# Stem Cell Impairment Associated with Type 2 Diabetes Mellitus: Investigating the effects of obesity-associated inflammation on Mesenchymal Stem Cell function

by
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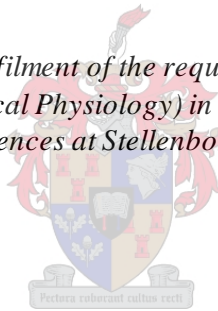
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Declaration

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Abstract

Background: South Africa has the highest prevalence of obesity in sub-Saharan Africa, particularly in Black women. This population is thus at a higher risk of developing obesity-associated type 2 diabetes mellitus (T2DM) and its associated co-morbidities such as non-healing wounds. Adipose tissue-derived mesenchymal stem cells (ADSCs) have been widely utilized in the treatment of chronic wounds, however, autologous stem cell therapies using endogenous ADSCs from T2DM patients have proven unsuccessful. Metabolic disorders such as T2DM are thus thought to compromise the functional capacity of mesenchymal stem cells. The underlying molecular mechanisms that contribute to the functional decline of mesenchymal stem cells is still unclear and it is not yet known at which stage of disease progression ADSCs become compromised. In this research study, it was hypothesised that the progressive worsening of chronic systemic inflammation during disease progression from obesity towards T2DM, contributes to the decline of ADSCs' multifunctional properties.

Methods: A total of forty-seven ($n=47$) reproductive aged (18-45 years) Black Xhosa women from peri-urban areas surrounding the Tygerberg hospital, were included in this study. Participants were subdivided into: a) healthy lean ($n=10$) (BMI ≤ 25 kg/m^2); b) healthy obese ($n=11$) (BMI ≥ 30 kg/m^2); c) obese metabolic syndrome ($n=19$) and d) previously diagnosed T2DM ($n=7$) groups. Health, lifestyle and dietary questionnaires were completed by participants. Anthropometric measurements and a dual energy x-ray absorptiometry (DXA) scan were performed in order to assess body composition. Blood samples were collected in order to assess each participant's metabolic- (fasting blood glucose, total cholesterol, HDL, LDL, triglycerides) and inflammatory (CRP, SDF-1α, IL-6, IL-8, IL-10, TNF-α, IFN-γ) profiles. To establish whether a relationship exists between systemic inflammation at different stages of disease progression and stem cell impairment, *in vitro* experiments were performed in which ADSCs (Poietics cell line) were exposed to participant-derived serum for a period of

48h. Changes in cellular viability (MTT-based assay), proliferation (BrdU) and migration (wound healing assay) were assessed using standard tissue culture techniques.

Results: Systemic inflammation was evident in the healthy obese (CRP 29.8 ± 8 pg/mL) and obese metabolic syndrome (CRP 50.8 ± 24 pg/mL) participants. Additionally, circulating levels of the anti-inflammatory cytokine IL-10, were significantly reduced in T2DM participants (0.42 ± 0.63 pg/mL) ($p < 0.05$) compared to the healthy lean and obese groups. Due to individual variability within the different groups, there were no significant differences observed in circulating levels of IL-6, IL-8, TNF-α and IFN-γ. However, there was a significant correlation between circulating levels of IL-6 and the proliferation of ADSCs, particularly in the healthy lean ($p < 0.01$) and metabolic syndrome ($p < 0.01$) groups. Furthermore, serum levels of IL-8 significantly correlated with the migration of ADSCs ($p < 0.01$). Healthy lean participant serum had a mitogenic effect on ADSCs, which was not observed in the obese groups.

Conclusion: This study demonstrated for the first time, that the disruption in the delicate systemic inflammatory balance as a result of obesity, regardless of metabolic syndrome, may have an adverse effect on the functional capacity of ADSCs.

Opsomming

Agtergrond: Suid Afrika het die hoogste voorkoms van vetsug (obesiteit), veral in swart vrouens, van al die Afrika-lande suid van die Sahara. Hierdie populasie het dus 'n hoë risiko om tipe II (insulien-weerstandige) diabetes mellitus (T2DM) en verwante sekondêre toestande soos kroniese wonde te ontwikkel. Vetweefsel afkomstige mesenchiem stam selle, word dikwels gebruik vir die behandeling van kroniese wonde, maar stamsel-terapie wat gebruik maak van diabetiese pasiënte se eie stam selle (liggaamseie sel terapie), is onsuksesvol. Daar word vermoed dat metaboliese siektes soos T2DM die funksionering van vetweefsel-stamselle (ADSCs) aantas, alhoewel dit nog nie duidelik is op watter stadium van die siekte dit gebeur nie. In hierdie navorsingstudie het ons die hipotese ondersoek dat die kroniese inflammasie wat gepaardgaan met vetsug en T2DM bydra tot die verswakte funksionering van ADSCs.

Metodes: Altesaam sewe-en-veertig (n=47) vrouens tussen 18 en 45 jaar oud, woonagtig in die voorstedelike gebiede naby Tygerberg hospitaal, het aan die studie deelgeneem. Die deelnemers is in vier groepe verdeel: a) gesond en skraal (n=10) (liggaamsmassa-indeks (LMI) ≤ 25 kg/m2); b) gesond, nie-diabeties maar vetsugtig (n=11) (LMI ≥ 30 kg/m2); c) vetsugtig met metaboliese sindroom (n=19) en d) reeds gediagnoseerde T2DM (n=7). Pasiënte het gesondheid-, leefstyl- en voedingsvraelyste voltooi. Antropometrie en 'n "dual energy x-ray absorptiometry" (DXA) skandering is uitgevoer om die liggaamsamestelling te assesseer. Bloedmonsters is versamel om die metaboliese (vastende bloedsuiker, totale cholesterol, HDL, LDL, triglycerides) en inflammatoriese profiele (CRP, SDF-1α, IL-6, IL-8, IL-10, TNF-α en IFN-γ) van pasiënte te analiseer. Om die vas te stel of daar 'n verwantskap bestaan tussen sistemiese inflammasie tydens die verskillende stadiums van siekte-progressie en ADSC beskadiging, is *in vitro* selkultuur eksperimente uitgevoer waarin ADSCs (Poetics sellyn) behandel is met die bloedsera van individuele deelnemers. Veranderinge in sel-

lewensvatbaarheid (MTT toets), selverdeling (BrdU toets) en sel migrasie (as 'n aanduiding van wondheling) is gemeet onder standaard selkultuur-kondisies.

