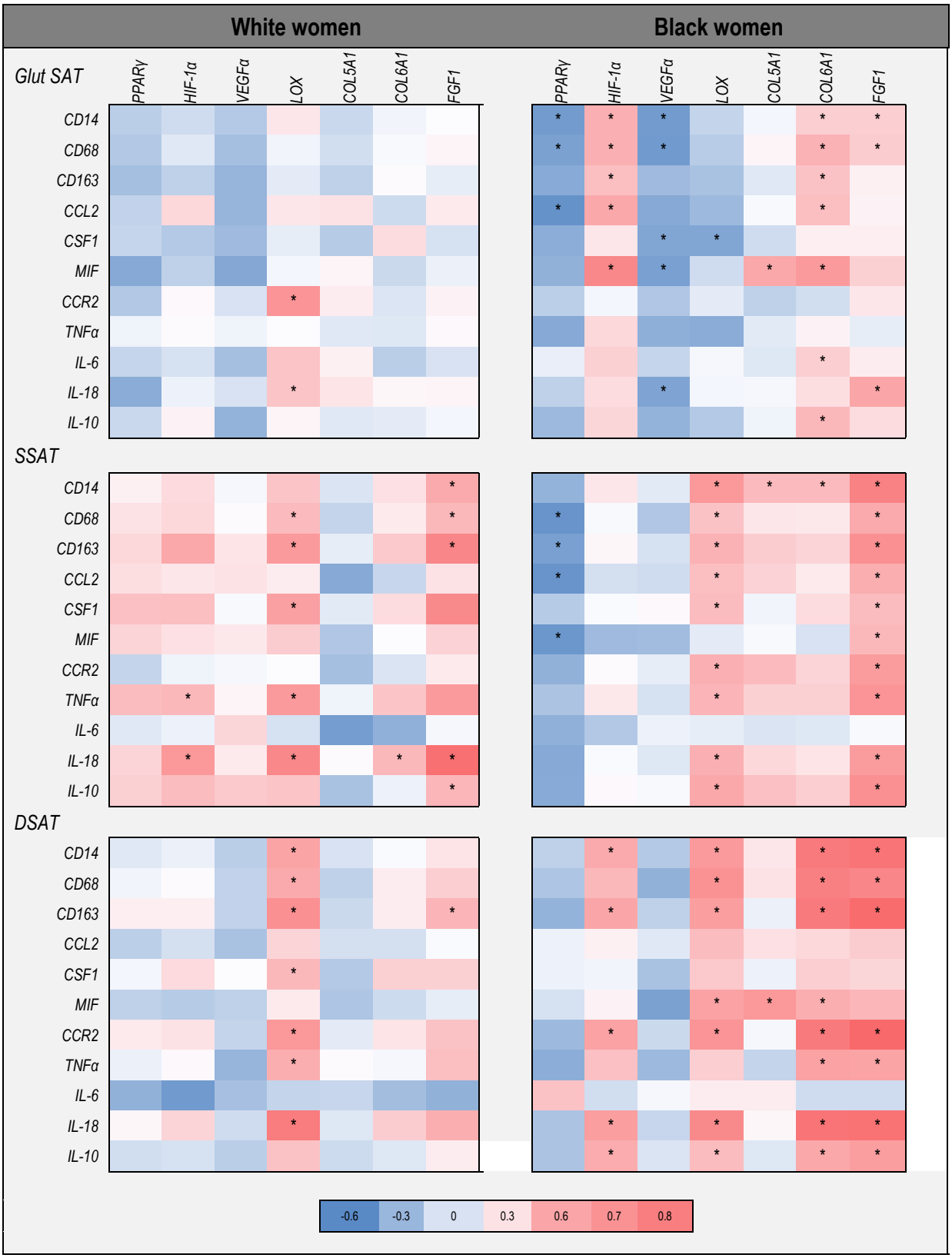
Values are correlation coefficients (r). Values for fasting insulin, S_I and AIR_g are adjusted for age and body fat %. P values: a, $p < 0.05$ and b, $p < 0.01$.; Cent/leg fat ratio, Central-to-leg fat mass ratio; S_I, Insulin Sensitivity; AIRg, acute insulin response to glucose

3.3.5 Associations between the expression of hypoxia and ECM related genes and inflammatory genes in abdominal and gluteal depots of black and white SA women.

Correlations between hypoxia and ECM related gene expression and inflammatory gene expression in gluteal and abdominal (SSAT and DSAT) depots of black and white women are presented in Table 3.3. In white women, with the exception of the association between *LOX* and the expression of *CCR2* and *IL-18* genes in the gluteal depot, hypoxia and ECM gene expression associated with higher inflammatory gene expression mainly in the abdominal SAT (SSAT and DSAT) depots. Specifically, *LOX* expression associated with higher expression of macrophage markers (*CSF1*, *CD14*, *CD68* and *CD163*), pro-inflammatory cytokines (*TNFα* and *IL-18)* in both abdominal depots. Similarly, *FGF1* expression associated with higher *CD14*, *CD68* and *CD163* and *IL-18* gene expression in the abdominal depots. *HIF-1α* expression was associated with higher *TNFα* and *IL-18* expression in the SSAT depot. *PPARγ*, *VEGFα* and *COL5A1* did not associate with inflammatory gene expression in white women.

In the black women, *PPARγ* expression associated with lower *CD14*, *CD68* and *CCL2* in the gluteal depot and with lower *CD68*, *CD163*, *CCL2* and *MIF* in the SSAT depot. *VEGFα* expression associated with lower macrophage marker and pro-inflammatory cytokine gene expression in the gluteal depot only. *HIF-1α* expression was associated with higher macrophage marker gene expression, *CCL2* and *MIF* expression in the gluteal depot and with higher *CD14*, *CD163*, *CCR2* and *IL-18* in the DSAT, with no associations in the SSAT depot. Similarly, *COL6A1* expression associated with higher macrophage marker and pro-inflammatory cytokine- and chemokine gene expression in mainly the gluteal and DSAT depots. *COL5A1* only associated with higher *MIF* gene expression in the gluteal and DSAT depots. Interestingly, *LOX* and *FGF1* expression

Table 3.3 Associations between the expression of hypoxia and ECM related genes and inflammatory genes in abdominal and gluteal depots of black and white SA women



Heat map of correlation coefficients between ECM and hypoxia gene expression and inflammatory gene expression. P < 0.05 *. Glut SAT, Gluteal subcutaneous adipose tissue, SSAT DSAT deep subcutaneous adipose tissue; SSAT, superficial subcutaneous adipose tissue

associated with higher macrophage marker gene expression, pro-inflammatory cytokines and pro-inflammatory chemokines, mainly in the abdominal depots.

3.4 DISCUSSION

The main findings of this study were that obese black women had higher gluteal expression of *HIF-1α*, *COL5A1* and *COL6A1* and reduced *VEGFα* expression compared to obese white women. Higher *HIF-1α*, *COL5A1*, *COL6A1* and *FGF1* expression in the gluteal and SSAT depots of black women were associated with their reduced S_I and higher fasted insulin concentrations. However, in white women, *LOX* and *FGF1* expression in the abdominal, but not in the gluteal depot, were associated with increased fasted insulin concentrations. Further, the expression of the hypoxia and ECM genes associated with inflammatory gene expression in both the gluteal and abdominal SAT depots of black women, while the expression of these genes associated with the inflammatory gene expression in the abdominal SAT, but not the gluteal SAT depot of white women.

Hypertrophic obesity is more closely associated with insulin resistance than hyperplastic obesity [214, 351]. Our group has recently shown that the adipogenic and lipogenic (*PPARγ*, FASN and PEPCK) gene expression in the gluteal depot were down-regulated to a greater extent in obese black women compared to obese white women [105], and that the reduced adipogenic capacity was also associated with adipocyte hypertrophy (Keswell et al., unpublished data, 2013). The expanding adipocyte diameter has been proposed to induce localised hypoxia in the adipocytes furthest removed from the vasculature [67, 335]. Hypoxic micro-environment remodelling is mediated by the oxygen sensitive transcriptional regulators such as the *HIF-1α* proteins. Studies done in tumour biology have shown that *HIF-1α* expression is

dependent on the availability of oxygen and is broken down under normoxic conditions [352, 353]. *HIF-1*α accumulates in hypoxic tissues and drives angiogenesis through the expression of pro-angiogenic proteins such as *VEGFα* [352, 353]. However, during adipose tissue expansion, *HIF-1α*-stimulated *VEGFα* expression remains low and has been shown to be associated with increased body fat %, reduced capillary density, and a reduction in adipose tissue oxygen partial pressure (AT PO_2, mmHg) [70, 116, 307], the magnitude of which is currently under investigation [70, 299, 300, 309]. Accordingly, higher *HIF-1α* and reduced *VEGFα* expression in the gluteal depot of obese black women suggests increased adipose tissue hypoxia and reduced vascularization in this depot.

Previous studies have shown that higher *HIF-1α* mRNA expression in obese mice fed a high fat diet associated with an increase in collagen and matrix remodelling enzymes [307]. Increased collagen and matrix remodelling enzymes in adipose tissue is described as fibrosis [307, 323, 327, 341], which has been associated with reduced insulin sensitivity. While *COL6A3* has previously been shown to be higher in Asian Indians compared to Caucasians [307, 323], this study was the first to show higher *COL5A1* and *COL6A1* expression in black compared to white women, and this associated with reduced insulin sensitivity in black women only. Type V collagen protein is localized around the large blood vessels in adipose tissue of obese individuals and can disrupt endothelial adherence to vascular structures [341, 354]. In contrast, type VI collagen is localized around adipocytes, and is proposed by several studies to restrict adipocyte expansion by limiting lipid storage capacity [226, 327, 328]. These studies provide insight towards a potential mechanism whereby reduced vascularization through *COL5A1* expression, and reduced lipid storage capacity through *COL6A1* expression, may reduce insulin

sensitivity/signalling either through inhibition of the insulin signalling pathway and/or through higher inflammatory signalling, and increased ectopic lipid deposition [323].

Adipose tissue hypoxia is associated with macrophage infiltration [294], and the associated increased-inflammatory response has been shown to reduce insulin sensitivity [294, 302]. Studies from our laboratory have shown that obese black women have higher SAT expression of inflammatory chemokines and cytokines in abdominal and gluteal SAT depots compared to white women [55]. Interesting, hypoxia and ECM genes expression levels were positively associated with higher expression of inflammatory genes in both the abdominal and gluteal SAT depots of black women whereas hypoxia and ECM gene expression associated with inflammatory gene expression mostly in the abdominal depots of the white women. Hypoxia has been shown to induce the transcription of nuclear factor-kappa β which regulates the inflammatory response as reviewed elsewhere [355, 356]. Thus, it may be suggested higher inflammatory signalling in the gluteal depot of black women may be mediated by hypoxia and increased *HIF-1α* signalling. During hypoxia, there is a greater infiltration of (M1) activated pro-inflammatory macrophages relative to the (M2) anti-inflammatory phenotype into adipose tissue and a concomitant reduction in insulin sensitivity [357]. These findings are in agreement with previous studies that show that the abdominal SAT depots contribute more to the development of insulin resistance compared to a protective effect shown for gluteal fat distribution [50, 61] . However, in black women, the gluteal depot does not seem to have the same protective effect but rather associates with their higher inflammatory profile [55] and lower insulin sensitivity [105], potentially mediated by increased hypoxic and ECM gene expression in this depot.

LOX, another transcriptional target of *HIF-1α*, facilitates collagen cross-linking and stabilization, and thereby increases tensile strength of the ECM components [334].

Although *LOX* expression has been reported to be higher in the adipose tissue of normal-weight compared to obese humans [358, 359], this investigation is the first to describe ethnic and depot differences in adipose tissue *LOX* expression. Specifically, this thesis reported increased *LOX* expression in the abdominal compared to the gluteal depots of obese black and white women, and that *LOX* expression in the abdominal depots associated with reduced S_I in white women only. Transgenic mice over-expressing *HIF-1α* have increased *LOX* expression and fibrotic deposition in adipose tissue and reduced glucose tolerance [307]. As a transcriptional target of *HIF-1α*, *LOX* expression in the gluteal depot was expected to be higher in black compared to white women, but *LOX* expression did not differ between black and white women. This may be explained by a previous observation in our laboratory of increased expression of TNFα, a pro-inflammatory cytokine, in the gluteal depot of black women compared to white women, which was independent of differences in BMI [55]. TNFα has been shown to reduce *LOX* mRNA expression in endothelial cells in a dose dependent manner [360, 361]. It is possible that the lower *LOX* expression may be due to the high inflammatory profile as observed in the gluteal SAT of black women.

In addition, higher *FGF1* expression in the gluteal SAT depot of black women and both abdominal SAT depots of white women, associated with reduced insulin sensitivity, independent of body fatness. *FGF1* under the regulation of *PPARγ*, has been shown to be critical for adipose tissue remodelling during metabolic changes [343, 349]. Under excess caloric conditions, *FGF1* knockout mice develop an aggressive fibrotic and diabetic phenotype that fails to resolve following normal calorie intake [343].[343]. In contrast, pharmacological treatment of insulin resistant, obese mice with *FGF1* results in insulin dependent glucose lowering and whole body insulin sensitization [349]. Along with its ECM remodelling function, elevated *FGF1*, primarily expressed by

inflammatory and fibroblast-like cells, has been shown to be essential for cardiac angiogenesis following myocardial infarction [362]. Thus, *FGF1* expression may be increased in obese adipose tissue as a compensatory mechanism to resolve the adverse metabolic effects of excessive ECM accumulation, such as increased inflammatory signalling and reduced S_I. In line with this hypothesis, *FGF1* expression associated closely with higher inflammatory signalling in the abdominal SAT depots of both black and white women.

This study has several limitations. Firstly, the sample size of the cohort is small. However, this study may be viewed as a pilot study, from which some novel relationships that require verification have been identified. Secondly, due to the small size of the fat biopsies obtained, protein expression in the adipose tissue samples could not be measured. Further studies are warranted to evaluate the hypothesis that greater adipose tissue hypoxia and the accumulation of ECM components in peripheral body fat plays a role in the development of insulin resistance in black women

In conclusion, this study showed that obese black women had higher expression of *HIF-1α*, *COL5A1* and *COL6A1* and reduced *VEGFα* expression in the gluteal depot compared to obese white women, independent of differences in body fat % and central fat mass. Higher *HIF-1α* and reduced *VEGFα* expression in the gluteal depot of obese black women suggests increased adipose tissue hypoxia and reduced vascularization that may contribute to higher inflammatory gene expression as previously observed in this depot. Based on these findings, it is proposed that reduced adipogenic capacity in the gluteal depot of black women may result in increased adipose tissue hypoxia and increased *HIF-1α* expression, which in turn induces a fibrotic and inflammatory response, rather than a compensatory pro-angiogenic response.

CHAPTER FOUR

HYPOXIA AND EXTRA-CELLULAR MATRIX GENE EXPRESSION IN THE GLUTEAL ADIPOSE TISSUE DEPOT ASSOCIATES WITH INSULIN SECRETION IN BLACK SOUTH AFRICAN WOMEN

- A 5 year pilot study -

Data from this chapter have been published in part in Kotzé-Hörstmann L M, Keswell D, Adams K, Dlamini T and Goedecke J H. Hypoxia and Extra-Cellular Matrix gene expression in adipose tissue associates with reduced Insulin Sensitivity in black South African women. 2017 *Endocrine* (55) p144-152

4.1 INTRODUCTION

In the previous chapter (Chapter 3), it was demonstrated that at comparable BMI, normal-weight black women had greater gynoid fat mass (%fm) compared to their normal-weight white counterparts, whereas there were no differences in gynoid fat mass (%fm) between obese black and white women. When the black participants of the larger cohort were followed-up after 5 years of free-living, they gained an average 9 % body fat, but the increase in fat mass was associated with a relative increase in central (trunk) fat mass (%fm), and a concomitant decrease in leg fat mass (%fm) [106]. These findings suggest that the development of obesity in black women is associated with a relative redistribution of fat from the periphery to the central fat depots.

Peripheral fat distribution appears to play a protective role in the maintenance of metabolic health [50, 51, 363], whereas accumulation of fat in the VAT depot has been implicated in the development of metabolic disturbances leading to insulin resistance [45, 100, 214, 364]. However, this relationship has been shown to be altered in black women in whom abdominal SAT correlates more closely with lower S_I than VAT[57, 120]. This ethnic specific relationship between abdominal adipose tissue depots and measures

of insulin sensitivity and secretion might be explained, in part, by factors that influences the accumulation of fat in the different adipose tissue depots.

Increasing adipose tissue mass during the development of obesity is characterised by altered extracellular matrix expression and increased M1-activated macrophage infiltration [226, 365]. The excess generation and deposition of ECM components during adipose tissue expansion in obesity may represent a mechanical limitation to adipose tissue expandability (section 2.4) [58, 366].

Given the previous findings of higher hypoxia and ECM component gene expression in the gluteal depot of obese black compared to white women, and the ethnic-specific associations with reduced insulin sensitivity (Chapter 3), this study hypothesises that higher hypoxia- and ECM-related gene expression is associated with higher central fat mass accumulation in black women and that the expression of these genes are more closely associated with changes in insulin sensitivity and secretion in black compared to white women. Therefore, the aims of this study are to i) compare expression of ECM and matrix remodelling components and genes relating to the cellular hypoxia response at baseline and after 5 year follow-up in free-living black and white women; and to ii) explore the associations between changes in expression of these genes and changes in body composition, insulin sensitivity and secretion in black and white women.

4.2 MATERIALS AND METHODS

4.2.1 Participants

Fifteen women (black, n=10; white, n=5) from the cross-sectional study described in Chapter 3, were followed up after a 5-year free-living period. Inclusion criteria for the follow-up study were similar to those described in Chapter 3, and included: no previous

diagnosis of, or not taking chronic medication for T2DM, hypertension, HIV, or other metabolic diseases; and not currently pregnant or lactating. Of our original sample of 56 women, 9 women were not contactable because they had relocated, 13 had altered contact details, 9 were not willing to participate, 3 were pregnant, 2 declined to participate due to illness, 1 woman was deceased and 4 women were excluded due to incomplete data. Therefore, 15 women were included in this follow-up study. Ethics approval was obtained from the Human Research Ethics Committee of the Faculty of Health Science at the University of Cape Town and written informed consent was obtained from all participants prior to testing

4.2.2 Testing procedures

4.2.2.1 Body composition assessment

Basic body composition assessment including height, weight, waist and hip were the same as described at baseline (Chapter 3). Total body fat mass, central (trunk) fat mass, gynoid and android fat mass regions of interest, were measured by DXA (Discovery- W, Software version 12.7.3.7, Hologic Inc., Bedford, MA, USA), as described previously (Chapter 3). The change in the variables over the follow-up period were calculated by subtracting the baseline measurement from the follow-up measurement. The body fat distribution variables (trunk, gynoid, android, and leg fat mass), as proportions of total fat mass (%fm), were calculated by dividing the body fat distribution variable by the total body fat mass (kg) and then multiplying by 100, as describe in Chapter 3.

4.2.2.2 Insulin sensitivity measures (S_I)

A frequently sampled intravenous glucose tolerance test was used to estimate insulin sensitivity and secretion in the follow-up sample, as described in Chapter 3.

4.2.2.3 Adipose tissue biopsies

From the right upper quadrant of the gluteal depot, approximately 2 ml of fat was collected via mini-liposuction method, as described in Chapter 3. Biopsy samples were then frozen and stored at -80˚C until analysis.

4.2.3 RT-PCR

RT-PCR on baseline and follow-up samples were carried out simultaneously on a StepOnePlus™ Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), with inventoried gene-specific TaqMan™ primer/probes gene expression assays for *PPARγ* (Hs01115513_m1) *HIF-1α* (Hs00153153_m1), *VEGFα* (Hs00900055_m1), *LOX* (Hs00942480_m1), *COL5A1* (Hs00609088_m1), *COL6A1* (Hs00242448_m1) and *FGF1* (Hs00265254_m1), using the Applied Biosystems StepOnePlus™ Real-Time PCR software Version 2, as described in Chapter 3.

4.2.4 Statistics Analysis

Assumptions of normality were verified for continuous data by Shapiro-Wilk test. Non-normally distributed variables (weight, BMI and $S_{I)}$ were $\log_{10}$ transformed prior to parametric analysis. Normal data are presented as mean and SD and non-normal data as median and interquartile range. If a parametric test was performed with non-normally distributed variables, Levene's test of variation was used to determine homogeneity of continuous data. Changes from baseline to follow-up in gene expression, body composition variables and measures of insulin sensitivity and secretion were assessed with paired-samples *t*-test in black and white women, separately. Pearson's correlations (r) were used to explore the relationships between the change in gene expression and the change in body composition, and insulin sensitivity and secretion over the follow-up period in black and white women,

separately. DATA were analysed using STATISTICA version 13 (Statsoft Inc., Tulsa, OK).

4.3 RESULTS

4.3.1 Participant characteristics

The baseline and follow up participant characteristics are shown in Table 3.1. Of the 10 black participants, 2 women reduced their body fat mass [-6.0kg to -13.1kg] over the 5-year follow-up period, while 6 women increased their body fat mass [+4.3kg to 23.5kg]. Of the 5 white participants, 3 women increased their body fat mass [+1.0kg to 2.4kg], while 2 women reduced their fat mass [-6.3kg to -7.0kg]. Over the 5-year follow-up period, in black women, BMI, android fat mass (%fm) and central/leg fat ratio ($p< 0.05$) increased, while gynoid fat mass (%fm) decreased, whereas in white women, there were no changes in body fatness and body fat distribution.

Despite changes in body composition, there were no changes in fasting glucose and insulin concentrations, or insulin sensitivity and secretion in the black women over the follow-up period. In contrast, in white women, both fasting insulin and glucose concentrations increased over time, while insulin sensitivity and secretion did not change.

4.3.2 Changes in Hypoxia and ECM related gene expression in gluteal SAT of black and white women over the 5-year follow-up period

The changes in the transcript levels of hypoxia and ECM related genes in gluteal SAT of white and black women over the 5-year follow-up period are presented in Figure 4.1. In both black and white women, *PPARγ, VEGFα, HIF-1α*, *LOX* and *FGF1* expression increased from baseline to follow-up. In contrast, *COL5A1* expression decreased in black women over time, but did not change in white women. *COL6A1*

Table 4.1 Change in body composition and insulin sensitivity measures over 5 year follow-up in white and black SA women

	White women			Black women		
	Baseline	**Follow-up**	**p-value**	**Baseline**	**Follow-up**	**p-value**
Age (years)	26 ± 4.3	32 ± 4.5		27 ± 8.4	32 ± 8.3	
Body composition						
Weight (kg)	67.2 (62 - 90)	65.3 (63-91)	0.75	91.0 (61 - 100)	92.4 (70- 108)	0.06
Waist (cm)	95.4 ± 4.8	102.4 ± 8.4	0.33	106.2 ± 6.8	109.3 ± 5.9	0.45
BMI (kg/m^2)	23.8 (22 -24)	22.8 (20- 24)	0.75	36.7 (24 -40)	36.9 (29 -47)	0.03
Body fm (%)	30.6 ± 12.9	29.6 ± 12.8	0.27	42.5 ± 9.4	44.2 ± 7.0	0.54
Cent fm (%fm)	43.7 ± 8.1	40.7 ± 7.9	0.40	48.6 ± 5.6	50.1 ± 4.3	0.22
Leg fm (%fm)	43.6 ± 8.3	44.6± 7.5	0.29	37.4± 5.7	36.02 ± 5.8	0.22
Cent/leg fat ratio	2.04 ± 0.7	1.93 ± 0.5	0.23	2.82 ± 0. 8	3.11 ± 0.9	0.03
Gynoid fm (%fm)	20.1 ± 2.4	21.4 ± 2.0	0.27	17.9 ± 2.8	16.9 ± 3.1	0.01
Android fm (%fm)	6.5 ± 2.7	6.4 ± 2.1	0.81	8.4 ± 2.0	9.2 ± 1.7	0.04
Insulin sensitivity						
Glucose (mmol/l)	4.3 ± 0.3	4.9 ± 0.6	0.01	4.6 ± 0.4	4.9 ± 0.7	0.21
Insulin (mU/l)	6.1 ± 3.2	14.9 ± 9.7	<0.01	13.9 ± 6.9	15.5 ± 8.9	0.52
S_I (x10^{-4} min^{-1}/[pmol/l])	5.2 (3.9– 7.0)	4.7 (2.3 – 6.9)	0.97	1.1 (0.8 - 1.9)	1.4 (1.1 – 1.9)	0.69
AIR_g (µU/ml)	393 ± 208	407 ± 210	0.42	2834 ± 2575	2277 ± 1078	0.68

Values represent unadjusted mean ± SD or median (interquartile range). Fm, fat mass; %fm, a percentage of fm; Cent, Central fat mass; S_I, Insulin Sensitivity; AIR_g, Acute Insulin Response to Glucose.

expression was not different between baseline and follow-up in both black and white women.

4.3.3 Associations between changes in gluteal SAT Hypoxia and ECM gene expression and changes in body composition in black and white SA women over the 5-year follow-up

The Pearson correlations coefficients (r), between the change in gluteal SAT gene expression and body fat distribution parameters from baseline to follow-up in black and white women are shown in Table 4.2. An increase in body fat mass (kg) from baseline to follow-up was associated with an increase in *PPARγ* expression in white

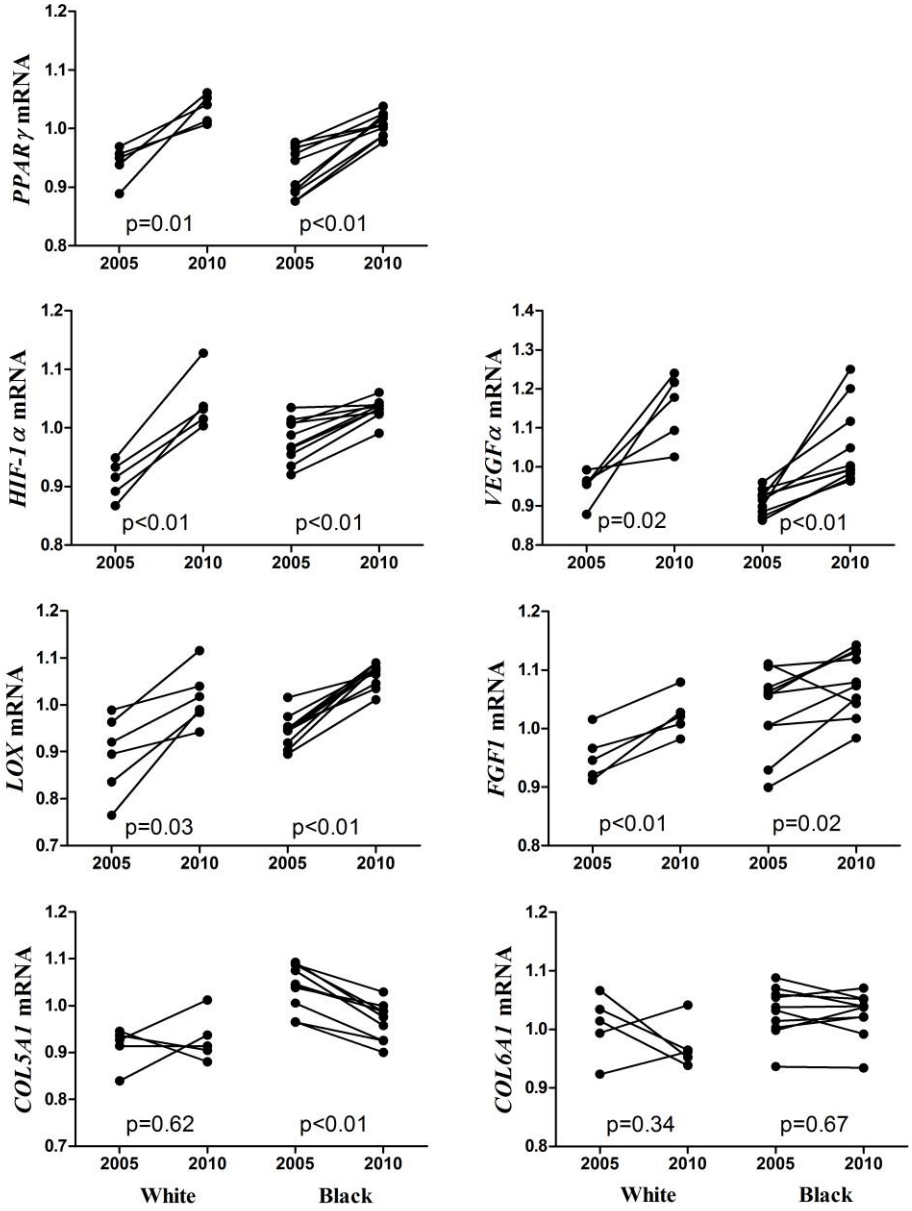


Figure 4.1 Changes in gene expression from baseline to follow-up in gluteal SAT of white and black women.
Circles represent unadjusted means for gene expression levels at baseline and at 5-years follow-up. P-value for paired samples Student's *t*-test- baseline vs follow up, in black and white women separately. mRNA expression relative to internal control genes and expressed in arbitrary units (AU).

women only, whereas an increased in body fat mass (kg) was associated with a decrease in *COL5A1* expression in black women only. Further, an increase in the central fat mass (kg) over the follow-up period was associated with an increase in *PPARγ* and *COL5A1* mRNA expression in white women only.

4.3.4 Associations between the changes in gluteal SAT hypoxia and ECM gene expression and changes in insulin sensitivity measures in black and white women over the 5-year follow-up

The Pearson correlation coefficients (r) between the change in gluteal SAT gene expression levels from baseline to follow-up and measures of insulin sensitivity and secretion are shown in Table 4.2. In black women, an increase in fasting insulin concentrations were associated with an increase in *HIF-1α* and *COL6A1* mRNA expression, whereas an increase in AIR_g was associated with an increase in *LOX* and *COL5A1* expression. These relationships remained significant when adjusting for baseline body fat mass and changes in body fat mass over time. Further, after adjusting for change in fat mass, an increase in fasting insulin concentrations was also associated with an increased *COL5A1* expression, (partial correlation r= 0.71; $p<0.033$) in black women. In white women, there were no significant associations between the change in insulin sensitivity and secretion and changes in gluteal SAT gene expression.

4.4 DISCUSSION

This study investigated the changes in gluteal SAT hypoxia and ECM related gene expression and their associations with changes in body composition and insulin sensitivity and secretion of black and white South African women over a 5-year free-living period. The main findings of this study were that *PPARγ*, *HIF-1α*, *VEGFα*, *LOX* and *FGF1* expression increased over the follow-up period in both black and white women, whereas *COL5A1* mRNA expression decreased in black women only. Notably, the increase in *PPARγ* expression in white women associated with an increase in total and central fat mass, whereas the decrease in *COL5A1* mRNA expression in black women correlated with an increase in their total fat mass. Changes

Table 4.2 Associations between the change in hypoxia and ECM gene expression and the change in body fat distribution- and insulin sensitivity measures over the 5-year follow-up period in gluteal SAT of black and white SA women.

	Body Composition				Insulin Sensitivity					
	Δ Body fm		Δ Cent fm		Δ Insulin		Δ S_I		Δ AIR_g	
	White	Black	White	Black	White	Black	White	Black	White	Black
Δ PPARγ	0.89[a]	-0.54	0.92[a]	-0.47	0.61	-0.24	-0.14	0.16	-0.26	0.35
Δ HIF-1α	0.80	0.25	0.87	0.23	0.39	0.77[b]	-0.13	-0.15	-0.36	0.36
Δ VEGFα	-0.81	0.01	-0.87	0.09	-0.79	0.39	0.50	-0.40	0.02	0.43
Δ LOX	0.05	-0.01	0.07	0.01	-0.34	0.16	0.16	-0.15	0.27	0.71[a]
Δ COL5A1	0.86	-0.71[a]	0.89[a]	-0.57	0.24	0.30	0.19	-0.30	-0.40	0.71[a]
Δ COL6A1	0.70	-0.04	0.60	0.03	0.49	0.81[b]	0.32	-0.50	-0.18	0.59
Δ FGF1	0.12	0.16	0.21	0.18	0.41	0.27	-0.59	-0.19	0.46	-0.15

Values are Pearson correlation coefficients (r). P-values: a, p<0.05; b, p<0.01. Δ, Change from baseline to follow-up; Fm, fat mass S_I, insulin sensitivity; AIR_g, acute insulin response to glucose.

in gene expression were not associated with changes in insulin sensitivity and secretion in white women. In black women, however, increases in *HIF-1α* and *COL6A1* mRNA transcription associated with increases in fasting insulin concentrations and changes in *LOX* and *COL5A1* mRNA associated with changes AIR_g.

Similar to the black women from the larger South African cohort [106], weight gain in the black participants of this study associated with a relative redistribution of body fat from the periphery (gynoid) to the central (android) depot. Cross-sectional studies in pre-menopausal women have shown that black women have greater peripheral fat distribution and a smaller central fat mass compared to white women [23, 108]. However, these longitudinal findings suggest that with increasing age and weight gain, black women may be equally at risk of the metabolic abnormalities associated with the centralization of body fat as white women. Similar findings were reported from the

IRAS Family study [367] in which a cohort of young African American (n=389) and Hispanic (n=844) women were followed up after a 5-year interval. Additionally, this study showed that the greater central fat accumulation of the African American compared to the Hispanic women was attributed to greater increases in both VAT and SAT area. The changes in body fat distribution may however be dependent on the age and or /starting BMI of the participants studied, as in an older cohort of African-American women with a higher BMI, Lara-Castro et al [46] demonstrated that the increased body fat mass over a 5 year follow-up period was associated with an overall increase in body fat mass, without proportional changes to either central or peripheral fat mass.

Change in body fat partitioning may be explained, in part, by adipogenic and lipogenic capacity in the gluteal depot of black and white women. In this study, it was shown for the first time that a gain in body fat mass in the black women did not associate with a proportional increase in *PPARγ* expression, as was shown in the white women. Pervious work in this cohort has demonstrated a greater down-regulation of *PPARγ* and other adipogenic and lipogenic genes in the gluteal depot of obese black compared to obese white women [105]. The lower expression of these genes in the gluteal SAT of obese black women associated with their lower S_I. Studies implementing thiazolidinediones (TZD's), known *PPARγ*-agonists, have shown that higher expression levels of *PPARγ* during adipose tissue expansion are associated with the generation of smaller, more insulin sensitive adipocytes, without changes to the overall adipose tissue depot size [368]. Taken together, these findings suggest that the reduced adipogenic capacity in the gluteal depot of black women may underlie the generation of more hypertrophied adipocytes, as was previously observed in this depot (Keswell et al., unpublished data) and the redistribution of fat to the central depots.

In this study, the gluteal expression of *HIF-1α* and its transcriptional target, *VEGFα*, were higher at follow-up compared to baseline of both black and white women, independent of their respective changes in body fat mass. An increase *in HIF-1α* mRNA expression is a well-known physiological response to the development of obesity and is hypothesised to stimulate increased *VEGFα* expression to increase angiogenesis in order to alleviate tissue hypoxia [307, 369, 370]. In addition to their role in hypoxia signalling, increased *HIF-1α* and *VEGFα* may also contribute to normal tissue maintenance and development by inducing adipocyte differentiation- and maturation processes [371] [372]. Together, these results may be indicative of an adaptive increase in vasculature formation during fat accumulation and normal tissue maintenance in the gluteal depot of black and white women over the follow-up period. However, higher *HIF-1α* and *VEGFα* mRNA expression in the white women was surprising given that no changes in fat mass were observed which suggests that the expression of these genes in adipose tissue is not simply a consequence of increased obesity.

In this study, higher *HIF-1α* expression in the gluteal SAT of black women associated with higher fasting insulin concentrations over the follow-up period. Insulin stimulation has previously been reported to induce *HIF-1α* protein and hypoxia-related gene expression in retinal epithelial cells *in vitro* [373]. However, it is not known whether higher circulating insulin may also increase the *in vivo HIF-1α* mRNA expression in human adipose tissue. Studies from tumour biology have shown a direct correlation between activity of the phosphatidylinositol 3-kinase (PI3K) / AKT (protein kinase B) pathway, increased *HIF-1α* expression, *HIF-1α* mediated transcription and *VEGFα* expression [374, 375]. It is thus plausible that high circulating insulin (hyperinsulinaemia) may increase *HIF-1α* mRNA transcription in adipose tissue. Importantly, the association between the higher *HIF-1α* expression and increased fasted insulin levels in the gluteal depot was

significant only in the black women. A potential explanation for this finding may be the small sample size of the white women.

Another unique finding of this study was that higher fat mass accumulation associated with a reduced *COL5A1* mRNA expression over the follow-up period in black, but not white women. Given that pericellular collagen is proposed to result in increased mechanical constraint during cellular hypertrophy [358], it stands to reason that the lower *COL5A1* mRNA expression in the gluteal SAT of black women may reflect the remodelling of the collagen network surrounding the adipocytes towards lowering these mechanical forces, and thereby restoring FFA storage capacity. In support, Abdenour et al., [366] recently suggested that diminished SAT stiffness of humans (i.e. reduced collagen accumulation) enables fat mass enlargement, which may protect against ectopic fat distribution [366].