Resultate: Sistemiese inflammasie was duidelik waarneembaar in die gesond vetsugtige (CRP 29± 8 pg/mL) en metaboliese sindroom vetsugtige (CRP 50.8 ± 24 pg/mL) groepe. Serumvlakke van die anti-inflammatoriese sitokien IL-10 was beduidend laer in T2DM deelnemers (0.42 ± 0.63 pg/mL) ($p < 0.05$). Serumvlakke van IL-6, IL-8, TNF-α en IFN-γ het gevarieer, as gevolg van individuele variasie in die deelnemers van die verskillende groepe. Betekenisvolle verband tussen IL-6 vlakke en sel-verdeling is waargeneem, veral in die gesond skraal ($p < 0.01$) en metaboliese sindroom ($p < 0.01$) groepe. 'n Veband tussen serumvlakke van IL-8 en sel-migrasie, was ook duidelik. Die stimulerende effek van serum op selverdeling was afwesig in serum afkomstig van vetsugtige deelnemers.

Gevolgtrekking: Hierdie studie is die eerste om te bewys dat die funksionering van vetweefsel-stam selle negatief beïnvloed kan word deur vetsug-gedrewe versteurings in die delikate sistemiese inflammatoriese balans, onafhanklik van die metaboliese sindroom.

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Abbreviations

ADSC – adipose tissue-derived mesenchymal stem cell

AGE – advanced glycated end-product

BMD - bone mineral density

BMI – body mass index

BMMSC – bone marrow-derived mesenchymal stem cell

CCL – CXC (chemokine) ligand

cm^2 – centimetre squared

CRP – C-reactive protein

CV - coefficient of variance

CVD – cardiovascular diseases

DMSO - dimethyl sulfoxide

DNA – deoxyribonucleic acid

DPP4 – dipeptidyl peptidase 4

DXA - dual energy x-ray absorptiometry

EC - endothelial cell

EDTA - ethylenediaminetetraacetic acid

EPC – endothelial progenitor cell

FACS – fluorescence-activated cell sorting

FBS - fetal bovine serum

FFA – free fatty acids

FMO – fluorochrome minus one

GM-CSF – granulocyte macrophage – colony stimulating factor

GRα – glucocorticoid receptor alpha

HbA1c – glycated haemoglobin

HDL – high density lipoprotein

hrG-CSF – human recombinant granulocyte – colony stimulating factor

hsCRP – high sensitivity C-reactive protein

IL – interleukin

kg – kilogram

L – litre

LDL – low density lipoprotein

LIF - leukemia inhibitor factor

LMIC – low-to-middle income countries

LPS – lipopolysaccharide

M1 – classically activated type macrophage

M2 – alternatively activated type macrophage

m^2 – meter squared

MACS – magnetic-activated cell sorting

MAPK – mitogen activated protein kinase

MCP – monocyte chemoattractant protein

mL – millilitre

mmol – millimole

mRNA – messenger ribonucleic acid

MSC – mesenchymal stem cell

NCD – non-communicable diseases

NFκB – nuclear factor kappa B

ng – nanogram

OSM - oncostatin M

PBMCs – peripheral blood mononuclear cells

PBS - phosphate buffered saline

pen/strep - penicillin streptomycin

pg – picogram

RAGE – receptor for advanced glycated end-product

RBC – red blood cell

ROS – reactive oxygen species

RPM – revolutions per minute

RT – room temperature

SA - South Africa

SAT – subcutaneous adipose tissue

SCF - stem cell factor

SDF-1α – stromal derived factor 1 alpha

SES – socio-economic status

SGM - standard growth medium

SI – insulin sensitivity index

SOD – superoxide dismutase

SST - serum separating tube

STAT3 – signal transducer and activator of transcription 3

T2DM –type 2 diabetes mellitus

TNF – tumour necrosis factor

VAT – visceral adipose tissue

VCAM – vascular cell adhesion molecule

VEGF – vascular endothelial growth factor

vs - versus

WAT – white adipose tissue

WBC – white blood cell

WHR – waist-to-hip ratio

°C – degrees celsius

μL – microlitre

Chapter 1

Introduction

In societies that are burdened with an increased incidence of lifestyle related diseases *(such as obesity-induced type 2 diabetes)*, there is a clear need for preventative interventions against disease progression. It is estimated that over 80% of deaths from lifestyle diseases worldwide occur in low- and middle-income countries (Abegunde *et al.*, 2007; Holmes *et al.*, 2010) and projections indicate that as a source of morbidity and mortality, lifestyle diseases will overtake infectious diseases by 2030 (Mathers & Loncar, 2006).

The literature review (chapter 2) will discuss the prevalence of obesity in South Africa, and highlight the predictions that in 2030 over 140 million people in developing countries *(including South Africa)* will be suffering from obesity-induced type 2 diabetes mellitus (T2DM) and its associated secondary complications (such as non-healing wounds) (Wild *et al.*, 2004). The literature review will also discuss disease progression from obesity to metabolic syndrome and ultimately the development of T2DM with the focus on body composition and low-grade systemic inflammation. It is well known that individuals who eventually develop T2DM, display inflammatory characteristics long before developing clinical symptoms (Alexandraki *et al.*, 2006; Pradhan *et al.,* 2003; Freeman *et al.*, 2002). The prominent role that inflammation plays in disease progression, taken together with the recent discovery that the pathogenesis of T2DM can be detrimental to the function of endogenous stem/progenitor cells *(endothelial progenitor cells, hematopoietic stem cells, mesenchymal stem cells)* (Fadini *et al.*, 2013; van de Vyver, 2017), suggests a potential link between systemic inflammation and the functional decline of stem cells.

The implications of long term disease-associated changes in the systemic (circulation) and localized stem cell niche environments are however ill defined and the resultant stem cell impairment could potentially be a contributing factor to the development of co-morbidities. Furthermore, autologous stem cell therapies performed using mesenchymal stem cells (MSCs) from diabetic patients have proven unsuccessful, despite numerous remarkable reviews of successful allogeneic stem cell therapy in the treatment of diabetic co-morbidities such as foot ulcers.

The overall purpose of this research was thus to improve our understanding of the high sensitivity of stem/progenitor cells to the pathological micro-environment associated with T2DM, and to assess the relationship between lifestyle choices, systemic inflammation and the functional impairment of stem cells.