Interestingly, higher *HIF-1α* and *COL6A1* mRNA expression associated with higher fasting insulin concentrations, whereas changes in *COL5A1* mRNA associated with lower AIR_g, in black, but not white women, independent of differences in baseline body fat mass (kg). Insulin has been reported to regulate the secretion of ECM components of 3T3-L1 adipocytes through the transcriptional regulation of the ECM processing enzymes [376]. This suggests that high levels of circulating insulin in the black women, could contribute to ECM dysregulation in adipose tissue, even in the absence of obesity. ECM remodelling involves the induction of matrix remodelling enzymes, such as *FGF1* and *LOX*, the induction of which were also observed in black and white women in this study. Accordingly, the increase in gluteal *LOX* and *FGF1* expression suggest increased collagen cross-linking and matrix remodelling in this depot at follow-up. *LOX* expression in SAT has been found to be higher in obese compared to normal-weight humans and mice [358] [377]. Moreover, up-regulation of *LOX* expression in obese

rats fed a high-fat diet was associated with increased pericellular fibrosis, whereas *LOX* inhibition by BAPN treatment ameliorated these alterations [377]. These findings suggest a role for this enzyme during obesity development [307]. However, the expression of these components have been shown to be altered in insulin resistant compared to insulin sensitive humans and mice [332, 378, 307] in a manner that is independent of obesity. In this study, increased *LOX* mRNA expression associated with increased AIR_g in the black women, but not in the white women. Taken together, it may be hypothesised that increased collagen and matrix remodelling components in the gluteal SAT may represent an ethnic specific mechanism of increased adipose tissue insulin resistance.

This study has some important limitations to consider. Firstly, the sample size of the follow-up cohort is small. Loss to follow-up is problematic in most cohort studies but nonetheless, has implications for the statistical power and for the down-stream generalizability of the results. With this in mind, statistical test were only conducted within each ethnic group. However, this study may be viewed as a pilot study, from which some novel relationships have been identified and that require further verification. Secondly, due to the small size of the fat biopsies obtained, protein expression in the adipose tissue samples could not be measured. In addition, methodological issues relating to measurement of insulin at different time points, and storage of baseline adipose tissue for ± 5 years may have confounded the results. However, the same technique was used for insulin analyses at both time points, and the quality of the adipose tissue was similar between baseline and follow-up tissue samples.

Importantly, to my knowledge, this is the first study to show that the changes in mRNA expression levels of hypoxia- and fibrosis-related genes correlated with changes in

fasting plasma insulin concentrations (*HIF-1α,* and *COL6A1*) and insulin secretion (*LOX* and *COL5A1*) over time. It may be hypothesised that hyperinsulinaemia in black women may not simply be a consequence of increased insulin resistance but may also contribute to the exacerbation of insulin resistance during obesity development by inducing the expression of hypoxia- and fibrosis-related genes in adipose tissue. However, these findings warrant further investigation in a larger cohort of black and white women.

CHAPTER FIVE

EFFECT OF HYPOXIA ON THE REGULATORY EFFECTS OF INSULIN ON ECM COMPONENT PRODUCTION IN *3T3-L1* ADIPOCYTES

5.1 INTRODUCTION

In Chapter 3 of this thesis it was demonstrated that obese black women were more insulin resistant than obese white women, and had higher fasting insulin levels and a greater insulin response to glucose in order to maintain normoglycaemia. The obese black women had higher mRNA expression levels of *HIF-1α*, *COL5A1* and *COL6A1* and lower *VEGFα* expression in the gluteal SAT depot compared to obese white women. Further, in Chapter 4 it was reported that changes in gluteal *HIF-1α* and *COL6A1* mRNA expression levels over the 5-year follow-up period in the black women, correlated positively with changes in fasting plasma insulin concentrations. In addition, changes in gluteal *LOX* and *COL5A1* mRNA expression levels correlated positively with changes in insulin secretion in response to a glucose load. Based on these findings, it may be hypothesised that in black women, gluteal SAT *HIF-1α* expression may be increased by their high circulatory insulin concentrations, resulting in a concomitant increase in *COL5A1* and *COL6A1* mRNA expression levels, and a consequent decrease in insulin sensitivity. This hypothesis is based on murine [294, 302] and human [70, 301] models of obesity, which have shown that adipose tissue of obese individuals is less vascularized [328] and has lower oxygen partial pressure (PO_2) compared to adipose tissue of their non-obese counterparts [70, 301]. The reduction in PO_2, also referred to as hypoxia [67], has been attributed to increased cellular hypertrophy and is proposed to be a result of lower proliferation and/or differentiation

capacity of SAT adipocytes (section 2.4.1). Although the molecular mechanisms are not yet fully understood, hypoxia in adipose tissue of obese individuals does not result in a pro-angiogenic response aimed at alleviating low PO_2 in adipose tissue, but produces a pro-fibrotic transcriptional program through the induction and stabilization of *HIF-1α* protein [307, 379]. The generation of excess ECM and matrix remodelling components in adipose have been linked to the development of insulin resistance in murine [307] and human models [295]. Several studies hypothesize that increased mechanical stress from a rigid fibrotic network of collagen leads to chronic low grade inflammation, which consequently, underlies the development of insulin resistance (For review see [71] and section 2.3.2). Ultimately, to maintain glucose homeostasis, β-cells secrete more insulin which in turn result in increased circulating hormone levels.

Insulin treatment has been shown to stabilize *HIF-1α* expression via a PI3K/mTOR dependent pathway [373] where it results in an increased accumulation of *HIF-1α* protein [372, 373, 380] and mRNA [372] in a time-dependent manner. Therefore, chronically elevated circulating insulin may contribute to the generation of fibrosis through increased *HIF-1α* protein expression in adipose tissue, in a manner that is independent of hypoxia. Further, insulin has been reported to regulate the secretion of ECM components of 3T3-L1 adipocytes through the transcriptional regulation of ECM processing enzymes [376]. *Col5a1* expression, along with the ECM processing enzymes, matrix metalloproteinase 2 (MMP2), metalloproteinase inhibitor 2 (TIMP2) and procollagen C-endopeptidase enhancer protein were significantly increased during insulin treatment of 3T3-L1 adipocytes in a microarray study [376]. This suggests that high levels of circulating insulin, commensurate with insulin resistance, could contribute to ECM dysregulation in adipose tissue, and consequently reduce insulin sensitivity further.

Although the independent effects of hypoxia and insulin treatment on *HIF-1α* expression have been investigated, less is known about the concurrent effects of these conditions, such as those that may occur in adipose tissue of hyperinsulinaemic obese individuals. It is not clear whether high circulating insulin may directly increase *HIF-1α* expression and contribute to the formation of excess ECM, or whether increased insulin may simply be a concomitant downstream effect of increased insulin resistance as a consequence of increased fibrosis and the generation of inflammation. This study hypothesises that the expression of *HIF-1α, Col5a1* and *Col6a1* will increase in a dose-dependent manner in insulin-treated 3T3-L1 adipocytes under hypoxic- compared to normoxic conditions. Therefore, the aims of this study are to i) determine the effects of insulin on *HIF-1α*, *Col5a1* and *Col6a1* mRNA and protein expression in 3T3-L1 adipocytes, and to ii) determine whether these effects may be different during hypoxic vs. normoxic conditions.

5.2 MATERIALS AND METHODS

5.2.1 Cell culture

The 3T3-L1 mouse pre-adipocyte cell line Cl-173 (American Type Culture Collection (ATCC) number CL-173, Manassas, VA, USA) was seeded in CELLBIND 6-well plates (Corning, MA, USA) at 7.5 x 10^4 cells/well and grown to confluence in growth medium (Dulbecco's modified Eagle's medium (DMEM) containing 10% new-born calf serum (NCS) (The Scientific Group, JHB, SA) and 4 mM glutamine) at 37°C in humidified air with 5% CO_2 for three days. When cells reached 80% confluence, growth media was removed and differentiation was induced by substituting with adipocyte differentiation medium (DMEM containing 10% fetal bovine serum (FBS) (Biochrom, Berlin, Germany), 1 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine and 1µg/ml insulin). After 72 hrs of culture, the differentiation medium was replaced with adipocyte

maintenance medium (AMM) (DMEM containing 10% FBS, and 1 µg/ml insulin) and cells were cultured for 2 days, at the end of which the 3T3-L1 adipocytes were fully differentiated [381]. AMM was then replaced by DMEM containing 10% FBS and the cells cultured for a further 3 days. On day 8, cells were refreshed in either DMEM containing 10% FBS (control treatment), DMEM containing 10% FBS and 175 nM insulin (medium treatment) or DMEM containing 10% FBS and 1 µM insulin (high treatment) prior to incubation and assays.

5.2.2 Cell viability in the hypoxia and insulin treatments

Control, medium [175 nM] and high [1 µM] insulin treatment concentrations and treatment duration were selected based on previous reports that have investigated acute insulin stimulation [315, 372, 373, 382] and chronic high insulin treatment [376, 383] in 3T3-L1 adipocytes. (See supplementary Table 4.a). Hypoxia was induced by incubating cells in the GasPak EZ Campy pouch system (BD Biosciences, San Diego, USA) which creates an atmosphere of >10% CO_2 and 5% O_2, according to the manufacturer's guidelines. Cells under the normoxic condition remained in the incubator at humidified air with 5% CO_2 and 21% O_2 at 37˚C.

Cells were evaluated for cell viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St Louis, MO, USA) based colorimetric assay. Cells were incubated with 2 µg/ml of MTT in PBS for 1 h at 37˚C, and the produced formazan dissolved in Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO, USA) and Sorensen's phosphate buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) before quantification at 570 nm using a BioTek® ELX 800 plate reader and Gen 5® software (BioTek Instruments Inc., Winooski, VT, USA).

5.2.3 RNA extraction and reverse transcription

Total RNA was extracted from whole cell-lysates under hypoxic and normoxic conditions from control, medium and high insulin treatments. After incubation, culture media was aspirated from the cells after which the cells were washed in phosphate buffered saline (PBS) (Lonza, Walkersville, MD, USA) and collected by scraping in TriPure Isolation Reagent (Roche Diagnostics Ltd, Burgess Hill, UK). Three wells from the 6 well culture plates were pooled into a 2 ml Eppendorf tube and stored at -20°C until extraction. The collected cells were homogenized using the Qiagen Tissue lyser at 25Hz for 2 min and this was repeated five times. Total RNA was then purified using the RNeasy Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Genomic DNA was removed using the Turbo DNase kit (Ambion Inc., Austin, TX, USA). RNA concentration (A260) and purity (A260/A280) were quantified spectrophotometrically (Nanodrop Technologies, Wilmington, DE, USA) and integrity was assessed using the Agilent bioanalyser (Agilent Technologies Inc., Santa Clara, CA) (Figure 5.1). One microgram of DNase treated total RNA was reverse transcribed to cDNA using the High Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The reaction mix consisted of reaction buffer, dNTPs, random primers, RNase-inhibitor (5000 units/mL), reverse transcriptase (RT) enzyme and nuclease-free water. A duplicate set of reactions were prepared without RT enzyme and was used to calculate the amount of genomic DNA contamination (Supplementary Table 4.b for reaction conditions).

5.2.4 Quantitative Real-time PCR for gene expression analyses

Quantitative Real-Time PCR was conducted with Taqman® gene expression assays for *HIF-1*α (Hs00153153_m1), *Col5a1* (Hs00609088_m1) and *Col6a1* (Hs00242448_m1), according to the manufacturer's protocol (Applied Biosystems

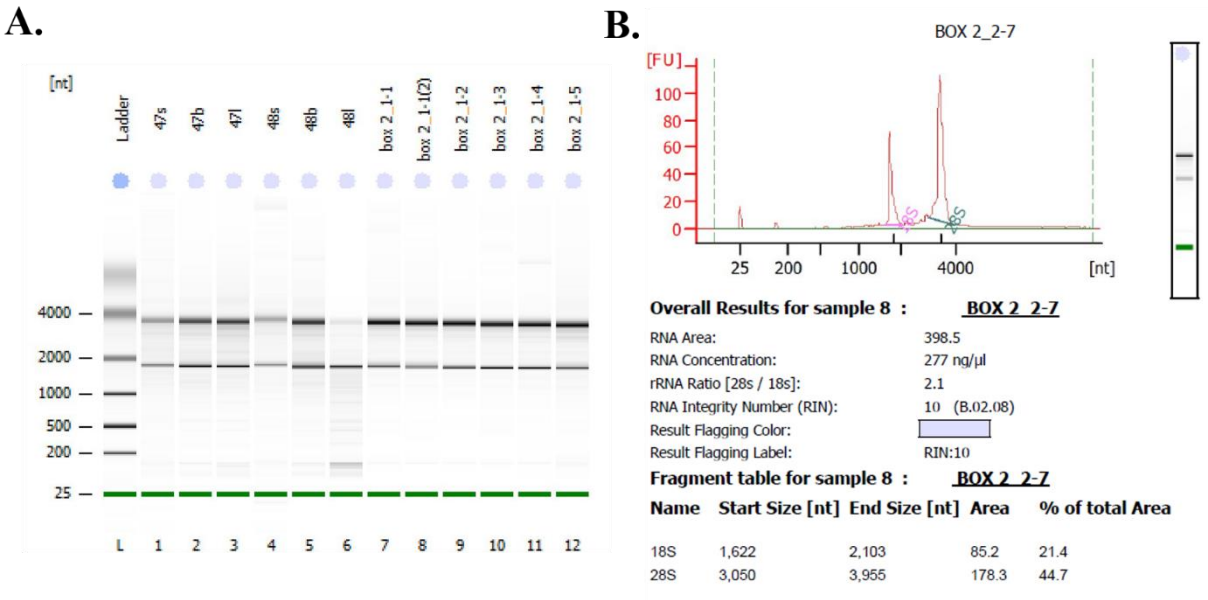


Figure 5.1 RNA quality and integrity analysis with Agilent Bio-analyser.
Typical representation of A) Bio-analyser gel result for samples extracted from insulin and hypoxia treated 3T3-L1 adipocytes. From the left, (L) represents the ladder followed by RNA samples in lane 1-12. On the gel photo, two bands are clearly visible in each sample lane and represents the 28S and 18S ribosomal subunits. B) Electropherograms generated by the Agilent Bio-Analyser. For each RNA sample, a profile is generated that reports RNA integrity and concentration. RNA integrity number (RIN) value is generated to indicate the quality of the RNA samples and can range between 10 (for intact RNA) – 1 (most degraded).

Foster City, CA, USA). Briefly, 1µl cDNA was added to 0.5µl of the respective TaqMan probes, 5 µl of TaqMan® Universal PCR Master Mix and water in a final reaction volume of 10 µl. Reactions were run in duplicate on a 7500 Real Time PCR system (Applied Biosystems) using universal cycling conditions of 50ºC for 2 min and 95ºC for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min.

Gene expression was determined by extrapolating against a standard curve defined by serial 1:10 dilutions of pooled cDNA. Tyrosine 3/tryptophan 5-monooxygenase activation protein (*Ymhaz*), TATAA-box binding protein (*Tbp*) and betaglucuronidase (*Gusb*) were evaluated as endogenous controls, based on previous studies of hypoxia [384]. From these, NormFinder was used to identify the most suitable endogenous

control genes [350]. The combination of *Gusb* and *Tbp* was identified as the most stable for use during hypoxic treatment conditions (stability value= 0.038).

5.2.5 Protein extraction and Western blotting

Whole cell-lysates were harvested from the control, medium and high insulin treated 3T3-L1 adipocytes incubated under hypoxic and normoxic conditions. Three wells of the 6 –well culture plates were pooled by scraping each well in 150 µl lysis buffer (50 mM KCl, 0.5%, Nonidet P-40, 25 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] [pH 7.8], 1 mM phenylmethylsulfonyl fluoride, 10 µg leupeptin/ml, 20 µg aprotinin/ml, 100 µM dithiothreitol) and combining in a 2 ml Eppendorf tube and placing on ice within 1 min. Samples were homogenised in a Tissue Lyser (Qiagen, Hilden, Germany) at 25 Hz in 60 sec increments which were repeated 5 times. After centrifugation at 16 000 x g for 1 min (4˚C), supernatant was collected.

Protein concentrations were determined using Reducing Agent and Detergent Compatible (RC DC) protein assay kit (Bio-Rad, Hercules, CA, USA). Heat denatured protein was separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane for 90 min at 160 V (4˚C). Non-specific protein labelling was blocked by incubating the membrane in 5% (w/v) low-fat milk powder in Tris-buffered saline with Tween 20 (TBST) (TBS pH 7.2; 0.001% Tween 20) at room temperature for 2 hrs. Membranes were labelled with the relevant primary antibody at 4˚C over-night and followed by horseradish peroxidase (HRP) conjugated secondary antibodies (Supplementary Table 4.c for list of antibodies used). Proteins were visualized by chemiluminescence with Clarity™ Western ECL Substrate (Bio-Rad Hercules, CA, USA) using a Chemidoc-XRS imager, (Bio-Rad Hercules, CA, USA) and quantified using Quantity One 1-D software (Bio-Rad, Hercules, CA, USA). β-Actin was used as internal reference control.

5.2.6 Intercellular lipid content determination

On day 8 of differentiation, fully differentiated adipocytes were washed and fixed in neutral buffered formalin 10% (v/v in PBS) and stained with 0.7% (w/v) Oil-Red O solution (Sigma-Aldrich, St Louis, MO, USA) for 30 min at room temperature. The stained lipid was quantified by extracting the dye with 100% isopropanol and measuring the absorbance at 510 nm using a BioTek® ELX 800 plate reader and Gen 5® software (BioTek Instruments Inc., Winooski, VT, USA). Plates were then de-stained with 70% Ethanol. Lipid content was normalized to cell density according to a protocol adapted from Gillies *et al* [385]. Briefly, the decolourised fixed cells were re-stained with 0.5% Crystal Violet, the Crystal Violet extracted with 70% ethanol, and the absorbance read at 570 nm using the BioTek® ELX 800 plate reader.

5.2.7 Statistical analysis

Results are expressed as mean ± standard deviation (SD) of three independent biological experiments, unless otherwise stated. Two-way analysis of variance (ANOVA) was used to examine differences in cellular viability, mRNA- and protein expression and triglyceride content between normoxic and hypoxic conditions in response to three different insulin treatments (control, medium- [175 nM] and high-insulin [1 µM] concentrations) with Tukey *post hoc* test. Differences were considered significant at $p \leq 0.05$. Data were analysed using STATISTICA version 12 (Statsoft Inc., Tulsa OK, USA).

5.3 RESULTS

5.3.1 Cell viability in the normoxic and hypoxic conditions and insulin treatments.

Cell viability was evaluated under different durations of hypoxia exposure. Compared to the normoxic condition, cell survival increased similarly after 4, 8 and 12 hrs of hypoxia exposure (Figure 5.2), showing that hypoxia exposure (5% O_2) did not have cytotoxic effects on the differentiated 3T3-L1 adipocytes. Eight hours exposure was selected as appropriate hypoxia exposure duration, as exposure beyond 8 hrs treatment did not result in further significant affects beyond 4 hrs exposure. Cell viability was evaluated in control, medium- [175 nM] and high- [1 µM] insulin concentrations in normoxic vs. hypoxic conditions. Under normoxic conditions, medium- and high-insulin treatment increased cell viability (treatment effect: $p < 0.001$), whereas under hypoxic conditions, insulin treatment did not alter cell viability (condition x treatment interaction, $p < 0.001$).

5.3.2 Effect of insulin treatment on *HIF-1α*, *Col5a1* and *Col6a1* mRNA and protein expression under normoxic and hypoxic conditions.

To determine the effect of insulin on the abundance of *HIF-1α*, *Col5a1* and *Col6a1* mRNA and protein levels under normoxic and hypoxic conditions, mature 3T3-L1 adipocytes were incubated under control, medium- [175 nM] and high- [1 µM] insulin concentrations (Figure 5.3). In the untreated control groups (no-insulin- normoxia vs. hypoxia), hypoxia resulted in a significant increase in *HIF-1α* mRNA transcript levels compared to the normoxic condition. Insulin treatment was associated with higher *HIF-1α* mRNA expression (treatment effect, $p<0.001$) in hypoxic and normoxic conditions. In the normoxic condition, *HIF-1α* mRNA levels were increased in the medium- and high-insulin treatments compared to the untreated control. However, the effect was attenuated in the hypoxic condition (condition × treatment interaction, $p= 0.001$) where

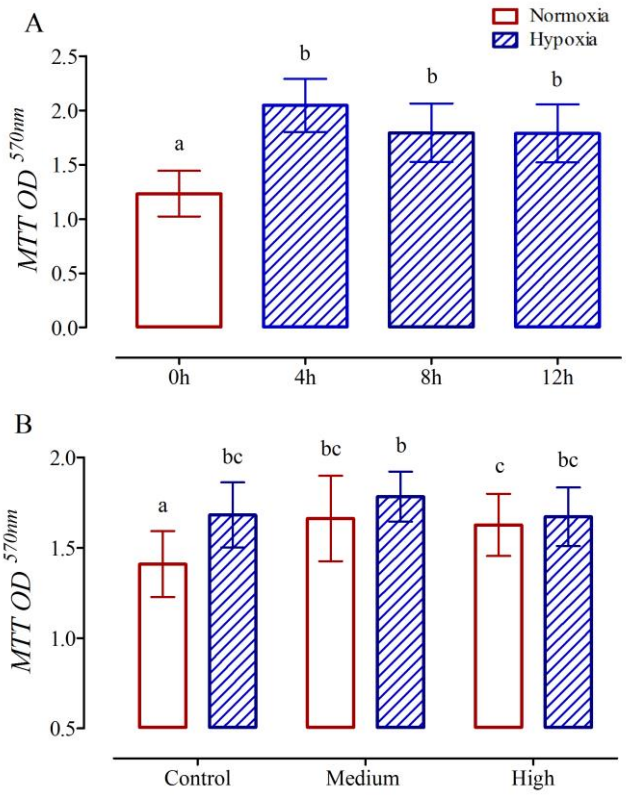


Figure 5.2 MTT assay of insulin and hypoxia treated 3T3-L1 adipocytes.
Cell viability of fully differentiated 3T3-L1 adipocytes was measured by MTT assay in A) 0-, 4-, 8- and 12 h hypoxia treatment (5% O2) (n = 60) and B) Control, medium [175 nM] and high [1 µM] insulin treatment in normoxic (21% O2) and hypoxic (5% O2) conditions (n = 16). Values are mean OD ± SD (read at 570 nm). OD, optical density.

HIF-1α mRNA expression was increased with high-insulin but not with medium-insulin treatment, compared to the untreated hypoxic control. *HIF-1α* protein expression was higher in the hypoxic vs. normoxic condition (condition effect, p=0.008). However, insulin treatment had no further effects on *HIF-1α* protein expression under the hypoxic condition compared to the normoxic condition (condition × treatment interaction, p= 0.03), whereas *HIF-1α* protein expression was increased in the high-, but not the medium-insulin treatment compared to the untreated normoxic control. Whilst insulin treatment was associated with lower *Col5a1* mRNA expression under the normoxic

condition, similar affects were not observed under the hypoxic condition (condition × treatment interaction, p=0.01). In contrast, hypoxia exposure was associated with higher *COL5A1* protein expression, but its expression was not altered by insulin treatment under either normoxic or hypoxic conditions. *Col6a1* mRNA expression was not significantly different between any of the insulin treatments under either condition.

In contrast, hypoxia exposure was associated with higher *COL6A1* protein levels (condition effect, p< 0.001) but its expression was not altered by the insulin treatments under either normoxic or hypoxic condition.

5.3.3 Effects of insulin treatment on lipid accumulation under normoxic and hypoxic conditions

The effect of hypoxia and insulin treatment on lipid accumulation was measured in mature 3T3-L1 adipocytes (Figure 5.4). In the untreated control groups, hypoxia resulted in a significant increase in lipid content compared to the normoxic condition. However, after 8 hrs insulin treatment under normoxic conditions, lipid content was significantly reduced in response to the medium- [175 nM], but not the high- [1 µM] insulin concentration. This effect was exacerbated in the hypoxic condition where insulin treatment reduced lipid content in the medium- and high-insulin treatments (condition x treatment interaction, p < 0.001).

5.4 DISCUSSION

The aim of this study was to determine the effects of chronic insulin treatment in normoxic (21% O_2) vs. hypoxic (5% O_2) conditions on the expression of *HIF-1α* and ECM components in differentiated 3T3-L1 adipocytes. The main findings of this study were that chronic high insulin and hypoxia treatment significantly elevated *HIF-1α* mRNA and protein levels but that observed effects were not additive. In addition,

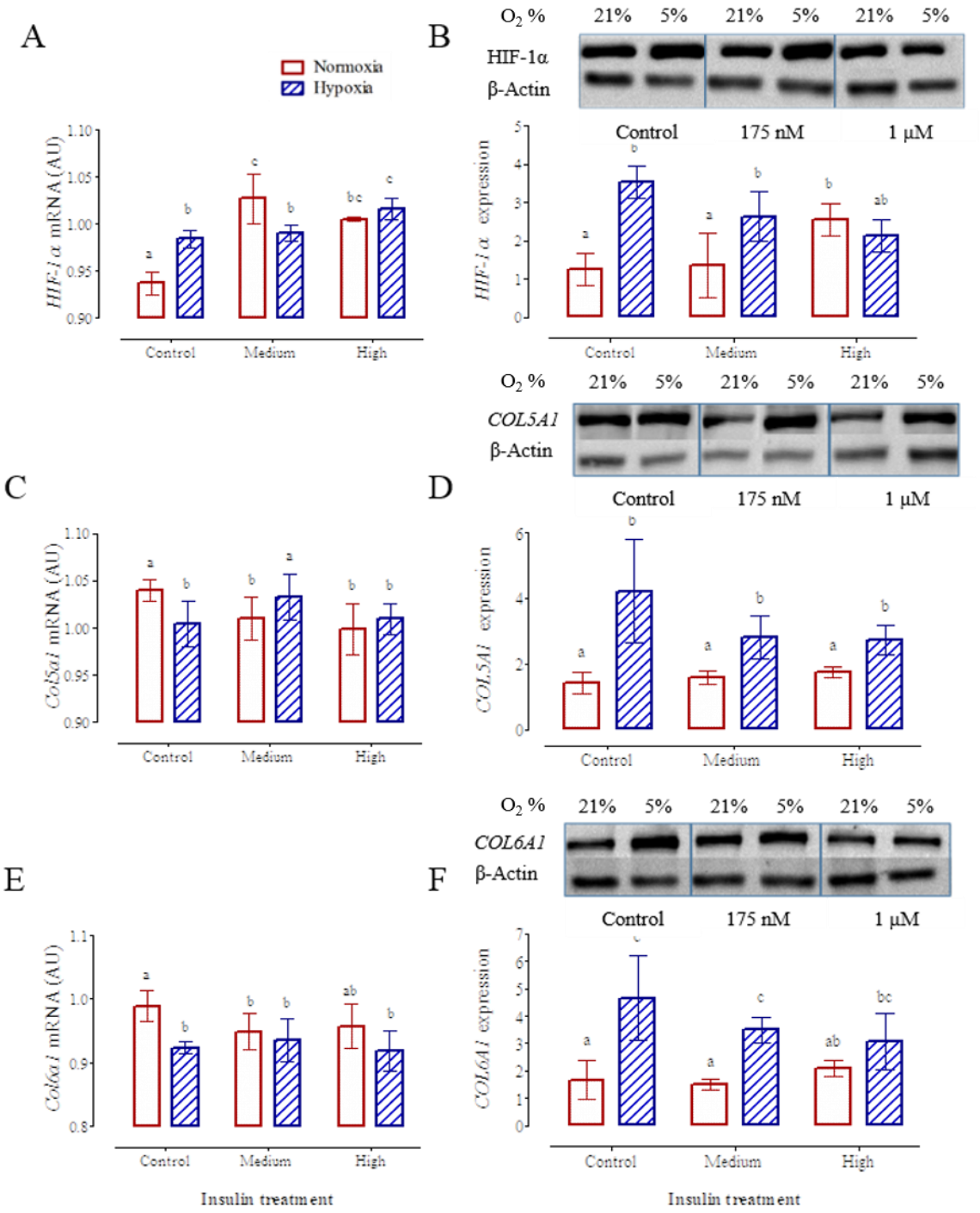


Figure 5.3 HIF-1α, *Col5a1* and *COL6a1* mRNA and protein expression in hypoxia and insulin treated 3T3-L1 adipocytes. The mRNA and protein levels for *HIF-1α* (A and B), *COL5A1* (C and D) and *COL6A1* (E and F) were measured in mature 3T3-L1 adipocytes in control, medium [175 nM] and high [1 μM] insulin treatments under normoxic (21% O_2) and hypoxic (5% O_2) conditions. Values are mean ± SD. mRNA expression relative to internal control genes and expressed in arbitrary units (AU). Protein expression relative to internal control, β-Actin. Results are representative of three independent experiments. Bars with different letters denote statistical differences between results ($p<0.05$).

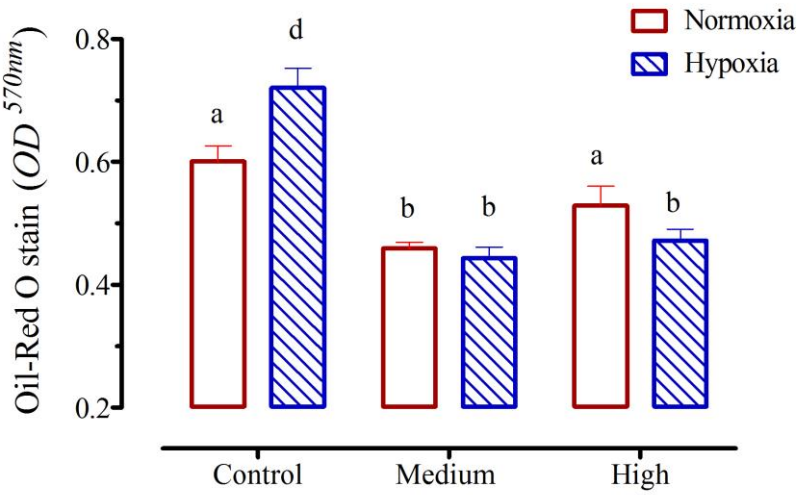


Figure 5.4 The effect of 8 hrs insulin treatment on lipid content in mature 3T3-L1 adipocytes under normoxic and hypoxic conditions.
Adipocytes were subjected to control, medium [175 nM] and high [1 μM] insulin treatments under normoxic and hypoxic conditions. Lipid was stained with Oil-Red O, and the intensity of the extracted stain was measured at 570 nm and results were normalized to cell density, as measured by crystal violet staining. Values are mean ± SD. Bars with different letters denote statistical differences between results ($p<0.05$).

hypoxia but not insulin treatment was associated with higher *COL5A1* and *COL6A1* protein- but not mRNA expression. Further, exposure of adipocytes to 8 hrs insulin treatment resulted in significantly reduced lipid staining and the effect was exacerbated in the hypoxic- compared to the normoxic condition.

Cellular adaptation to hypoxia is accomplished through the activation of an array of oxygen-sensing transcription factors, including *HIF-1α* [303, 304]. Under normoxic conditions, *HIF-1α* is targeted for proteasomal destruction by proline hydroxylation by an E3 ubiquitin ligase complex containing the von Hippel-Lindau tumour suppressor protein (pVHL) [306]. During hypoxic conditions, the lack of molecular oxygen, an absolute requirement for *HIF-1α* hydroxylation, results in increased *HIF-1α* protein accumulation. Accordingly, the association between hypoxia treatment and higher *HIF-1 α* protein accumulation observed in this study is in line with this oxygen-sensing

function of the *HIF-1 α* protein- a finding also reflected in other studies in 3T3-L1 adipocytes [294, 315, 370, 382]. In contrast to the well-characterised post-transcriptional regulation of *HIF-1α* protein in response to hypoxia, the regulation of *HIF-1α* mRNA during low oxygen levels remain controversial and unexplored. Several studies [357, 372, 386, 387], but not all [294, 307, 352, 370, 388, 389], report no change of *HIF-1α* mRNA in response to hypoxia *in vitro*. In this study, the expression of *HIF-1α* mRNA levels was higher in the hypoxic vs normoxic condition. Notably, increased *HIF-1α* mRNA expression in adipose tissue has been implicated as a consequence of a high fat diet (HFD)[357] and has also been associated with its related metabolic abnormalities. Specifically, in human [301, 390] and rodent [310, 370, 372, 391] models, increased *HIF-1α* mRNA expression in adipose tissue has been associated with reduced adipose tissue PO_2, increased BMI, increased macrophage infiltration, insulin resistance and the development of adipose tissue fibrosis. Moreover, higher *HIF-1α* mRNA expression has been demonstrated to have relevant implications in adipose tissue metabolism (Chapter 3 and 4) [301, 310, 370, 372, 390, 391] although the effect of these remain largely uninvestigated.

Although *HIF-1α* expression has mostly been investigated for its functions in the cellular response to tissue hypoxia, results from this study demonstrated a strong induction of *HIF-1α* mRNA expression with medium [175 nM] and high [1 µM] insulin treatment in the absence of hypoxia. Similar results have previously been noted in 3T3-L1 adipocytes where 100 nM insulin treatment significantly elevated *HIF-1α* mRNA expression within 4 hrs after treatment [372] but the physiological significance of this insulin-induced *HIF-1α* mRNA expression has not yet been investigated. Surprisingly, although *HIF-1α* (mRNA and protein) expression was increased by both insulin treatment and during exposure to hypoxia (5% O_2), duel treatment did not result in an additive effect. Therefore, these findings imply that the induction of *HIF-1α*

through insulin and hypoxia exposure may result from two different pathways. Interestingly, recent evidence has indicated that certain growth factors such as insulin and IGF-1 may induce the transcriptional activity of *HIF-1α* in the absence of tissue hypoxia [380]. Insulin induces the expression of genes containing the hypoxia response element (HRE) by formation of a *HIF-1*α /ARNT complex [380]. These HRE occurs in the 3' regions of genes that regulate glycolytic metabolism such as glucose transporters (Glut1 and Glut3), several glycolytic enzymes in addition to genes such as erythropoietin (EPO) and vascular endothelial growth factor (*VEGF*α) which aim to restore tissue oxygen levels [380, 392-394]. It may be hypothesised that the link between insulin signalling and its activation of gene transcription through HRE represents an evolutionary adaptive response [312] to enhance glucose utilization through the glycolytic pathway rather than producing ATP through the oxygen dependent β-oxidation in order to reduce adipocyte O_2 consumption. In support, it was recently demonstrated that early in the course of high-fat diet (HFD) feeding and obesity, adipocyte respiration becomes uncoupled, leading to increased oxygen consumption and a state of relative adipocyte hypoxia [357]. Under these circumstances, insulin may induce *HIF-1α* transcriptional activity through HRE aimed at inducing glycolysis and thereby lower oxygen utilization by the adipocytes.

It has been suggested that hypoxia in adipose tissue may represent the driving phenomenon behind the development of fibrosis. Interestingly, *Col5a1* and *Col6a1* mRNA levels were slightly decreased or unchanged in response to hypoxia vs. normoxia, while *COL5A1* and *COL6A1* protein expression were significantly increased. Discordant mRNA and protein expression has also been observed in other studies [395, 396]. It may be suggested that differences in *COL5A1* and *COL6A1* protein and mRNA expression levels in this study reflect a time dependent mRNA response

where mRNA expression stabilized at a lower level after an initial increase. Nevertheless, several other studies suggest that hypoxia, through the induction of *HIF-1α* protein, is a major initiator of excess ECM expression in adipose tissue and may underlie the development of inflammation and concomitant insulin resistance [307, 379, 390]. Conversely, genetic ablation of the *HIF-1α* protein through PX-478-treatment in a mouse model of obesity demonstrated a down-regulation of fibrillar collagens (and collagen type VI) and *LOX* (which plays an important role in collagen fibre formation) [397]. Although collagen type VI has been previously characterised for its effects during hypoxia [295, 323], no such reports have been found for collagen type V. Results from this study show that lower oxygen availability may result in higher levels of both type V and VI collagen expression in adipose tissue. Functionally, collagen type V has a structural role in collagen type I fibre diameter [237, 398]. Collagen type I forms the major structural component of the interstitial ECM in adipose tissue and exists as a well organised and dense cobweb-like framework. In addition, adipocytes are also individually surrounded by a basement membrane [225]. Collagen type VI has been shown to function as an anchor between the adipocyte basement membranes and interstitial ECM [399, 400]. Taken together, these results demonstrate that hypoxia may result in alterations to the adipocyte microenvironment through altered collagen V and VI expression. Surprisingly, in this study insulin treatment did not result in changes to the expression of either *COL5A1* and *COL6A1* mRNA- or protein levels. These results are in contrast to a study done in 3T3-L1 adipocytes which demonstrated that insulin stimulates the gene expression of ECM processing enzymes and their regulators, such as the MMPs, but not the target ECM proteins themselves [376]. This discrepancy may be explained by the longer duration of insulin treatment (48h) compared to 8hrs treatment as implemented in this study.