Chapter 2

Literature Review

The dynamic shift to urbanisation has had various implications on the lives of people globally, with an astounding increase in the prevalence of obesity and obesity-related co-morbidities (Kelly *et al*., 2008). According to the World Health Organisation (WHO), in 2014 over 500 million people worldwide were considered obese (WHO World Health Statistics, 2014 http://www.who.int/gho/publications/world_health_statistics/2014/en/), and it is predicted that by the year 2030, approximately 1 billion people will be affected by this lifestyle related disorder. In developing countries, such as those in Sub-Saharan Africa, despite the majority of people living in poverty and undernutrition, obesity has fast become an epidemic (Ng *et al*., 2014), with the highest prevalence evident in South Africa (Micklesfield *et al*., 2013).

In 2014, the World Health Organization (WHO) indicated that over 70% of South African females were overweight (body mass index: BMI > 25 kg/m^2), with 37.3% of females being obese (BMI > 30 kg/m^2) and at risk for the development of T2DM (Dalal *et al*., 2011). Obesity is however less pronounced in South African males (15.7%) than females. The growing obesity epidemic in South Africa can be attributed to numerous factors such as poor dietary habits, a sedentary lifestyle, genetics and gender-related socio-cultural norms (Okop *et al*., 2016; Puoane *et al*., 2002; Stephens *et al*., 2017).

o *Poor dietary habits:* Increasingly busy lifestyles contribute to poor nutritional habits due to the inclination of people to eat ready-made foods (Parfitt *et al*., 2010; Puoane *et al*., 2002) with a high calorie content. In low-to-middle income countries (LMIC) like South Africa, there is furthermore an inverse relationship between socio-economic

status (SES) and obesity (Chantler *et al*., 2016; Egbujie *et al*., 2016). Whilst the traditional western idea that lifestyle and dietary habits influence weight gain remain true, SES is another major contributor to the obesity epidemic. This is partly because in LMICs, the food that is affordable to the majority of people, is often unhealthy, lacks real nutritional value and contain large amounts of saturated fats and starch which ultimately lead to weight gain (Okop *et al*., 2016).

- o *Sedentary lifestyle:* Low physical activity is another factor associated with weight gain (Blundell *et al*., 2017; Cassidy *et al*., 2017). Obesity is driven by an energy intake versus energy expenditure factor (Blundell *et al*., 2017), thus, a balance in the amount of physical activity and calories consumed is required to maintain a healthy BMI (Cassidy *et al*., 2017). Increased physical activity on its own, however, does not guarantee the prevention of obesity (Blundell *et al*., 2017; Dickie *et al*., 2014). Highlighting that increased physical activity as well as calorie restrictions, are necessary for weight management.

- o *Socio-cultural norms:* Following an investigation into the perception of body size, obesity threat and the willingness to lose weight, Okop *et al*. (2016) demonstrated that in Black South African cultures, being overweight is seen as a sign of happiness and wealth. Consequently, men desire overweight women because it creates the illusion of prosperity (Mbochi *et al*., 2012; Okop *et al*., 2016). It is thus not surprising that the prevalence of obesity is greater in Black South African women than in men (Keswell *et al*., 2016).

These factors, together with the rapid urbanisation of developing countries, are thus contributing to a much higher proportional increase in the prevalence of obesity compared to developed countries (Kelly *et al*., 2008). This exacerbates the burden of disease (Kelly *et al*., 2008), especially since obesity predisposes an individual to develop metabolic syndrome.

Metabolic syndrome is a group of risk factors (dyslipidaemia, hyperglycaemia, hypertension and a large waist-to-hip ratio) (Xu *et al.*, 2003), which increases an individual's risk for developing non-communicable diseases (NCDs) such as cardiovascular disease (CVD), non-alcoholic fatty liver disease, stroke and insulin resistant T2DM (Grundy, 2004; Ritchie & Connell, 2007). Globally, NCDs account for about 65% of all deaths and in developing countries, NCDs are responsible for 80% of deaths (Keswell *et al.*, 2016). Type 2 diabetes in particular is associated with numerous co-morbidities, and given the increasing burden of disease, a better understanding of the molecular mechanisms underlying disease progression can aid prevention and treatment strategies.

2.1. Disease progression: Obesity → Metabolic syndrome → T2DM

2.1.1. *Ethnic variations in body composition and the role of adipose tissue depots in the development of insulin resistance*

There are two main types of adipose tissue in the human body: brown adipose tissue (abundant in infants, heat producing function) and white adipose tissue (function as fat storage) (Nedergaard *et al.*, 2007; Wensveen *et al.*, 2015). White adipose tissue (WAT) is characterized by adipocytes with a single large lipid storage vesicle and may be distributed throughout the body at multiple visceral and subcutaneous locations. WAT provides plentiful capacity for storage of lipids, in the form of triglycerides (Ahmadian et al., 2007) and can be further subdivided depending on its location, type of precursor cells from which it originated and/or its metabolic profile (Sanchez-Gurmaches & Guertin, 2014). Visceral white adipose tissue (VAT) is located around internal organs and in abundance as central abdominal fat, whereas the peripheral fat located around the gluteo-femoral region is known as subcutaneous white adipose tissue (SAT). Numerous studies conducted to elucidate the role of different adipose tissue depots, suggest that not all fat stores contribute equally towards the development of

metabolic complications (Goedecke *et al.*, 2009; Goedecke *et al.*, 2011; Hanley *et al.*, 2007; Keswell *et al.*, 2016; Sanchez-Gurmaches & Guertin, 2014; Ritchie & Connell, 2007). The topic of whether all adipose tissue stores play a role in the development of metabolic syndrome, has however been controversial.

For years, researchers thought that VAT is more closely associated with the development of metabolic syndrome. This was based on evidence from studies conducted on Caucasian participants, showing that VAT in obese individuals is a predominant source of chronic systemic inflammation, which is associated with metabolic diseases (Brochu *et al.*, 2000; Ross *et al.*, 2002; Weisberg *et al.* 2003; Hayashi *et al.*, 2008) (refer to section 2.1.2, pg. 24). Hence, indices of VAT such as the threshold for waist circumference or waist-to-hip ratio (WHR) have been used as the gold standard to identify high risk individuals who are more prone to metabolic diseases. However, it is now known that ethnic variations exist in the relationship between waist circumference, VAT, and the development of metabolic diseases (Caroll *et al.*, 2008; Considine *et al.*, 2008; Deboer, 2010; Evans *et al.*, 2011; Hamdy *et al.*, 2006; Hayes *et al.*, 2013; Lovejoy *et al.*, 1996).