The main function of insulin in adipose tissue is to store energy in the form of triacylglycerol. However, increasing concentrations of insulin treatment reduced the lipid content in the 3T3-L1 cells (as measured by Oil-Red O staining) over 8 hrs incubation in both the hypoxic and normoxic conditions. MTT analysis indicated that the reduction in lipid content was not because of decreased cell-viability, but rather suggests an increase in lipolysis and/or an inhibition of lipogenesis. Insulin, a potent antilipolytic hormone [401] controls the accumulation of TG by inducing the expression of lipoprotein lipase (LPL) [151, 402] and inhibiting the activation of hormone sensitive lipase (HSL). However, chronic hyperinsulinemia, as opposed to its acute effects, has been shown to increase rather than decrease rates of lipolysis in vitro [403-406]. Acute insulin treatment activates phosphodiesterase 3B, reduces cyclic adenosine monophosphate (cAMP) levels and quenches β-adrenergic receptor signalling [407]. However, chronic insulin treatment amplifies cAMP production by downregulating β-arrestin1 protein- a process that leads to lipolysis through cAMP production and enhanced protein kinase A (PKA) activation [408] and subsequent activation of HSL [405]. In this study, the increased lipolytic effect of chronic (8 hrs) insulin as observed in the normoxic condition was further exacerbated by hypoxia exposure, resulting in a condition × treatment interaction effect. By using an *HIF-1α* inhibitor, digoxin, Zhao et al demonstrated a reduction in cAMP accumulation paired with suppression in PKA activity in hypoxic adipose tissue of HFD fed mice. These findings show an important inductive role for *HIF-1α* in lipolysis. These data may, in part, support why hyperinsulinemia may coexists with increased circulating nonesterified FFAs and increased adiposity in some obese and/or T2DM patients [409].

Conversely, in absence of insulin treatment, TAG content was increased in hypoxic treatment (5% O_2) compared to the normoxic condition. Hypoxia treatments in tissue

culture mostly range between 1% O_2 and 2% O_2 with 1% O_2 the most widely employed O_2 concentration in *in vitro* investigations [67]. However, 5% O_2 as used in this study may be more closely related to normal physiological O_2 concentrations. For example, in obese mice, adipose tissue PO_2 has been recorded to range between 15-22 mm Hg, which is equivalent to 3-5% O_2 depending on the depot of measurement [294], while abdominal SAT in obese humans may range between 5-11% (23-84mmHg) [70, 309]. In support, Weiszenstein et al.[410], recently showed that adipocytes are sensitive to small changes in O_2 concentration and that 3T3-L1 adipocytes cultured in 4% O_2 not only had improved cell survival but also had increased triglyceride accumulation when compared to cells grown at 1% O_2 or 20 % O_2. This finding underscores the need to establish experimental protocols that more closely reflect human physiological ranges. As such, pH and temperature are tightly controlled to resemble physiological conditions in *in vitro* experiments. It stands to reason that the implementation of more physiological oxygen concentration ranges (4 - 6% O_2,) as implemented in this study in contrast to the regularly implemented abnormal (1% O_2) level of oxygen may provide more consistent results. Under these conditions, it may be hypothesised that the low oxygen condition (5% O2,) in obese adipose tissue may not be adequate to induce the proangiogenic response through *VEGF*α and pyruvate dehydrogenase kinase-1 (PDK1) expression [312] as it is often reported from adipocyte- and tumour cell culture experiments conducted at 1% O_2. In tumour biology, *VEGF*α expression in tumour cells [313] allows for vascular development and tumour expansion. However, in hypertrophic obese adipose tissue, even though *HIF-1α* protein increases, *VEGF*α does not increase [70, 294, 301, 307] and is rather accompanied by reduced capillary density [116, 294].

Evaluation of hypoxia treatment on cell-survival duration in this study showed that 4 hrs hypoxia treatment increased cell viability, which did not change after 8 and 12 hrs. When the effect of hypoxia and insulin was tested on the survival of these mature 3T3-L1 adipocytes, it was found that both insulin stimulation and hypoxia treatment was able to increase cell survival compared to the untreated control. These results are in agreement with previously established functions of insulin on cell survival and anti-apoptosis [411, 412]. Notably, reduced cell survival and increased apoptosis have been described as hallmark effects of hypoxia treatment in some earlier studies [294, 310, 386] but not all [315, 382]. This discrepancy may be explained by differences in cell culture O_2 concentrations. In conclusion, the aim of this study was to determine the effects of insulin on *HIF-1α*, *Col5a1* and *Col6a1* mRNA and protein expression in 3T3-L1 adipocytes and to determine whether these effects may be different under hypoxic vs. normoxic conditions. It was demonstrated that insulin was able to induce *HIF-1α*- but not *COL5A1* or *COL6A1,* mRNA and protein expression in the absence of hypoxia and that co-treatment did not result in an additive effect. Chronic hyperinsulinemia is characteristic of insulin resistance and the metabolic syndrome. Assuming that these *in-vitro* findings may be generalised, it is tempting to speculate that the expansion capacity of adipose tissue is reduced by i) hypoxia induced alterations to the pericellular basement membranes by induction of ECM expression around the adipocytes resulting in mechanical stress-induced inflammation and ii) by the exacerbation of the lipolytic effect of chronic hyperinsulinemia under hypoxic conditions resulting in higher circulating nonesterified FFAs and reduced insulin sensitivity.

CHAPTER SIX

ASSOCIATIONS BETWEEN *COL5A1* AND *COL6A1* GENE POLYMORPHISMS AND MEASURES OF BODY COMPOSITION AND INSULIN SENSITIVITY IN BLACK AND WHITE SOUTH AFRICAN WOMEN

6.1 INTRODUCTION

In this thesis (Chapter 2) and research from others [65, 366], it is argued that the excess generation and deposition of ECM components during excessive adipose tissue expansion in obesity may represent a mechanical limitation to adipose tissue expandability. However, factors which trigger the dysregulated ECM response that may lead to reduced adipose tissue insulin sensitivity are not known. As a structural tissue, adipose tissue protects organs and muscles from mechanical- and sheer stresses that are generated through movement. Similar to other connective tissues such as ligaments, tendons, fascia and bone [257], repeated mechanical loading may also result in alterations to the cellular components and ECM composition which would affect adipocyte physiology and metabolism in itself. Specifically, increasing adipose tissue mass during the development of obesity is indeed characterised by altered ECM expression and increased M1-activated macrophage infiltration (section 2.4.3).

The expression/remodelling of structural proteins in soft connective tissues are regulated by signalling cascades in response to external stimuli such as repetitive mechanical loading and is often reported as a risk factor for soft tissue injuries [257] such as those that occur during sporting activities that involve sudden deceleration or change in direction [262]. Although the mechanisms of these overuse injuries are poorly

understood, the expression of collagens, proteoglycans and/or glycoprotein content have been demonstrated to be altered in tendinopathy and ligament injuries [248]. The degenerative fibrotic changes in tendinopathy is suggested to be preceded by a chronic inflammatory state. [413, 414].

Results from previous population studies published online in the NCBI database have shown significant differences in *COL5A1* (rs12722) and *COL6A1* rs35796750 allele frequencies between Europeans and populations of African or East Asian descent. The ancestral C- allele and the minor T- allele of the *COL5A1* (rs12722) were reported to be equally distributed (45%-55%) in European populations whilst in the African and Asian populations, the C- allele was found to be more frequent (77% and 86%, respectively) than the T- allele (14 and 22%, respectively). In Europeans and East Asian populations, the T- allele of *COL6A1* rs35796750 was more frequent than the C- allele (60% and 72%, respectively) whilst in the African populations, the C- allele was more frequent that the T- allele (67% vs 33%). (www.ncbi.nlm.nih.gov, accessed April 2017).

Although these polymorphisms and their associated risk factors have been extensively studied for their associations with increased risk for developing soft tissue injury, to my knowledge, no such studies have been undertaken to determine whether associations exist between these polymorphisms and measures of adipose tissue fat mass and its distribution and/or insulin resistance that may be implicated in the risk of T2DM and cardiovascular disease. Given the similarities of ECM expression in adipose- and other soft connective tissues during their respective adaptive responses to mechanical stresses and the functional relevance of these two polymorphisms on the development of connective tissue diseases, it is hypothesised that the *COL5A1* rs12722 and *COL6A1* rs35796750 polymorphisms are associated with differences in

body fat mass and its distribution in black and white women, and consequently their differences in risk for insulin resistance. Thus, the aims of this study are to i) examine differences in the frequencies of the rs12722 polymorphism within the *COL5A1* gene and the rs35796750 polymorphism within the *COL6A1* gene between black and white South African women, and to ii) examine the ethnic-specific associations with body fat, its distribution and insulin resistance.

6.2 MATERIALS AND METHODS

6.2.1 Participants

A sample of 229 black and 188 white apparently healthy premenopausal South African women between the ages of 18 to 49 years were recruited from community centres, universities and church groups. Inclusion criteria were: no previous diagnosis of, or not taking chronic medication for T2DM, hypertension, HIV, or other metabolic diseases; and not currently pregnant or lactating. Ethics approval was obtained from the Human Research Ethics Committee of the Faculty of Health Science at the University of Cape Town and written informed consent was obtained from all participants prior to testing.

6.2.2 Testing procedures

6.2.2.1 Body composition assessment

All testing procedures, including assessment of body composition (BMI and DXA) and body fat distribution (waist circumference and CT) have been described in Chapter 3.

6.2.2.2 Insulin resistance (IR)

Blood samples were collected after an overnight fast for determination of fasting plasma glucose and serum insulin concentration as described in Chapter 3. These

values were used to estimate homeostatic model of assessment of insulin resistance (HOMA-IR) using an online computer model available from (http://www.dtu.ox.ac.uk/homacalculator/) [415]. For simplicity, this thesis uses the term HOMA-IR as a proxy for the frequently used term "HOMA2-IR" (which is mainly used to differentiate this algorithmically-adjusted estimation of insulin resistance from its predecessor, HOMA1-IR [415]).

6.2.3 Genotyping Analysis

The two polymorphisms (*COL5A1* rs12722 and *COL6A1* rs35796750) were selected based on their functional relevance in soft tissue injuries [272, 277], position in and around the gene and usage in previous studies [269, 416, 417]. *COL5A1* rs12722, (C/T) B*st*U1 restriction fragment length polymorphism (RFLP) within the *COL5A1* 3′-UTR was polymerase chain reaction (PCR) amplified according to a method described by Greenspan and Pasquinelli [418] and modified by Mokone et al.[267]. Each PCR reaction was performed in a final volume of 60 µl containing at least 100 ng DNA, 20 pmol of each primer (forward, 5'- GAA GAC GTT TCT GGA GGA TC-3' and reverse, 5'-GAA GGC ACC TGC AGA ATG AC-3'), 20 mM Tris-HCl (pH8.4), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM of each dNTP (dATP, dTTP, dCTP and dGTP), and 2.5 units of Taq polymerase (New England Biolabs, Ipswitch, Massachusetts, USA). PCR conditions were as follows: 3 min initial denaturing step at 94˚C followed by 35 cycles of 1 min denaturing at 94˚C, 1 min annealing at 53˚C and 1.5 min extension of the strand at 72˚C, and the final extension step for 8 min at 72˚C (Thermal cycler, Hybaid, PCR Express, Middlesex, UK). The 667-bp PCR products were digested with the restriction endonucleases BstUI, to produce 351-bp and 316-bp fragments for the T- allele and 316-bp and 271-bp and 80-bp fragments for the C- allele (Figure 6.1 A). The resultant fragments were separated on 6 % non-denaturing poly-acrylamide gels, together with

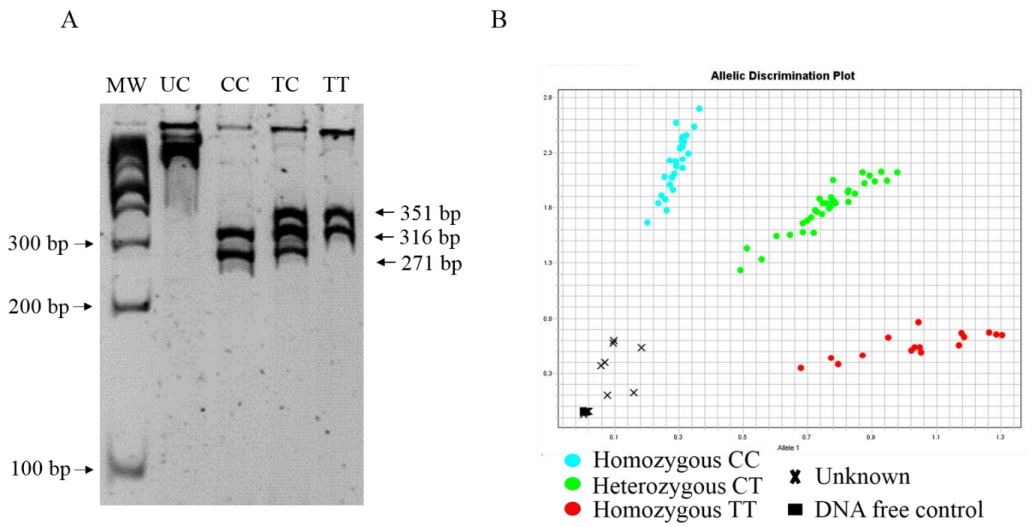


Figure 6.1Genotyping COL5A1 rs12722 and COL6A1 rs35796750.
Typical representation of **A**) 6% non-denaturing poly-acrylamide gel showing the genotypes of COL5A1 BstU1 RFLP. Digestion of the 667-bp PCR product with BstU1produced 351-bp and 316-bp fragments for the T-allele and 316-bp and 271-bp and 80-bp fragments for the C-allele. Left lane contains the 100-bp molecular weight marker (MW) with appropriate fragment sizes indicated. The second lane contains the 667-bp uncut (UC) fragment, followed by CC-, TC-and TT-genotypes in the lanes thereafter and **B**) Allelic discrimination plot using Taqman® genotyping assay for *COL6A1* rs35796750 (C/T) on the StepOnePlus™ Real-time PCR system

a 100-bp molecular weight marker, and were visualized under UV light with Ethidium Bromide nucleic acid gel stain. *COL6A1* rs35796750 (C/T) was genotyped using custom designed Fluorescence-based Taqman® PCR assay probes and flanking primer sets (Assay ID: AH89JPA, Applied Biosystems, Foster City, CA, USA), and used along with a pre-made PCR mastermix containing ampliTaq® DNA polymerase Gold (Applied Biosystems, Foster City, CA, USA) in a final reaction volume of 8 µl. The PCR conditions were as follows: a 10 min heat activation step at 92ºC followed

by 40 cycles of 15 sec at 92ºC and 1 min at 60ºC. The PCR reactions were performed on an Applied Biosystems StepOne Plus™ Real-Time PCR system (Life technologies, Applied Biosystems, Foster City, CA, USA), using the Applied Biosystems StepOnePlus™ Real-Time PCR software Version 2.1 (Applied Biosystems, Foster City, CA, USA) (Figure 6.1 B).

6.2.4 Statistical Analysis

Values are presented as median and inter-quartile range. Where appropriate, skewed data (VAT (cm^2), total SAT (cm^2), SSAT, fasted insulin and HOMA-IR) were log transformed. Normal data are presented as mean and SD and non-normal data as median and interquartile range. If a parametric test was performed with non-normally distributed variables, Levene's test of variation was used to determine homogeneity of continuous data. Chi-squared (χ^2) tests were used to determine significant differences in genotype and allele frequency distributions between black and white women. In order to determine whether body fat mass, fat distribution and measures of fasting glycaemia by genotype/allele categories were different in black and white women, a two-way ANCOVA was performed that included an ethnicity × genotype interaction term, adjusting for age (or age and fat mass), with Fisher least significant difference (LSD) *post hoc* analysis. In order to determine statistical power, *post hoc* power calculations were conducted in black and white women to determine the power to detect a significant interaction term between genotype and ethnicity on HOMA-IR. This was conducted for both *COL5A1* rs12722- and *COL6A1* rs35796750 polymorphisms using G*Power software V.1.2.4 ((http://www. gpower.hhu.de/) using an alpha of 0.05 and effect size (Cohen's f^2) [419] of 0.32-0.65 (HOMA-IR), and a sample size of 117-196 (Supplementary Table 6.1. 1). Based on these calculations, the sample size of used in this study was found to have 99.9 % power to detect a significant interaction between genotype and ethnicity on HOMA-IR values. Tests for Hardy-Weinberg proportions (HWE) were performed using Gene-POP web version 4.0.10. (http://genepop.curtin.edu.au/). Data were analysed using STATISTICA version 13 (Statsoft Inc., Tulsa OK, USA) and GraphPad Prism (version 5, GraphPad Software, San Diego, CA, USA).

6.3 RESULTS

6.3.1 Participant characteristics

Body composition and fasting glycaemia in black and white women are shown in Table 6.1. Black women were significantly younger, had a higher BMI and body fat mass (kg and %). However, as a percentage of total fat mass (%fm), black women had less central fat mass (%fm) but greater leg fat mass (%fm) compared to white women. Black women had greater total SAT (cm^2) and SSAT, but similar DSAT (cm^2) and VAT (cm^2) compared to the white women. However, when adjusting for differences in total fat mass (kg) between black and white women, VAT (cm^2) and DSAT (cm^2) were lower and SSAT (cm^2) was higher in the black women but total SAT (cm^2) was similar between ethnicities.

Black women had lower fasting glucose, higher fasting insulin but similar HOMA-IR values compared to the white women independent of differences in body fat mass between black and white women.

6.3.2 Genotype distribution and allele frequency of *COL5A1* rs12722 and *COL6A1* rs35796750 polymorphisms in black and white women

There were significant differences in the genotype distributions and allele frequencies between black and white women for the *COL5A1* rs12722 and *COL6A1* rs35796750 polymorphisms (Figure 6.2). Specifically, the T- allele for *COL5A1* rs12722 was significantly more frequent in white compared to black women (44% vs 13%, respectively). Accordingly, the genotype distribution for *COL5A1* rs12722 in white women (CC- genotype 35%, TC- genotype 42% and TT- genotype 23%) was significantly different to the genotype distribution of the black women (CC- genotype 76%, TC- genotype 22% and TT- genotype 2%) (black vs white, χ^2=71.88, p<0.001).

Table 6.1 Participant characteristics of white and black women

	White		Black		
	n		n		p-value
Age (years)	188	31.7 ± 8	229	27.3 ± 7.5	<0.001
Body composition					[a] p-value
BMI (kg/m²)	186	27.8 ± 6.6	226	30.2 ± 8.1	<0.001
Waist (cm)	186	90.1 ± 15.1	225	90 ± 18.2	0.073
Body fat mass (%)	185	35.5 ± 8.8	210	38.7 ± 8.9	<0.001
Central fat mass (%fm)	185	47.8 ± 5.3	210	43.6 ± 5.3	<0.001
Leg fat mass (%fm)	185	41.2 ± 6	210	45.8 ± 6.5	<0.001
VAT (cm²)	114	80.4 (60 - 124)	135	66.0 (45 - 102)	0.142
Total SAT (cm²)	110	290.8 (180 - 455)	132	340.7 (182 - 554)	0.002
SSAT (cm²)	140	145.0 (86 - 235)	166	216.4 (117 - 320)	<0.001
DSAT (cm²)	140	155.0 ± 93.6	166	165.2 ± 114.3	0.112
Fasting glycaemia					[b] p-value
Fasting glucose (mmol/l)	173	4.7 ± 0.4	212	4.4 ± 0.5	<0.001
Fasting Insulin (mU/l)	174	6.9 (5.1 - 10.5)	217	8.6 (5.0 – 15.6)	0.003
HOMA-IR	173	0.9 (0.6 - 1.3)	213	1.0 (0.6 - 1.7)	0.532

Values are mean ± SD or median (interquartile range). [a] p-values for body composition valuables adjusted for age; [b] p-values for fasting glucose, fasting insulin and HOMA-IR are adjusted for age and body fat mass (kg). BMI, body mass index; DSAT deep subcutaneous adipose tissue; SSAT, superficial subcutaneous adipose tissue; VAT, visceral adipose tissue; % fm, a percentage of fat mass; HOMA-IR, homeostatic model of assessment of insulin resistance

Because of the rarity of the TT- genotype in the *COL5A1* rs12722 polymorphism in black women (2%), the TT- and TC- genotypes, were combined in all subsequent analysis.

The frequency of the T- allele of *COL6A1* rs35796750 was higher in the white women compared to the black women (59% vs 30%, respectively). Genotype distribution for *COL6A1* rs35796750 in white women (CC- genotype 20%, TC- genotype 43% and TT- genotype 37%) was significantly different to the genotype distribution of the black women (CC- genotype 49%, TC- genotype 41% and TT- genotype 10%) (black vs. white, χ^2 =59.07, p<0.001). The genotype distributions were in Hardy-Weinberg equilibrium for *COL5A1* rs12722 (HWE, p=0.49 and p=1.00) and *COL6A1* rs35796750

(HWE, p=0.73 and p=0.35) polymorphisms, in both black and white women, respectively.

6.3.3 Differences in body composition and fat distribution and fasting glycaemia in *COL5A1* rs12722 and *COL6A1* rs35796750 polymorphisms in black and white women

When body fatness and body composition measures were compared between CC- and TC + TT genotype groups of the *COL5A1* rs12722 polymorphism in black and white women (Table 6.2), participants with the T- allele had a lower BMI, lower body fat mass (kg) and less central fat mass characterised by smaller waist circumference (cm^2), central fat mass (%fm), VAT and DSAT, compared to those in the CC-genotype, irrespective of ethnicity (allele effect, $p< 0.05$). Differences in waist circumference and VAT mass between CC- and TC + TT genotype groups were independent of differences in body fat mass in both black and white women. In addition, participants carrying the T- allele had lower insulin resistance, characterised by lower HOMA-IR and fasting insulin concentrations, but similar fasting glucose concentrations (allele effect, $p< 0.05$). These relationships remained significant after adjusting for differences in body fat mass.

Post-hoc tests indicated that waist circumference (cm) and VAT (cm^2) was significantly different between CC- and TC + TT groups in both the black and white women, whereas fasted insulin levels and HOMA-IR were lower in the TC + TT group of the white women only. Despite ethnic differences in body fatness and body fat distribution and genotype/allele frequencies between black and white women, the associations between genotype and body comp or HOMA-IR did not differ by ethnicity (no genotype x ethnicity interactions).

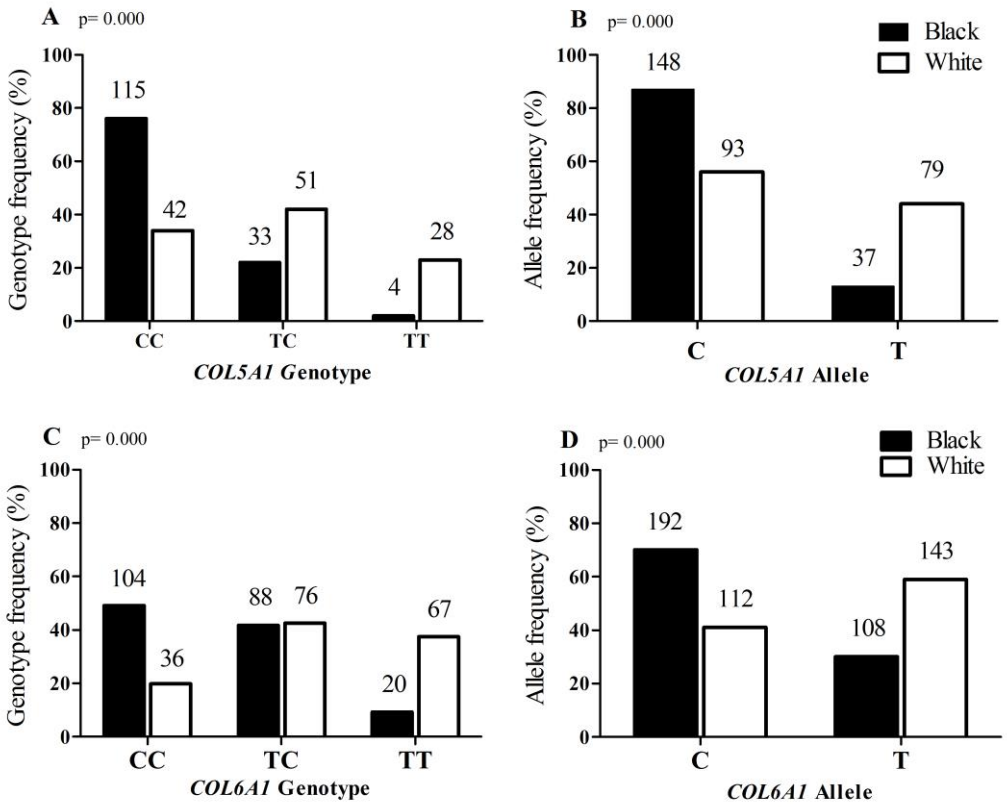


Figure 6.2 Genotype distribution and allele frequency of *COL5A1* (rs12722) and *COL6A1* (rs35796750) polymorphisms in black and white women.

Top: genotype distributions A) and allele frequencies B) of COL5A1 rs12722 (C/T) polymorphisms in black and white women. Because of the small sample size of the TT-genotype in the black women, the CC-genotype was compared with the combined CT- and TT- genotype groups in subsequent analyses. Bottom: Genotype distributions C) and allele frequencies D) of COL6A1 rs35796750 (C/T) polymorphisms in black and white women. The number of participants in each group is indicated. P-value for χ^2 shown

There were no significant associations between *COL6A1* rs35796750 and any of the body fat mass or body fat mass distribution parameters or measures of fasting glycaemia or insulin resistance in black and white women (Table 6.3).

Table 6.2 Body composition and fasting glycaemia according to *COL5A1* rs12722 C/T genotype in black and white women

	White women				Black women						
	CC		CT+TT		CC		CT+TT		p-value		
	n		n		n		n		Genotype	Ethnicity	G × E
Age (years)	42	30 ± 8.4	79	31.5 ± 7.3	115	24 (21 - 31)	37	24 (21 - 28)	0.81	<0.01	0.19
Body composition									[a] p-value		
BMI (kg/m²)	42	29 ± 7.2	79	26.8 ± 5.6	113	30.3 ± 8.2	37	27.8 ± 6.8	0.01	0.01	0.77
Waist (cm)	42	93.1 ± 16.5	79	87.8 ± 13*	112	91.3 ± 19.5	37	84.4 ± 15.7*	<0.01	0.88	0.93
Body fm (%)	42	37.5 ± 9.3	79	35 ± 8.7	105	38.7 ± 8.9	36	36.9 ± 7.9	0.06	0.02	0.55
Body fm (kg)	42	30.9 ± 13.7	79	27.1 ± 12.3	105	30.9 ± 14.6	36	26.6 ± 12.1	0.02	0.27	0.88
Central fm (%fm)	42	47.9 ± 5.8	79	46.8 ± 4.9	105	44.1 ± 5.4	36	42.6 ± 4.4	0.05	<0.01	0.95
Leg fm (%fm)	42	41.1 ± 5.9	79	42.4 ± 5.7	105	45.5 ± 6.6	36	46.6 ± 5.7	0.10	<0.01	0.62
VAT (cm²)	30	88.0 (58 - 153)	51	77.2 (57 - 104) *	72	67.8 (52 - 102)	28	57.0 (42 - 77)*	0.02	0.13	0.55
Tot SAT (cm²)	30	313.0 (180 - 535)	49	297.0 (174 - 404)	69	380.8 (159 - 588)	28	325.7 (178 - 542)	0.26	0.05	0.59
SSAT (cm²)	37	182.0 (90 - 270)	63	152.3 (77 - 216)	90	230.8 (118 - 327)	29	145.5 (108 - 300)	0.22	<0.01	0.48
DSAT (cm²)	37	170.9 ± 106.5	63	144.8 ± 81.7	90	175.9 ± 118.5	29	135.6 ± 89.5	0.02	0.42	0.83
Fasting glycaemia									[b] p-value		
Glucose (mmol/l)	42	4.7 ± 0.4	75	4.6 ± 0.3	114	4.4 ± 0.5	35	4.4 ± 0.5	0.90	<0.01	0.25
Insulin (mU/l)	42	10.1 (5.6 – 18.0)	75	6.3 (4.9 – 8.4) *	114	9.9 (5.4 – 16.9)	37	8.4 (5.1 – 12.7)	0.01	0.05	0.25
HOMA-IR	42	1.2 (0.7 – 2.1)	75	0.8 (0.7 - 1.2) *	114	1.1 (0.6 - 1.8)	35	1.0 (0.6 - 1.3)	0.01	0.87	0.13

Values are mean ± SD or median (interquartile range); [a] p-values for body composition valuables adjusted for age; [b] p-values for fasting glucose, fasting insulin and HOMA-IR are adjusted for age and body fat mass (kg). DSAT, deep subcutaneous adipose tissue; SSAT, superficial subcutaneous adipose issue; VAT, visceral adipose tissue; % fm, a percentage of fat mass, HOMA-IR, homeostatic model of assessment of insulin resistance. *, $p < 0.05$ from *post-hoc* analysis.

Table 6.3 Body composition and fasting glycaemia according to *COL6A1* rs35796750 T/C genotype in black and white women

	White women						Black women								
	CC		CT		TT		CC		CT		TT		p-value		
	n		n		n		n		n		n		Genotype	Ethnicity	G *E
Age (years)	36	29.3 ± 6.5	76	32.8 ± 8.7	67	32 ± 7.8	104	27.8 ± 7.6	88	27.4 ± 7.8	20	25.4 ± 6.7	0.20	<0.01	0.06
Body composition														[a] p-value	
BMI (kg/m2)	36	28.5 ± 7.6	75	27.8 ± 6.3	66	27 ± 6.2	103	30.9 ± 7.8	87	29.9 ± 8.2	20	28.8 ± 8.7	0.19	<0.01	0.84
Waist (cm)	36	90.4 ± 16.9	75	90.7 ± 15.3	66	88.8 ± 14	102	91.3 ± 16.5	87	88.6 ± 17.9	20	90 ± 24	0.52	0.11	0.66
Body fm (%)	36	36.4 ± 9.6	75	35.3 ± 8.7	65	34.8 ± 8.5	96	39.9 ± 8.1	79	38.1 ± 9.1	18	35.4 ± 11.1	0.08	<0.01	0.86
Cent fm (%fm)	36	30.3 ± 15.3	75	28.1 ± 12.5	65	27 ± 12.4	96	32.4 ± 14.2	79	29.9 ± 14.7	18	27.3 ± 17.1	0.90	<0.01	0.22
Leg fm (%)	36	46.7 ± 4.8	75	48.6 ± 5.8	65	47.6 ± 4.7	96	44.1 ± 5	79	43.0 ± 5.4	18	42.4 ± 6	0.67	<0.01	0.13
VAT (cm^2)	15	75.7 (61 - 104)	49	90.2 (61 - 146)	45	82.8 (59 - 111)	63	70.2 (48 - 90)	52	61.0 (44 - 94)	10	59.9 (41 - 80)	0.56	0.03	0.22
Tot SAT (cm^2)	15	372.1 (196 - 497)	49	333.2 (167 - 450)	44	264.4 (182 - 423)	61	339.6 (190 - 560)	52	395.7 (192 - 549)	10	208.0 (144 - 517)	0.54	0.12	0.63
SSAT (cm^2)	26	182.1 (93 - 290)	55	152.3 (83 - 230)	53	143.6 (79 - 238)	78	200.9 (120 - 309)	63	258.1 (120 - 337)	15	136.5 (87 - 322)	0.51	<0.01	0.18
DSAT (cm^2)	26	161.8 ± 95.8	55	162.1 ± 100.6	53	145.7 ± 86.8	78	161.4 ± 102.6	63	173.5 ± 117.1	15	153.2 ± 150.8	0.72	0.11	0.59
Fasting glycaemia														[b] p-value	
Glucose (mmol/l)	35	4.6 ± 0.3	68	4.7 ± 0.4	63	4.6 ± 0.3	97	4.4 ± 0.5	80	4.4 ± 0.5	19	4.3 ± 0.4	0.11	<0.01	0.36
Insulin (mU/l)	35	6.2 (4.2 - 10.5)	68	7.3 (5.6 - 11.1)	64	6.4 (4.5 - 11.6)	100	9.3 (5.0 - 16.0)	82	8.0 (5.0 - 15.5)	19	7.2 (5.2 - 15.3)	0.72	0.23	0.08
HOMA-IR	35	0.8 (0.5 - 1.3)	68	0.9 (0.7 - 1.4)	63	0.8 (0.6 - 1.5)	96	1.2 (0.6 - 1.8)	81	0.9 (0.6 - 1.7)	19	0.8 (0.6 - 1.3)	0.31	0.47	0.06

Values are mean ± SD or median (interquartile range). [a] p-values for body composition valuables adjusted for age; [b] p-values for fasting glucose, fasting insulin and HOMA-IR are adjusted for age and body fat mass (kg). DSAT deep subcutaneous adipose tissue; SSAT, superficial subcutaneous adipose issue; VAT, visceral adipose tissue; % fm, a percentage of fat mass, HOMA-IR, homeostatic model of assessment of insulin resistance. *, $p < 0.05$ for *post-hoc* analysis.

6.4 DISCUSSION

The main findings were that although allele and genotype distributions of the *COL5A1* rs12722 polymorphism, as well as body fat distribution were significantly different between black and white women, the T- variant of the *COL5A1* rs12722 polymorphism was associated with significantly less central fat mass, characterised by a smaller waist circumference and lower VAT, and this effect was independent of ethnicity and overall body fat mass. In addition, T- variant of the *COL5A1* rs12722 polymorphism was associated with lower fasted insulin concentrations and HOMA-IR in white but not in black women. In contrast, no genotype associations between *COL6A1* rs35796750 and any of the body fat mass, its distribution and insulin resistance measures in black or white women were reported.

Numerous studies have investigated *COL5A1* rs12722 for its role in musculoskeletal soft tissue pathologies including Achilles Tendinopathy (AT) [267, 268], carpal tunnel syndrome (CTS) [258] and exercise associated muscle cramping [420], where the T-allele has been associated with higher risk of developing these conditions. This *COL5A1* 3′ UTR rs12722 polymorphism, among others within this region [421], has been hypothesised to alter *COL5A1* mRNA stability and thereby lead to increased α1 (V) chain production and by implication, type V collagen accumulation [272]. As collagen type V regulates fibril diameter and packing density of collagen type I and III [422], it is reasonable to assume that this polymorphism may alter the mechanical properties of the connective tissues it functions in. Indeed, patients with cEDS have mutations within the *COL5A1* gene that lead to significantly reduced type V collagen content in connective tissues and present with structural tendon pathology and low tendon stiffness [423]. Conversely, individuals who carry the *COL5A1* rs12722 TT- genotype and present with a higher incidence of tendon pathology and increased injury risk, have

higher connective tissue stiffness compared to individuals who carry at least one copy of the C- allele [422]. Although an exact mechanism whereby increased type V collagen may influence adipose tissue metabolism has not been identified, it could be hypothesised that the T- allele of *COL5A1* rs12722 polymorphism that associates with increased tensile strength of the collagen network in tendons and ligaments, may represent a mechanical limitation to adipose tissue expandability with a commensurate effect on body fat distribution. As each adipocyte is surrounded by a pericellular basement membrane, it might also be reasonable to suggest that increased tensile strength of the basement-membrane collagen-components may limit the lipid storage capacity of the adipocyte itself, resulting in smaller, more insulin sensitive adipocytes distributed within the depot. As the results of this study show an association between TT +TG- genotype groups and a propensity for smaller waist circumference and a lower VAT compared to individuals with the CC-genotype, it may be hypothesised that the T- allele of the *COL5A1* polymorphism may influence body fat distribution through limiting lipid storage capacity in VAT depot. In support of this finding, Minchin *et al* recently showed that VAT-specific increased collagen type V expression in *Plexin D1*(plxnd1)- deficient zebra-fish resulted in altered morphology of VAT adipocytes, leading to a reduced VAT:SAT ratio and better glucose tolerance [424]. As type V collagens are expressed in differentiating adipocytes and have effects on hyperplasia [246], these results suggest a role for *COL5A1* in hyperplastic adipocyte morphology. According to the adipose tissue expansion capacity hypothesis [58, 59], the establishment of an ECM microenvironment conducive to adipocyte hyperplasia would provide protection against the negative consequences of adipocyte hypertrophy such as increased inflammatory response and the exacerbating effects on insulin resistance. Indeed, women with the T- allele of *COL5A1* had lower insulin resistance independent

of differences in body fat mass. Surprisingly, the associations between *COL5A1* genotype measures of insulin sensitivity were significant only in the cohort of white women and suggests that other factors may also be involved. Conversely, this finding may also be explained by the rarity of the T- allele in the black women. Further studies that include higher TT-genotype representation in ethnically diverse populations are needed to test the hypothesis that alterations to the collagen-fibre mechanical properties, as a result of *COL5A1* (rs12722) genotype, modulate body composition and insulin sensitivity. Further, the effects of ECM expression on pre-adipocyte hyperplasia and its relationship with adipose tissue fibrosis and the development of insulin resistance requires further investigation.