Numerous studies (refer to Table 2.1, pg. 22 - 23) have demonstrated that BMI and WHR do not accurately reflect VAT in Black women and that Black women have more peripheral SAT than VAT in the abdominal region, compared to Caucasian women of the same BMI (Carroll *et al.* 2008, Hayes *et al.*, 2013, Keswell *et al.*, 2016). From the table (Table 2.1, pg. 22 - 23) it is clear that despite differences in body composition and less central fat mass, similar levels of insulin resistance were evident in Black and Caucasian women. Goedecke *et al.* (2009) furthermore showed that deep SAT is closely, if not equally associated with the development of metabolic syndrome in Black South African (SA) women.

SAT consists of the superficial SAT (subcutaneous layer in gluteal-femoral regions) as well as deep SAT (a third layer of adipocytes), which are histologically distinct and differ in metabolic

processes, with the deep SAT being more metabolically active (Brochu *et al.*, 2000; Lundbom *et al.,* 2013). Deep SAT has a protein profile which closely resembles that of VAT (known to be associated with metabolic syndrome) and may thus contribute to insulin resistance (Walker *et al.*, 2007). Walker *et al.* (2007) made use of adipose tissue biopsies from healthy men (*n=3*) and women (*n=7*) to investigate the difference in adipokine expression between superficial SAT, deep SAT and VAT. The overall results suggested that deep SAT displayed an independent association to the development of insulin resistance through its close resemblance to the protein profile observed in VAT (Walker *et al.*, 2007). These findings are supported by an earlier study by Lovejoy *et al.* (2001), in which the authors highlighted the need to distinguish between the superficial SAT and deep SAT in terms of their relationship to metabolic risk factors.

Table 2.1. Ethnic variations in fat distribution and their association with the development of metabolic syndrome

Author(s)	Population Studied	Findings
Lovejoy *et al.*, 1996	African-American (n=37) and Caucasian (n=22) women	African-American women are more insulin resistant than Caucasian counterparts and have less VAT.
van der Merwe *et al.,* 1998	Black (n=10) and Caucasian (n=10) South African women	Caucasian women have significantly more VAT compared to Black women; more plasma insulin and circulating FFA.
Lovejoy *et al.*, 2001	African-American (n=55) and Caucasian (n=103) women	African-American women have larger SAT depots, compared to Caucasian women. DSAT and VAT strongly correlates with metabolic risk factors.
Ferris *et al.*, 2005	Indian (female n=24, male n=19), Caucasian (female n=22, male n=19) and Black (female n=19, male n=8) subjects	Black individuals had smaller WHR compared to Indian and Caucasian individuals. White individuals had higher serum adiponectin levels than Black and Indian individuals.
Hanley *et al.*, 2007	African-American (female n=299, male n=223) and Hispanic (female n=662, male n=452) individuals	There is a stronger inverse association of VAT with adiponectin levels among African-American compared to Hispanic individuals. Age; female gender; HDL and SI were significant positive correlates of adiponectin in both ethnicities.

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Author(s)	Population Studied	Findings
Lee *et al*., 2006	African-American (female n=42, male n=41) and Caucasian (female n=37; male n=41) youth	African American youth have significantly lower adiponectin levels, less VAT and are less insulin sensitive compared to their Caucasian counterparts.
Carroll *et al*., 2008	African-American (female n=45, male n=21), Caucasian (female n=32, male n=15), Hispanic (female n=52, male n=20)	African-American women have higher overall body fat percentage compared to Hispanic and Caucasian women as well as lower VAT, despite similar BMI and WHR.
Goedecke *et al*., 2009	Black (n=26) and Caucasian (n=28) South African women	Black women are more insulin resistant; have less VAT than Caucasian women. Insulin resistance is associated with deep SAT in both Black and Caucasian women, but with only VAT in Caucasian women.
Goedecke *et al*., 2011	Black (n=28) and Caucasian (n=26) South African women	Black women have less VAT; SI and lipogenic and adipogenic genes in their SAT than Caucasian women. SI correlated with adipogenic genes expression.
Gardener *et al*., 2013	African-American (female n=481, male n=225), Caucasian (female n=356, male n=228), Hispanic (female n=975, male n=570)	Adiponectin levels are higher in Caucasian individuals than in African American and Hispanic individuals. Obesity is more strongly associated with adiponectin levels in African American individuals.
Hayes *et al*., 2013	Black (n=28) and Caucasian (n=26) South African women	Black women are more insulin resistant. DSAT & SSAT is associated with decreased insulin sensitivity in both ethnicities, but only with VAT in Caucasian women.
Goedecke *et al*., 2013	Black (n=28) and Caucasian (n=26) South African women	Caucasian women have more VAT; are more insulin sensitive and have elevated levels of GRα mRNA in SAT depots.
Keswell *et al*., 2016	Black (n=288) and Caucasian (n=197) South African women	Black women have more total body fat; less central fat and more lower-body fat than Caucasian women. There are ethnic differences associated with increased risk of CVDs.

Footnote: Included in the table are studies conducted in South Africa and the United States of America, demonstrating the differences in fat distribution and type of fat prevalent in Black and Caucasian women, as well as how these relate to the development of metabolic syndrome. **Abbr.**: CVD – cardiovascular diseases; DSAT – deep subcutaneous adipose tissue; FFA – free fatty acid; GRα – glucocorticoid receptor alpha; SA – South Africa; SI – insulin sensitivity index; SSAT – superficial subcutaneous adipose tissue; WHR – waist-to-hip ratio; VAT – visceral adipose tissue.