As COL6A1 is also one of the major adipocyte secretary proteins, and sequence variation in this gene is associated with multiple multifactorial conditions, this study investigated whether genetic variation in the *COL6A1* rs35796750 polymorphism may be associated with differences in body fat mass, its distribution and measures of fasting glycaemia in black and white South African women. In contrast to the *COL5A1* rs12722 polymorphism, there were no significant associations between *COL6A1* rs35796750 genotypes and body fat and its distribution or measures of fasting glycaemia in either black or white women. Results from Chapter 3 and 4 of this thesis demonstrated that *COL6A1* expression in the gluteal depot of black women associated with obesity and their higher insulin resistance. Both glucose intolerance and high BMI have been shown to be significant risk factors for the development of OPLL in two distinct Japanese populations where previous genome wide linkage studies have identified a T→C substitution at intron 32 [-29] (rs35796750) in *COL6A1* that occurs near the branch site of the intron as the most strongly associated with the development of OPLL [274] [273, 276]. These results indicate that the *COL6A1* (rs35796750)

polymorphism may not have significant effects on the Collagen VI expression levels between black and white women.

This novel study was the first to investigate the associations between *COL5A1* and *COL6A1* polymorphisms and body fat, its distribution and insulin resistance in black and white women. However, it is important to keep in mind that there are limitations to this study. Firstly, the use of a candidate gene approach, where only one polymorphism within each of *COL5A1* and *COL6A1* genes were investigated for their associations with body fat mass, its distribution and insulin resistance in South African women instead of a genome wide association approach (GWA) may represent a limitation to this study. However, GWA studies require large numbers, are expensive to undertake and may not cover all the regions of the genome. In addition, ethnic populations such as those of African descent have been shown to have differences in disease-allele frequency and linkage (LD) patterns, differences in phenotypic prevalence rates, differences in effect sizes and differences in rare variants compared to populations of European decent [425]. Although candidate gene association studies have identified several diverse risk factors for the development of insulin resistance, limited data is available for black South Africans. Moreover, despite the strong phenotypic relevance of these two polymorphisms in connective tissue biology, to my knowledge, these polymorphisms have not been previously investigated for the effects on adipose tissue biology and its possible link with insulin resistance in populations of either African or European descent. However, the relationships identified in this study warrant further investigation in larger multi-ethnic cohorts. Another limitation of this study was that HOMA-IR was used to estimate insulin resistance. Although HOMA-IR has been shown to correlate with the hyperinsulinemic-euglycemic clamp, the gold standard for assessing insulin sensitivity in white populations, the association is less

robust in a black population [426, 427], and therefore the results must be interpreted with caution. A strength of the study is the state-of-the-art measures of body composition (DXA) and body fat distribution (CT), in particular for the measure of VAT.

In conclusion, although no allele or genotype effects were identified for the *COL6A1* (rs35796750) polymorphism, this study has identified an association between the T-allele of the *COL5A1* rs12722 polymorphisms and lower central fat mass, in particular, less VAT, and lower insulin resistance. Further studies are required to verify these findings.

CHAPTER SEVEN

SUMMARY AND PERSPECTIVES

7.1 OVERVIEW OF RESEARCH QUESTION AND FINDINGS

The mechanisms underlying the higher prevalence of T2DM in black African women compared to white women are currently being examined. Studies in South Africa [22, 23] and the USA [109] have shown that the prevalence of insulin resistance, a major risk factor for T2DM, is higher in black compared to white women. Increased insulin resistance in black women is associated with an appropriately greater insulin response to maintain normoglycaemia [57, 428]. The resultant hyperinsulinaemia in these women are partly due to increased insulin secretion from β-cells and reduced hepatic insulin clearance [94 51, 405]. This compensation to higher insulin resistance has been proposed as a potential protective mechanism whereby black Africans can conserve β-cell function during prolonged exposure to insulin resistance, although it does not protect them from T2DM [15].

One of the major determinants of insulin resistance is the centralization of body fat, specifically, increased VAT or intra-abdominal adipose tissue, while the accumulation of peripheral adipose tissue (gluteal/femoral) is considered to be protective (section 2.2). However, contrary to the prevailing hypothesis, black women have less VAT and more SAT but are more insulin resistant compared to their white counterparts [57, 104]. Subsequently, studies have shown that the correlation of VAT with insulin resistance in black women was not as strong as that shown in white women but was more closely correlated with abdominal SAT, specifically the DSAT depot [23, 40, 104]. Moreover, lower-

body fat distribution in African women does not seem to confer similar protective effects as shown in larger cohorts of white women who have lower-body fat distribution [47, 105]. This suggests that the relationship between body fat distribution and the risk of insulin resistance may be ethnic-specific.

Although the exact mechanisms are still unclear, an increasing amount of evidence is showing that the differential relationships of body fat mass, its distribution and systemic insulin resistance has its origin in adipose tissue biology. In order to identify putative mechanisms that may explain the different insulin resistance phenotypes described in black and white women, the notion of *adipose tissue expandability* in relation to a model of insulin sensitive vs insulin resistant adipose tissue expansion was proposed (section 2.5). This model incorporated three major factors that may influence an insulin resistant (left) or insulin sensitive (right) mode of adipose tissue expansion:

(1) reduced adipogenic capacity (section 2.4.1),

(2) insufficient and inappropriate vascularization (section 2.4.2) and

(3) aberrant ECM remodelling (section 2.4.3).

Impaired/reduced adipogenic capacity in adipose tissue results in the partitioning of excess lipid into existing adipocytes and consequently results in the development of enlarged, insulin resistant adipocytes [429]. The increased cell diameter of hypertrophic adipocytes has been proposed to increase the distance that oxygen has to diffuse from the vasculature to distal adipocytes [221] resulting in clusters of adipocytes becoming relatively hypoxic [69]. Adipocytes respond to hypoxia by increased production and stabilization of *HIF-1α*, a transcriptional activator of pro-angiogenic genes such as *VEGFα*, with the aim of inducing neovascularization in order to restore

oxygen delivery to the adipose tissue. During obesity, however, chronically increased *HIF-1α* in adipose tissue is associated with the increased expression and deposition of ECM and lower angiogenic gene expression [307]. Adipose tissue fibrosis, through its mechanical constraints on adipocyte expansion, is one of the main triggers for the pro-inflammatory response [69] and concomitant insulin resistance that characterizes adipose tissue of obese humans [71]. Together, these factors constitute pathological SAT expansion, during which the development of metabolic defects in adipose tissue ultimately lead to higher systemic insulin resistance and higher risk of developing T2DM.

It is not known whether the expression of hypoxia and ECM genes may be differently expressed in black and white women. Previous studies have mostly been undertaken in Caucasian/white populations in whom the significant associations between body fat distribution variables and risk of insulin resistance relate almost exclusively to the levels of VAT- rather that SAT accumulation. In this context, this thesis hypothesised that the increased peripheral insulin resistance in black women may be a consequence of dysregulated gluteal SAT expansion as a function of higher adipose tissue hypoxia signalling, dysregulated ECM remodelling and higher pro-inflammatory (M1) macrophage infiltration which ultimately results from a lower TAG storage capacity in this depot with a concomitant redistribution of fat to the central depots.

Chapter 3

The first study in this thesis examined the hypothesis that ethnic-specific associations exist between insulin sensitivity measures and measures of body composition and the expression of ECM- and hypoxia-related genes in the SAT of black and white South African women. In addition, higher insulin resistance in black women may be associated with their higher insulin resistance compared to their white counterparts.

Given the reduced adipogenic capacity and greater adipocyte size observed in the gluteal SAT depot of black compared to white women [105], it was proposed that the gluteal SAT of black women has greater expression of ECM- and hypoxia-related genes than white women. Further, it was proposed that the expression of these genes differ between gluteal and abdominal SAT and differentially associated with insulin sensitivity in black and white women. Therefore, the primary aims of this study were to compare depot-specific expression of ECM and matrix remodelling components and genes relating to the cellular hypoxia response in normal-weight and obese black and white women, and to examine the ethnic-specific associations between these genes and measures of insulin sensitivity and secretion. The main findings of this study were that obese black women had higher hypoxia and ECM related gene expression in the gluteal SAT depot compared to the white women. Further, the expression of these genes was associated with higher insulin resistance in black, but not white women. These results suggest that the gluteal depot of black women may be more hypoxic than abdominal SAT and SAT of white women, which associates with lower insulin sensitivity in black women. Further, a secondary aim of this study was to investigate ethnic- and depot-specific associations between the expression of hypoxia and ECM related genes and the expression of a panel of inflammatory genes. The expression of the hypoxia and ECM genes associated with inflammatory gene expression in both the gluteal and abdominal SAT depots of black women, whereas the expression of these genes associated with the inflammatory gene expression mostly in the abdominal SAT depot of white women. Based on these findings, this study may suggest that reduced adipogenic capacity in the gluteal depot of black women may result in increased adipocyte hypertrophy, adipose tissue hypoxia and increased *HIF-1α* expression, which in turn induces a fibrotic and inflammatory response, rather than

a compensatory pro-angiogenic response. Only a few studies have investigated depot-specific expression of hypoxia and ECM components and the associations with insulin resistance. To my knowledge, this is the first study to show that the expression of these gene and their associations with reduced insulin sensitivity might also be ethnic-specific.

Chapter 4

Because of its distinct cellular and metabolic characteristics compared to abdominal SAT (i.e. lipid metabolism (Table 2.3), its adipo/cytokine production (Table 2.4) and structural composition (Table 2.5)), it has been proposed that the gluteal/femoral SAT depot may have a better long term lipid storage capacity and a weaker association with the development of systemic insulin resistance (section 2.3). However, during the development of obesity, adipose tissue hypoxia and increased fibrosis may influence body fat distribution patterns by lowering the storage capacity for excess lipid in the gluteal SAT depot with a resultant expansion of the abdominal fat depots (section 2.4). Based on the cross-sectional, ethnic-specific associations between hypoxia and ECM gene expression in the gluteal SAT depots and S_I shown in the previous chapter, this study hypothesised that over time, higher hypoxia and ECM related gene expression would associate with higher central fat mass accumulation in black women and that the expression of these genes would be associated with changes in the measures of insulin sensitivity in black women but not in white women. Thus, this longitudinal study aimed to determine whether changes in body composition and insulin sensitivity variables over a 5 year follow-up period associated with variations in hypoxia and ECM related gene expression in the gluteal SAT of black and white women. While previous cross-sectional studies have shown that black women may have a more favourable body fat distribution pattern compared to white women, this study showed that over

time, weight gain in the black women associated with a relative redistribution of body fat from their gluteal/femoral region to the central depots, highlighting that black women may be equally at risk of the metabolic abnormalities associated with the centralization of body fat mass with increasing age and fat mass. Importantly, increased fat accumulation associated with higher *PPARy* mRNA expression in the gluteal depot in white women, but not black women. This finding supports our previous finding that gluteal SAT of black women may have a lower adipogenic and lipogenic response to weight gain compared to that of equally obese white women. Further, to my knowledge, this is the first study to show that the changes in mRNA expression levels of hypoxia- and fibrosis-related genes (*HIF-1α, COL6A1* and *LOX*) correlated with changes in fasting plasma insulin concentrations and insulin secretion over time. From these results, it may be speculated that hyperinsulinaemia in black women may not simply be a consequence of increased insulin resistance but may also contribute to the exacerbation of insulin resistance during obesity development by inducing the expression of hypoxia- and fibrosis-related genes in adipose tissue. This notion, however, remains to be investigated. Moreover, in humans, changes to ECM expression in adipose tissue in response to changes in adipose tissue mass have only been investigated in response to extreme weight-loss interventions such as bariatric surgery or in response to short bouts of over- or under feeding (4 to 16 weeks). Less is known about the changes (if any) to structural components within adipose tissue after a period of free-living during which fluctuations in body fat mass and composition occur independent of an external intervention. In this study increased body fat mass associated with the reduced *COL5A1* mRNA expression in black women, whereas increased central fat mass associated with higher *COL5A1* expression in the white women. Previous research has shown that increased peri-adipocyte fibrosis

associated with less weight loss one year after bariatric surgery, and suggests that the ability to effectively remodel the ECM around the changing adipocyte diameter is essential for structural adaptation to changes in adipose tissue volume [327, 366] .

Chapter 5

Although the independent effects of hypoxia and insulin treatment on *HIF-1α* expression have been investigated, less is known about the concurrent effects of these conditions, such as those that may occur in adipose tissue of hyperinsulinaemic obese individuals. It is not clear whether high circulating insulin may directly increase *HIF-1α* expression and contribute to the formation of excess ECM, or whether increased insulin may simply be a concomitant downstream effect of increased insulin resistance, as a consequence of increased fibrosis and the generation of inflammation. Therefore, the third study in this thesis hypothesized that the expression of *HIF-1α, Col5a1* and *Col6a1* will increase in a dose-dependent manner in insulin-treated 3T3-L1 adipocytes under hypoxic- compared to normoxic conditions. The aims of this study were to determine the effects of chronic insulin treatment on *HIF-1α*, *Col5a1* and *Col6a1* mRNA and protein expression in 3T3-L1 adipocytes and to determine whether these effects were different during hypoxic vs. normoxic conditions. The main findings of this study were that chronic high insulin and hypoxia treatment significantly elevated *HIF-1α* mRNA and protein levels, but the observed effects were not additive. In addition, hypoxia, but not insulin treatment, was associated with higher *COL5A1* and *COL6A1* protein, but not mRNA expression. Further, exposure of adipocytes to 8 hrs insulin treatment resulted in significantly reduced lipid staining and the effect was exacerbated in the hypoxic compared to normoxic conditions. Chronic hyperinsulinemia is characteristic of insulin resistance and the metabolic syndrome. Assuming that these *in vitro* findings may be generalised, it is tempting to speculate

that the expansion capacity of adipose tissue is reduced by hypoxia-induced alterations to the pericellular basement membranes, a process also exacerbated by chronic hyperinsulinaemia during hypoxic conditions. Increased pericellular fibrosis would result in increased mechanical stress-induced inflammation, higher circulating nonesterified FFAs and reduced insulin sensitivity.

Chapter 6

Common genetic polymorphisms associated with the expression and remodelling of ECM components within soft connective tissue have previously been implicated in the development of connective tissue disorders (section 2.4.3). Higher susceptibility to these disorders is often dependent on the exposure to extrinsic risk factors such as repeated mechanical loading or overuse. The T-allele of *COL5A1* rs12722 has been associated with a higher risk for developing exercise-related musculoskeletal soft tissue pathologies (section 2.4.3), possibly due to effects on connective tissue stiffness. In contrast, the *COL6A1* rs 35796750 (T/C) SNP is strongly associated with OPLL and OLF, conditions characterised by ectopic bone formation in paravertebral ligaments, for which a high BMI after age 20 years, dietary habits and T2DM were independent risk factors for developing these disease states [274] [273], [276]. As in other structural connective tissues, adipose tissue ECM components and associated remodelling enzymes may also be regulated by signalling cascades in response to changes in mechanical and sheer stresses such as those associated with increased adipose tissue mass during obesity. In such a case, genetic variation associated with altered ECM and matrix remodelling gene expression in adipose tissue may result in ethnic-specific associations between ECM component gene expression and insulin resistance as described in chapters 3 and 4. Indeed, ethnic variation in the frequencies of the *COL5A1* rs12722 and *COL6A1* rs35796750 alleles have been such that the C-

allele for both were more frequent in the black/African population compared to Europeans. Although these polymorphisms have been extensively studied for their associations with increased risk for developing soft tissue injury, to my knowledge, no such studies have been undertaken to determine whether associations exist with measures of adipose tissue fat mass and its distribution and/or insulin resistance. Given the similarities of ECM expression in adipose and other soft connective tissues during their respective adaptive responses to mechanical stresses, and the functional relevance of these two polymorphisms for the development of connective tissue diseases, the fourth study in this thesis hypothesised that the *COL5A1* rs12722 and *COL6A1* rs35796750 polymorphisms were associated with differences in body fat mass and its distribution in black and white women and consequently their differences in risk for insulin resistance. Thus, the aims of this study were to examine differences in the frequencies of the rs12722 polymorphism within the *COL5A1* gene and the rs35796750 polymorphism within the *COL6A1* gene between black and white South African women and to examine the ethnic-specific associations with body fat, its distribution and insulin resistance. Although allele and genotype distributions of the *COL5A1* rs12722 polymorphism (CC- 35%, TC- 42% and TT- 23% in white women vs CC- 76%, TC- 22% and TT- 2% in black women), as well as body fat distribution were significantly different between black and white women, the T- variant of the *COL5A1* rs12722 polymorphism was associated with significantly less central fat mass, characterised by a smaller waist circumference and lower VAT, and that this effect was similar in black and white women. In addition, T- variant of the *COL5A1* rs12722 polymorphism was associated with lower fasted insulin concentrations and HOMA-IR in white, but not black women. This difference may also possibly be attributed to the significantly lower frequency of the T- variant of the *COL5A1* rs12722 polymorphism

in black women. In contrast, no genotype associations between *COL6A1* rs35796750 and body fat mass, its distribution and insulin resistance were reported in black or white women. Although no allele or genotype effects were identified for the *COL6A1* (rs35796750) polymorphism, this study identified a novel association between the T-allele of the C*OL5A1* rs12722 polymorphisms and lower central fat mass, in particular, less VAT, and lower insulin resistance. Further studies are required to verify these findings.