2.1.2. The relationship between metabolic dysregulation, chronic systemic inflammation and the progression of metabolic syndrome towards T2DM

Adipocytes secrete adipokines (proteins with paracrine/autocrine or endocrine functions) such as leptin and adiponectin, these factors contribute to metabolic regulation and insulin sensitivity (Hoffstedt *et al.*, 2004; Scherer, 2006). Leptin, which functions as a satiety hormone under normal conditions (King *et al.*, 2010), is significantly elevated in obese individuals and has been shown to be positively correlated with the hypertrophy of adipocytes (Weisberg *et al.*, 2003). Circulating leptin levels are thus often used as an indicator of adipose tissue mass (Johnson *et al.*, 2012). Chronic elevation in leptin levels results in leptin resistance causing distortion of appetite regulation, which in turn exacerbates obesity (Johnson *et al.*, 2012). Leptin also functions to regulate insulin secretion, which in extreme obesity, contributes to the development of insulin resistance (Osegbe *et al.*, 2016). It is hypothesized that the increased levels of leptin lead to impaired regulation of the adipocyte-insulin axis in pancreatic beta cells causing hyperinsulinemia and ultimately insulin resistance (Osegbe *et al.*, 2016).

In contrast to leptin, adiponectin (role in modulation of glucose control and fatty acid oxidation) levels are significantly decreased in obese individuals and have a strong inverse relationship with the risk of developing metabolic syndrome (Duncan *et al.*, 2004). This has been shown in both animal models and patient based studies (Choi *et al.*, 2007; Yamauchi *et al.*, 2001; Zhao *et al.*, 2016). Insulin resistant mice have significantly decreased serum adiponectin levels compared to controls (Duncan *et al.*, 2004), which is true for individuals who are insulin-resistant. Furthermore, reductions in adiponectin production (within subcutaneous adipose tissue) is synonymous with decreased serum concentrations of the protein (Hoffstedt *et al.*, 2004). In the study by Hoffstedt *et al.* (2004) the authors demonstrated that in healthy obese (not insulin-resistant) individuals, despite reduced subcutaneous adipose tissue adiponectin production, no change in the serum concentration of the cytokine was observed. Another

interesting observation in this study was the significantly higher levels of adiponectin production observed in the healthy obese compared to the non-obese subjects, although serum concentrations were the same in these two groups. This suggested that serum adiponectin levels as well as adipose tissue production of adiponectin, are influenced by insulin sensitivity (Hoffstedt *et al.*, 2004). The association between adiponectin levels and the development of insulin resistance, is however ethnically specific (Hanley *et al.*, 2007; Considine *et al.*, 2008). The ethnic variability in adiponectin levels is evident in studies demonstrating that normal weight African-American individuals have marginally lower levels of adiponectin compared to their Caucasian counterparts (Cohen *et al.*, 2011; Gardener *et al.*, 2013; Hanley *et al.*, 2007). It has therefore been suggested that adiponectin levels might be more closely associated with insulin resistance in the Black population (Schutte *et al.*, 2007) and might partially explain the higher prevalence of insulin resistance observed in this population.

It is well known that insulin resistant obese individuals who eventually develop T2DM, display inflammatory characteristics long before developing the disease (Alexandraki *et al.*, 2006; Freeman *et al.*, 2002; Pradhan *et al.*, 2003). There is an ongoing debate over whether the inflammation observed during obesity is due to macrophage infiltration into adipose tissue (Bourlier *et al.*, 2008; Cancello *et al.*, 2006; Curat *et al.*, 2004 & 2006) or due to changes in the inflammatory secretory profile of adipocytes as a result of hypertrophy (reviewed by Cinkajzlová *et al.*, 2017). Nonetheless, chronic low grade systemic inflammation is an independent risk factor for disease progression from obesity to metabolic syndrome and ultimately the development of T2DM (Christiansen *et al.*, 2010).

A persistent inflammatory environment such as that observed in extreme obesity results in an immune imbalance which is characterised by an accumulation of pro-inflammatory (classically activated M1) macrophages (Weisberg *et al.*, 2003) and increased release of pro-inflammatory cytokines such as: C-reactive protein (CRP), interleukin 6 (IL-6) and tumour necrosis factor -

alpha (TNF-α). This inflammatory environment has deleterious effects at multiple sites in the body (Ritchie & Connell, 2007) and exacerbates insulin resistance (Rask-Madsen *et al*., 2003). Numerous studies have been conducted to elucidate the mechanism involved in insulin resistance, and TNF-α has consistently been shown to decrease insulin sensitivity by distorting insulin signalling at the receptor site (Rask-Madsen *et al*., 2003; Stagakis *et al*., 2012; Tangvarasittichai *et al*., 2016; Wang *et al*., 2015). In addition to contributing to the development of insulin resistance, chronic adipose tissue inflammation and excessive M1 type macrophage infiltration into adipose tissue are associated with a lack of adipocyte turnover (Barbagallo *et al*., 2017). Together these changes in the adipose tissue cellular composition (adipocyte hypertrophy followed by macrophage infiltration) and altered inflammatory cytokine expression at tissue level is considered a major cause of metabolic dysregulation and disease progression (Martyniak & Masternak, 2017).

This shift in the inflammatory profile in response to adipocyte hypertrophy has been demonstrated in studies such as that of Briand *et al*. (2011). The authors used a diabetic rat (Zucker) model to show that in obese rats, an increase in adipocyte size is followed by a proportional increase in lipid droplet size, which in turn induce the expression of caveolin-1 (Briand *et al.*, 2011). The caveolin-1-dependent endothelium pathway has been shown to take part in the regulation of macrophage flow from the blood into adipose tissue (Ghigliotti *et al*., 2014), which further demonstrates the role of caveolin-1 in accumulation of macrophages in the adipose tissue. In another study, Weisberg *et al*. (2003) demonstrated that the size of adipocytes is directly proportional to macrophage accumulation, by using immunohistochemical analyses of human subcutaneous adipose tissue. Kanda *et al*. (2006) furthermore demonstrated that the expression of chemokines such as the monocyte chemoattractant protein-1 (MCP-1/CCL2), which is largely produced by macrophages and endothelial cells, is increased in the adipose tissue of obese mice. This suggests that during

obesity-induced adipocyte hypertrophy, the expression of cytokines and chemokines by either adipocytes and/or macrophages drives the inflammatory response into a chronic state (refer to Fig. 2.1 and Table 2.2).

From the table (Table 2.2, pg. 28) it is clear that the development of inflammatory disorders such as T2DM can be attributed to the shift in the cytokine profile to a pro-inflammatory state (Diaz-Flores *et al.*, 2013). However, the exact mechanism in which these pro-inflammatory cytokines contribute to the development of T2DM is not yet fully understood.
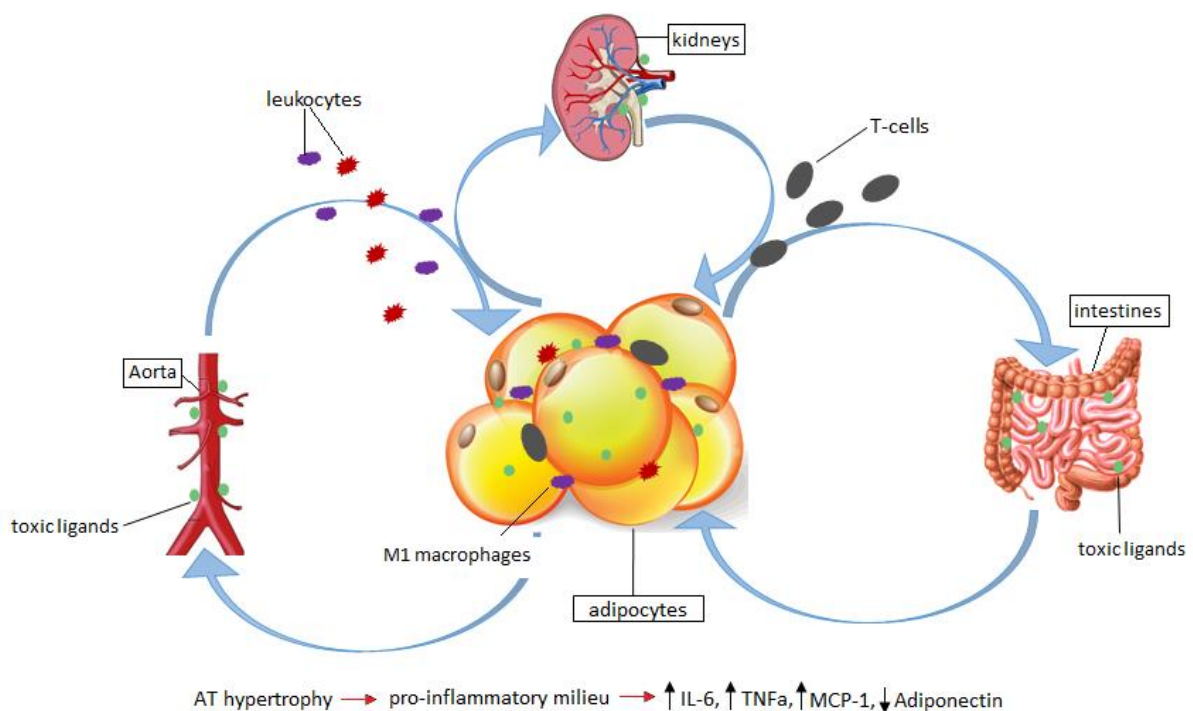
Figure 2.1. Adipose tissue hypertrophy triggers migration of innate and adaptive immune effector cells. The figure demonstrates how the increased size of adipocytes causes a cascade of events which trigger an immune response, resulting in the infiltration of pro-inflammatory cytokines (adapted from Ghigliotti *et al.*, 2014).

Table 2.2. Inflammatory cytokines and their association with metabolic syndrome and T2DM.

Author(s)	Purpose of study	Inflammatory cytokines measured				
		Baseline [control]	Baseline [obese/MS/T2DM]	Increased	Decreased	No change
Esposito *et al.* **(2003)**	intervention	**IL-6** (1.25 pg/mL), **CRP** (1.4 pg/mL); **IL-10** (1.2 pg/mL)	**IL-6** (3.08 pg/mL), **CRP** (5.8 pg/mL); **IL-10** (2.45 pg/mL)	-	**IL-6** (1.92 pg/mL); **CRP** (3.5 pg/mL); **IL-10** (2.1 pg/mL)	-
Hoffstedt *et al.* **(2004)**	correlation	Adiponectin (13.5 ug/mL)	Adiponectin (14 ug/mL)	-	-	-
Unek *et al.* **(2010)**	correlation	hs-**CRP** (2.03 mg/L), sCD40L (0.44 ng/mL)	hs-**CRP** (4.48 mg/L), sCD40L (0.86 ng/mL)	-	-	-
Derakhshan *et al.* **(2012)**	correlation	SDF-1 (60 pg/mL)	SDF-1 (205 pg/mL)		-	-
Lucas *et al.* **(2013)**	correlation	**IL-6** (1.39 ng/L), **IL-8** (1.83 ng/L), **IL-10** (5.42 ng/L), **TNF-α** (1.77 ng/L), GM-CSF (0.94 ng/L)	**IL-6** (2.61 ng/L), **IL-8** (2.47 ng/L), **IL-10** (8.58 ng/L), **TNF-α** (2.87 ng/L), GM-CSF (1.92 ng/L)	-	-	-
van Beek *et al.* **(2014)**	correlation	**IL-6** (0.43 pg/mL), **IL-8** (8.4 pg/mL), **IL-10** (1.8 pg/mL), **TNF-α** (5.9 pg/mL), **CRP** (1.9 mg/L)	**IL-6** (1.62 pg/mL), **IL-8** (8.2 pg/mL), **IL-10** (3.0 pg/mL), **TNF-α** (7.6 pg/mL), **CRP** (7.6 mg/L)	-	-	-
Al-Daghri *et al.* **(2015)**	intervention	-	Adiponectin (0.82 ug/mL), **CRP** (40 ng/mL), **IL-6** (35.8 pg/mL), **IL-10** (28.4 pg/mL), **TNF-α** (91.5 pg/mL)	Adiponectin (1.2 ug/mL)	**CRP** (20 ng/mL), **IL-6** (27.1 pg/mL), **IL-10** (21.1 pg/mL), **TNF-α** (79.4 pg/mL)	
Vargas *et al.* **(2015)**	intervention	**CRP** (19 mg/L)	**CRP** (61 mg/L)	-	-	
Tangvarasittichai *et al.* **(2016)**	correlation	**TNF-α** (2.78 pg/mL), **IL-6** (1.65 pg/mL), **hsCRP** (1.29 pg/mL)	**TNF-α** (3.98 pg/mL), **IL-6** (2.78 pg/mL), **hsCRP** (2.41 pg/mL)			