7.2 PROPOSED MODEL OF INSULIN SENSITIVE VS INSULIN RESISTANT ADIPOSE TISSUE EXPANSION IN BLACK AND WHITE WOMEN

The ethnic-specific associations of body fat mass, its distribution and the risk of insulin resistance between black and white women are not fully understood. In an attempt to identify putative mechanisms that could explain, at least in part, the different insulin sensitivity phenotypes described for the black and white women, the notion of *adipose tissue expandability* in relation to a model of insulin sensitive vs insulin resistant adipose tissue expansion was proposed. Thus, potential ethnic differences in the three major factors that could influence adipose tissue expansion in an insulin sensitive vs insulin resistant manner were investigated. These factors and their potential effects on insulin sensitivity are graphically represented in Figure 7.1 and summarised below:

(1) Cellular proliferation and differentiation capacity

During positive nutrient balance, excess energy is stored in SAT adipocytes by either the enlargement of the existing cell diameter (hypertrophy) and/or by the recruitment and differentiation of new adipocytes from the precursor cells from the SVF (hyperplasia). Increased *PPARγ* expression during obesity development is associated with the generation of smaller more insulin sensitive adipocytes (insulin sensitive AT

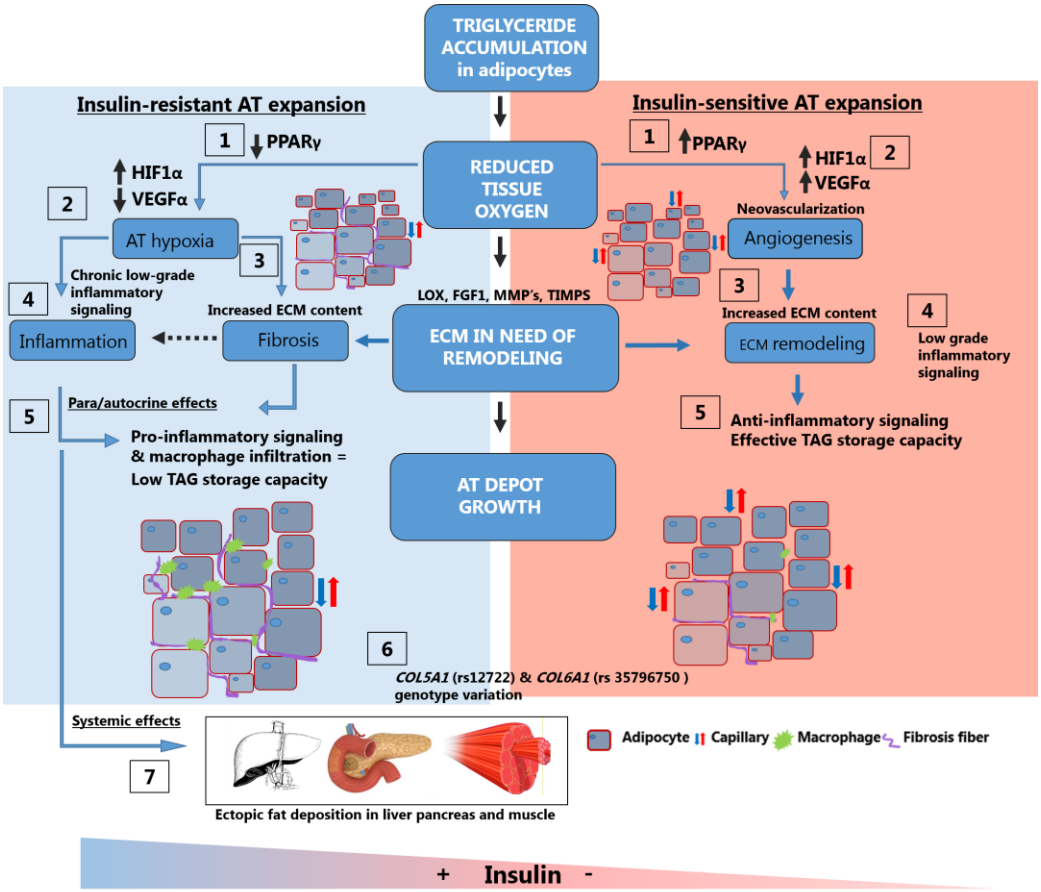


Figure 7.1 Model of insulin sensitive vs insulin resistant adipose tissue expansion. Insulin resistant adipose tissue expansion (Blue block to the left): Pathologic adipose tissue expansion results in local and systemic insulin resistance. Increased adipocyte cell size results in local hypoxic state in adipose tissue. Increased HIF-1α stabilization does not induce angiogenic response, but rather invokes an inflammatory state with a high degree of macrophage infiltration, limited vasculature development and fibrotic response. Such inflammatory activation ultimately results in systemic insulin resistance. Insulin sensitive adipose tissue expansion (**Red block to the right)**: Healthy, insulin sensitive adipose tissue expansion encompasses the enhanced recruitment of adipocyte progenitors that may differentiate into small insulin sensitive adipocytes, appropriate increased vascularization, minimal induction of ECM and minimal inflammation.

expansion, right panel of Figure 7.1) whereas adipose tissue expansion characterised by lower expression of *PPARγ* is associated with the enlarged, hypertrophic adipocytes which are more insulin resistant (insulin resistant AT expansion, left panel of figure 7.1). Results from our laboratory have shown lower *PPARγ* expression in the gluteal depot of obese black women compared to white women which suggests that differences may exists in adipocyte proliferation and differentiation capacity between black and white women.

(2) Adipose tissue PO_2 and vascularization

Increased cell diameter has been suggested to reduce adipose tissue PO_2 by increasing the distances that oxygen has to diffuse from the microvasculature to the cells causing a local hypoxic environment. Cellular adaptation to hypoxia involves the activation of the master regulator of the hypoxia response, *HIF-1α. HIF-1α* aims to induce angiogenesis through the expression of pro-angiogenic factors, such as *VEGFα,* in order to induce the formation of new vasculature in the adipose depot to quench the oxygen requirement of the expanding adipocytes. However, higher *HIF-1α* and reduced *VEGFα* expression in the gluteal depot of obese black women suggests increased adipose tissue hypoxia and reduced vascularization in this depot.

(3) Adipose tissue ECM expression

Increased *HIF-1α* activation, unable to elicit an effective pro-angiogenic response, results in the up-regulation of ECM components that underlie the formation of fibrosis in adipose tissue. Increased fibrosis may further reduce angiogenesis in the gluteal depot of black women by obstructing the formation of new vasculature in this depot. Notably, *HIF-1α* may also alter fibrosis through the induction of remodelling enzymes such as the collagen-crosslinking enzyme *LOX*.

(4) Inflammation

Hypoxia and ECM gene expression were positively correlated with higher expression of inflammatory genes in both the abdominal and gluteal SAT depots of black women, whereas hypoxia and ECM gene expression associated with inflammatory gene expression mostly in the abdominal depots of white women. Hypoxia, through the stabilisation of *HIF-1*α induces inflammatory gene expression from adipocytes and resident macrophages initially to induce angiogenesis and vasculature formation in adipose tissue in order to alleviate the effects of hypoxia.

(5) Limited adipose tissue expansion and insulin resistance

Increased fibrosis accompanied by reduced angiogenesis and inadequate vascularization perpetuates the unresolved hypoxic state, resulting in the continual influx of M1-activated macrophages and a sustained low-grade inflammation response. Thus, a chronic high inflammatory state (through its effects on adipocyte apoptosis and reduced insulin sensitivity) along with increased pericellular fibrosis (through its effect on limiting adipocyte size) may limit the TAG storage capacity of the gluteal SAT.

(6) Genetic factors

Differences in the allelic frequencies of *COL5A1* rs12722 and *COL6A1* rs35796750 polymorphisms between black and white women did not associate with ethnic differences in body fat mass, body composition, or variables of insulin sensitivity and secretion. However, the T- variant of the *COL5A1* rs12722 polymorphism was associated with significantly less central fat mass, characterised by a smaller waist circumference and lower VAT, and this effect was independent of ethnicity and overall body fat mass. In addition, T- variant of the *COL5A1* rs12722 polymorphism was associated with lower fasted insulin concentrations and HOMA-IR in white but not in black women. Although significant differences in allele frequencies were found in black and white women, genetic explanations for the phenotypic differences in body composition and disparity in metabolic disease risk between African and white populations remain to be elucidated.

(7) Hyperinsulinaemia

Finally, hyperinsulinaemia in black women may not simply be a consequence of increased adipose tissue insulin resistance but may also contribute to the exacerbation

of insulin resistance during obesity development by up-regulating the expression of *HIF-1α*, which may have exacerbating effects for ECM production as described in (3).

In summary, the model of insulin sensitive vs insulin resistant adipose tissue expansion is presented in an effort to explain (at least in part) the disproportionate prevalence of T2DM and insulin resistance in black African women compared to their white/Caucasian counterparts. Based on previous research, plausible adipose tissue specific mechanisms are suggested that may differently regulate body composition and fat distribution patterns and associations with insulin sensitivity in black and white women. It should be noted, however, that the studies in this thesis which were used to create this model are largely cross-sectional in nature and as such, no causality can be inferred. Further studies in a larger cohort of black and white women are needed to determine the validity of this model.

7.3 STERENGTHS AND LIMITATIONS

The role of adipose tissue biology in the risk of developing metabolic disease such as insulin resistance is now well established (section 2.2). However, most of the previously established relationships between body composition measures and adipose tissue biology leading to insulin resistance were identified from studies performed almost exclusively in Caucasian/white populations in whom higher levels of VAT explain the largest risk for insulin resistance. This contrasts to studies in African populations in whom the relationship between obesity and VAT and the associated risk for insulin resistance is attenuated. This thesis provides novel insight into the ethnic-specific associations between body fat mass, its distribution and measures of insulin sensitivity and secretion by presenting a novel hypothetical model that highlights plausible biological mechanisms which may explain, in part, the increased prevalence of insulin resistance in black women.

Additionally, three different methodological approaches were used to further investigate this model namely, a gene expression analysis in a cross-sectional (chapter 3) and follow-up (chapter 4) design, a cell-culture based study (chapter 5) and a genotyping analysis (chapter 6). Further, state-of-the-art techniques (DXA and CT) were used to provide accurate estimations of body composition and fat distribution in the black and white women who are known to have different levels of VAT and abdominal SAT at equal levels of obesity and waist circumference.

This thesis is the first to show ethnic differences in hypoxia and ECM related gene expression in three different SAT depots (SSAT, DSAT and gluteal SAT) and ethnic-specific associations between the expression of these genes and body composition variables and measures of insulin sensitivity and secretion in black and white women. Further, this study was the first to show changes in the expression levels of hypoxia and ECM related genes and the associations with changes in body composition variables and measures of insulin sensitivity and secretion over a 5 year, free-living follow-up period in black and white women.

The studies in this thesis also have some important limitations to consider. An important limitation may be that the generalizability of the findings are limited by the small sample size of the participants in the cohort (chapter 3), especially in the follow-up study (chapter 4). Therefore, no ethnic comparisons could be made for changes in hypoxia and ECM related gene expression over the follow-up period. However, these studies may be viewed as a pilot study from which some novel relationships have been identified and may be verified in future. Another limitation is that protein expression in the adipose tissue samples could not be measured due to the small size of the fat biopsies obtained (chapter 3 and 4). Moreover, I am aware that there exists a general belief that hypoxia does not affect *HIF-1α* mRNA levels in contrast to its protein level,

which is broadly accepted to be post-transcriptionally regulated by oxygen dependent protein stabilization complexes [430]. In contrast to *HIF-1α* protein expression which has consistently being shown to be higher in adipose tissue of obese humans and animals when compared to their normal-weight counterparts (for review see [67]), several studies have also shown increased mRNA levels in response to hypoxia in several cell types [307, 352, 388, 389]. Moreover, higher *HIF-1α* mRNA expression has been demonstrated to have relevant implications in adipose tissue metabolism (chapters 3 and 4) [301, 310, 370, 372, 390, 391] although the effect of these remain largely uninvestigated.

In addition, the magnitude of oxygen partial pressure in adipose tissue is currently the subject of debate. Studies showing reduced oxygen partial pressure in obese humans[70, 299-301] and animal models [72, 294, 310, 311] have recently been compared to a study showing increased oxygen partial pressure "hyperoxia" [309] in the SAT of obese compared to normal-weight individuals. However, similar to many other studies, PO_2 was only measured in one part of the adipose tissue depot and the authors conceded that the possibility exists that other parts of the same depot may still be hypoxic. Differences in PO_2 within and between adipose tissue depots may be explained by differences in blood flow delivery to these areas [309]. Further, factors such as age, gender, race, insulin sensitivity and adipose tissue depot volume may be confounding factors that affect PO_2 in different adipose tissue depots throughout the body. The contribution of these factors to the variability in PO_2 between lean and obese humans and its effects on adipocyte metabolism remains to be tested.

The cell-culture analysis (chapter 5) has some shortcomings to consider. Firstly, due to budget restrictions and a lack of laboratory resources, mRNA expression, protein analysis and lipid content determination assays were prioritized in this study whereas other metabolic analyses could not be performed. Future analyses may include for

example, carbohydrate metabolism assays (determination of 2-deoxy-[$_3$H]-D-glucose uptake) or intracellular ATP content determination. Secondly, the use of 5 % O_2 for hypoxia incubation of the 3T3-adipocytes rather than the more frequently used 1% O_2 may be viewed as a limitation to this study. As mentioned (Table 2.7), adipose tissue PO_2 has been recorded to range between 15-22 mm Hg, (equivalent to 3-5% O_2 [294]), while abdominal SAT in obese humans may range between 5-11% (23-84mmHg) [70, 309]. Thus, 5% O_2 as used in this study may be more closely related to normal physiological O_2 concentrations. However, in this context, the regular use of 21% O_2 as normoxia (control condition) may also be questioned as this PO_2 might be much higher (hyperoxia) than that to which even pulmonary cells are exposed to *in vivo*. Clearly, studies examining more physiological PO_2 (3-11% PO_2) ranges over longer periods of time are lacking and experimental protocols that more closely reflect human *in vivo* (physiological) PO_2 ranges are warranted.

Moreover, the use of a candidate gene approach (chapter 6), where only one polymorphism within each of *COL5A1* and *COL6A1* genes were investigated for their associations with body fat mass, its distribution and insulin resistance in SA women, instead of a GWA approach is another limitation to this study. However, GWA studies require large sample numbers, are expensive to undertake, and, due to limited resources and a lack of the appropriate technology, were not performed in this thesis. In addition, the use of HOMA-IR to estimate insulin resistance may represent another limitation to this study. Although HOMA-IR has been shown to correlate with the hyperinsulinemic-euglycemic clamp, the gold standard for assessing insulin sensitivity in white populations, the association is less robust in a black population [426, 427].

Finally, although the hypothetical model was proposed based on previous research findings and the work of this thesis, the suggested mechanisms might not be the only

factors contributing to increased risk of insulin resistance and the development of T2DM. Further studies are warranted to evaluate this hypothesis that i) greater adipose tissue hypoxia and the accumulation of ECM components in peripheral body fat plays a role in the development of insulin resistance and ii) that ethnic specific differences exist which could explain the increased prevalence of insulin resistance and higher risk for T2DM in black women.

7.4 CONCLUSION

The burden due to T2DM in developing countries such as South Africa is unacceptably high. While intensive efforts have been made to improve health care delivery in South Africa, the development and implementation of effective preventative care strategies remain crucial to reduce the immense fiscal burden of the high T2DM associated morbidity and mortality rates in this country. As part of the 2030 Agenda for sustainable Development, ambitious targets have been set to reduce pre-mature mortality from NCDs (including T2DM) by one third by 2030 [77]. However, to achieve any realistic change to the incidence and prevalence of T2DM, an improved understanding of the relevant physiological and environmental risk factors on population level are needed to develop evidence-based, cost-effective preventative-care strategies which are relevant to the modern South African demographic.

As such, this thesis provides preliminary evidence that physiological differences in adipose tissue between African and white populations may contribute to their increased risk of insulin resistance and T2DM prevalence. Specifically, it was shown that the gluteal depot of obese black women has higher expression of hypoxia and ECM genes compared to that of obese white women which also associates with their reduced insulin sensitivity and hyperinsulinaemia. Therefore, this thesis provides novel insight into the apparent paradox of reduced insulin sensitivity despite lower VAT

and greater peripheral SAT accumulation in black compared to white women. Based on these finding, future studies should investigate ethnic differences in the expression and deposition of other ECM components such as matrix remodeling enzymes and cell-surface integrins which may associate with other mechanosignal transduction pathways relevant in adipose tissue metabolism. Furthermore, prospective studies are required to determine whether the expression of these components are indeed associated with the development of insulin resistance and increased inflammation in larger cohors and whether an altered adipose tissue micro-environment could underlie ethnic-specific differences in metabolic disease risk

APPENDIX

SUPPLEMTARY TABLES

Supplementary Table 4. a. Insulin treatment concentration and duration as used in studies which have evaluated either acute or chronic insulin treatment

Cell type	Insulin concentration	Treatment duration	Reference
Acute insulin treatments			
HEK-293 and 3T3-L1-cells	0.01 - 100 nM	5 min	Regazzetti et al [315]
ARPE-19	100 nM	4h	Treins et al [373]
3T3-L1	100 nM	15 min	Yin et al [310]
3T3-L1	100 nM	0- 24 h (intervals)	He et al [372]
3T3-L1	0.02nM-1uM	2 min	Smith et al [431]
Chronic insulin treatment			
3T3-L1	1 µM	48h	Wang et al [376]
Rat adipocytes	500 nM	20 h vs 30 min	Pryor et al [383]
Human adipocytes	1-1000 nM	48 h	McTernan [405]
3T3-L1	100ng/ml	8h &12h	Hupfeld et al [408]
3T3-L1	17nM	8h	Zhang et al [432]

Supplementary Table 4. b. Reverse transcription reaction-mix used to generate cDNA with High Capacity RT-PCR (Applied Biosystems) kit

Kit component	RT (+) (ul)	RT (-)
10 RT buffer	2.0 µl	2.0 µl
25 x dNTP mix	0.8 µl	0.8 µl
10 x random primer	2.0 µl	2.0 µl
RNase inhibitor	1 µl	1.0 µl
Nuclease –free water	3.2 µl	4.2 µl
Reverse transcriptase	1 µl	- µl
RNA (1 µg)	10 µl	10 µl
Total Volume	20 µl	20 µl

PCR cycling conditions: 25˚C for 10 min, 37 ˚C for 120 min, 85 ˚C for 5 sec and followed by 4 ˚C until reaction completed.

Supplementary Table 4. c. List of antibodies and dilutions used for western blot

Santa Cruz Biotechnologies	Cat #	Protein size	Dilution
Primary antibodies			
Anti -COL5A1	sc-20648	220/140kD	1:1000
Anti -COL6A1	sc-20649	140kD	1:1000
Anti-HIF-1α	sc-53546	132kD	1:1000
Anti-β-Actin	sc-47778	43kD	1:500
Secondary antibodies			
Donkey Anti-mouse IgG-HRP	sc-2318		1:4000
Donkey Anti-rabbit IgG-HRP	sc-2317		1:4000

Supplementary Table 6.1. 1 Cohen's f^2 effect size for HOMA-IR values and power calculation in black and white woman as determined by multiple regression analysis F

	R^2_A			R^2_{AB}			$f^2 = \frac{R^2_{AB} - R^2_A}{1 - R^2_{AB}}$			Power (1-β err prob)		
	All	White	Black	All	White	Black	All	White	Black	All	White	Black
***COL5A1* rs12722 (C/T)**										(n=266)	(n=117)	(n=149)
HOMA-IR	0.052	0.098	0.017	0.283	0.338	0.270	0.322	0.362	0.319	1.000	0.999	0.999
***COL6A1* rs35796750 (T/C)**										(n=362)	(n=166)	(n=196)
HOMA-IR	0.004	0.001	0.005	0.292	0.394	0.241	0.407	0.648	0.310	1.000	1.000	1.000

f^2, effect size; R^2_A: Proportion of HOMA-IR variance accounted for by genotype alone; R^2_{AB}: Proportion of variance accounted for by genotype, age and fat mass (%) relative to model with no regressors. Power for given sample size calculated in G*Power by *post-hoc* analysis using linear multiple regression method for fixed model, single regression coefficient.

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