Footnote: This table displays studies demonstrating the difference in serum circulating cytokines in obese, pre-diabetic and diabetic individuals compared to healthy lean women. Analysis was done on plasma or serum from patients. The increase/decrease refers to changes observed in the study subjects (obese/prediabetic/diabetic). **Abbr.:** hsCRP – C-reactive protein; IL – interleukins; MS – metabolic syndrome; T2DM – type 2 diabetic; TNF – tumour necrosis factor; SDF – stromal derived factor

The following sections will discuss some of the key cytokines involved in disease progression from obesity to metabolic syndrome and the development of T2DM.

o *C-Reactive Protein (CRP)*

Numerous studies that investigated the role of inflammation in the development of T2DM, particularly in women (refer to Table 2.2), have showed a strong correlation between increased levels of serum CRP and the development of T2DM (Freeman *et al.*, 2002; Pradhan *et al.*, 2003; Castoldi *et al.*, 2007; Unek *et al.*, 2010). CRP is produced in the liver, leukocytes and adipose tissue, in response to increased levels of inflammatory cytokines (such as IL-6) (Tangvarasittichai *et al.*, 2016) and is thought to play a role in the amplification of the inflammatory response (Unek *et al.*, 2010). It is therefore defined as a biomarker of low-grade inflammation and has been implicated in metabolic diseases such as cardiovascular diseases (CVDs) and T2DM (Freeman *et al.*, 2002; Han *et al.*, 2002). In a study to determine whether CRP could be used as a predictor for the future development of T2DM, Freeman *et al.* (2002) recruited diabetic and non-diabetic middle-aged men and showed that high levels of CRP were associated with the development of T2DM, independent of established risk factors such as fasting plasma triglyceride, BMI, and glucose levels. Pradhan *et al.* (2003) conducted a similar study on middle-aged women, in order to investigate the association between insulin resistance, inflammation and an increased risk for the development of CVD and T2DM. The results confirmed that the correlation between serum CRP levels and the development of insulin resistance is independent of other metabolic risk factors (Chou *et al.*, 2010; Lu *et al.*, 2010; Pradhan *et al.*, 2003). This supported the findings of Freeman *et al.* (2002) indicating that serum CRP levels can be used as a marker to predict the development of T2DM. Park *et al.* (2005) suggested that the association between CRP and insulin resistance might be linked to obesity. In the study by Park *et al.* (2005), the authors showed that serum CRP levels are correlated

with overall body mass (BMI) whilst IL-6 levels were more closely associated with visceral adiposity.

    o *Interleukin-6 (IL-6)*

Research focussed on the association between CRP and inflammation have identified the cytokine IL-6 as a major role player in disease progression towards T2DM. IL-6 is a well-known pro-inflammatory cytokine secreted by adipocytes and macrophages (Carey *et al.*, 2004; Park *et al.*, 2005) and is thought to be one of the main inducers of CRP production in the liver (Park *et al.*, 2005). IL-6, much like CRP, is known to be elevated in obese individuals and has been linked to the development of T2DM. This was confirmed by Bertoni *et al.* (2010), who showed that elevated baseline serum IL-6 levels were a strong predictor for the incidence of T2DM in a multi-ethnic cohort over a period of 5 years. The same findings (elevated baseline serum IL-6) were later reported by Mirza *et al.* (2012) in both poorly controlled and well-controlled diabetic patients. The association between IL-6 and the development of T2DM is thought to be through the upregulation of CRP production (Pradhan *et al.*, 2003). There are however, some studies such as that of Hu *et al.* (2004), indicating that serum concentration of IL-6 is not as accurate as CRP when it comes to predicting T2DM. However, the unreliability of IL-6 as a predictor is because it displays both pro- and anti-inflammatory characteristics, due to a single nucleotide polymorphism in its promotor gene (Carey *et al.*, 2004; Wang *et al.*, 2015) and its expression can be influenced by numerous acute inflammatory signals.

    o *Tumour necrosis factor- alpha (TNF-α)*

TNF-α is overly expressed in obese individuals (Alexandraki *et al.*, 2006) and plays a key role in the development of T2DM (Hu *et al.*, 2004; Mirza *et al.*, 2012; Rask-Madsen *et al.*, 2003;) since it decreases insulin sensitivity by distorting insulin signalling at the receptor site (Rask-Madsen *et al.*, 2003). TNF-α furthermore has the functional capacity to influence the secretion

of other pro-inflammatory cytokines, and by doing so, amplifies the inflammatory response (Alexandraki *et al.*, 2006; Kern *et al.*, 2001). An imbalance of anti- and pro-inflammatory cytokines together with the environmental influences which result in a sustained inflammatory system, particularly in obesity, exacerbates disease progression (Wang *et al.*, 2015).

o *Interleukin – 10 (IL-10)*

IL-10 is an anti-inflammatory cytokine that is largely produced by immune cells including alternatively activated (M2) macrophages (van Exel *et al.*, 2002) and functions to modulate inflammatory responses by suppressing synthesis of pro-inflammatory cytokines (Cassatella *et al.*, 1993). In chronic inflammatory diseases such as T2DM, the balance between pro- and anti-inflammatory cytokines is disrupted and as a consequence results in low levels of serum IL-10 (Acharya *et al.*, 2015; Al-Daghri *et al.*, 2015; Pirola *et al.*, 2017). Van Exel *et al.* (2002) conducted a study to determine the influence of a patient's metabolic status on the ability of leukocytes to produce pro- (TNF-α) and anti-inflammatory (IL-10) cytokines. The authors assessed the cytokine production capacity in whole blood of T2DM patients after *ex vivo* stimulation with lipopolysaccharide (LPS) and observed a correlation between reduced IL-10 production capacity and high plasma glucose and HbA1c levels. Furthermore, a correlation was observed between an increase in IL-10 production and a gradual decrease in the total cholesterol; LDL cholesterol; triglycerides; glucose and HbA1c levels (van Exel *et al.*, 2002). TNF-α production had very little effect on the metabolic parameters of participants when IL-10 production capacity was taken into account (van Exel *et al.*, 2002). It has therefore been suggested that increased levels of serum IL-10 might have a protective effect against the development of T2DM and associated metabolic diseases by inhibiting the release of pro-inflammatory cytokines (Cassatella *et al.*, 1993; Esposito *et al.*, 2003; Hong *et al.*, 2009; Kelly *et al.*, 2015).

2.2. The pathogenesis of T2DM and its influence on progenitor / stem cell mobilization and function

Type 2 diabetes mellitus presents with numerous co-morbidities (including nephropathy, neuropathy, non-healing ulcers, and atherosclerosis) that occur as a consequence of pathological changes in the microenvironment that induce damage to various tissues and their microvascular network. Persistent hyperglycaemia in poorly controlled T2DM patients leads to the accumulation of advanced glycation end-products (AGEs) (Wang *et al.*, 2015). AGEs are modified proteins which have been non-enzymatically glycated through the reduction of glucose with their amino group, thus initiating a complex series of rearrangements and hydrations (Kume *et al.*, 2005; Serban *et al.*, 2016; Unoki *et al.*, 2007). On a cellular level, progenitor and stem cells are very susceptible to AGE-induced cellular damage (Basta *et al.*, 2002; Lu *et al.*, 2012; Yang *et al.*, 2010). The interaction between AGEs and their AGE-specific receptors (RAGEs) results in increased oxidative stress by inducing reactive oxygen species (ROS) production and activating the MAP kinase pathway, thus leading to downstream signalling activity through nuclear factor kappa B (NFκB) (Loeser *et al.*, 2005; Madonna & De Catterina, 2011). Activation of NFκB by RAGE signalling, results in activation of the inflammatory pathway associated with diabetic osteoarthritis (Loeser *et al.*, 2005).

In the micro-vasculature system, the interaction between AGEs and their AGE-specific receptors (RAGEs) results in the upregulation of adhesion molecules (such as vascular cell adhesion molecule 1, VCAM1), allowing for the adhesion of activated inflammatory cells (M1 macrophages) that in turn causes vascular stress (Schmidt *et al.*, 1994). The repair of micro-vascular damage is however compromised due to the lack of endothelial progenitor cell mobilization and function in the diabetic microenvironment (Fadini e*t al.*, 2013). These pathological changes in the microenvironment are furthermore known to cause remodelling of the bone marrow, affect the mobilization of mesenchymal stem cells (MSC) into peripheral

circulation and have detrimental effects on the regenerative function (maintenance of tissues) of these cells (Rezaie *et al.*, 2017; van de Vyver, 2017).

In healthy individuals, the mobilization of MSCs and endothelial progenitor cells is mediated by stromal derived factor – 1 alpha (SDF-1α) signalling (Li *et al.*, 2016). This SDF-1α signalling axis is however disrupted in T2DM (Albiero *et al.*, 2015; Karimabad *et al.*, 2015) and differences in the serum SDF-1α concentrations is evident between healthy and T2DM patients (Derakhshan *et al.*, 2012). Derakhshan *et al.* (2012) compared the expression levels of SDF-1α in the serum of T2DM patients and control subjects and suggested that the significantly elevated levels of SDF-1α in diabetic patients might be associated with the aetiology of the disease. Based on previous reports, Derakhshan *et al.* (2012) suggested that the observed elevated levels of SDF-1α may occur in response to other pro-inflammatory cytokines (TNF-α, IFN-γ, IL-17) associated with T2DM (Arababadi *et al.*, 2010; Hassanshahi *et al.*, 2008) or as a compensatory mechanism in an attempt to induce mobilization of MSCs and endothelial progenitor cells (EPCs). Based on these findings, it was hypothesised that SDF-1α may be a useful marker of the T2DM disease state and the likelihood for a patient to develop secondary complications.

In another study, Ling *et al.* (2012) compared SDF-1α and vascular endothelial growth factor (VEGF) expression levels in T2DM and non-diabetic subjects following acute myocardial infarction. Plasma levels of SDF-1α and VEGF peaked higher in diabetic patients compared to the non-diabetic subjects. The authors suggested that this peak in SDF-1α and VEGF levels may have been a protective mechanism by these cytokines, to recruit EPCs from the bone marrow, to facilitate the maintenance of myocardium integrity. However, circulating levels of EPCs were not proportional to the increase in these cytokines, suggesting a distortion in the signalling of the mobilization of EPCs in T2DM patients (Ling *et al.*, 2012). This was also evident in a study by Butler *et al.* (2005) demonstrating that increasing levels of SDF-1α

corresponds to worsening disease complications, such as retinopathy. Their results showed a reduction in EPC recruitment with SDF-1α inhibition, which in turn prevented pre-retinal neovascularization leading to retinopathy. Taken together these studies indicate a strong association between elevated levels of SDF-1α and disease progression. It is thus clear that impaired mobilization of stem/progenitor cells from the bone marrow contributes to the development of co-morbidities and that chronic exposure to pathological changes within the bone marrow microenvironment leads to dysfunction of these stem progenitor cells (refer to Table 2.3, pg. 35).

Table 2.3. Studies on the effect of inflammation and the pathogenesis of diabetes on endothelial progenitor and mesenchymal stem cell function.

Author(s)	Cell type	Findings	Possible mechanism
Kume *et al.,* 2005	Human MSCs	AGEs reduce cell viability, decrease proliferation and differentiation capacity of MSCs	AGEs induce MSC apoptosis through increased oxidation.
Egan *et al.,* 2008	Human PBMCs	Reduced expression of CXCR4 and CD34/CXCR4-positive cells is observed in PBMCs from diabetic patients.	Diabetes impairs proliferation of EPCs as well as their mobilization
Kang *et al.,* 2009		Decreased circulating levels of EPC due to suppressed mobilization following ischemia in a diabetic mice model	Impaired EPC mobilization in diabetes is associated with altered HIF-1 alpha/VEGF and MMP/TIMP regulation
Fadini *et al.* 2010	Human EPCs	Circulating levels of CD34+ are reduced in IFG, IGT and T2DM subjects	Increased apoptosis and reduced mobilization due to hyperglycaemia.
Prasanna *et al.,* 2010	Human MSCs, WJMSCs	Pretreatment with IFN-y and TNF-α resulted in changes in morphology and growth characteristics of BMMSCs	Increased inflammation distorts immodulatory properties of MSCs
Stolzing *et al.,* 2010	Rat MSCs	Increased apoptosis, reduced cell viability and reduced bone formation observed in diabetic environment.	AGEs induce oxidation resulting in apoptosis of MSCs.
Lombardo *et al.,* 2012	Human PBMCs, EPCs	No significant difference in number of confirmed EPCs in T2DM and control subjects. A reduced number of circulating EPCs was observed in T2DM, suggesting impaired mobilization of EPCs in T2DM.	Hyperglycaemia results in impaired mobilization of EPCs.
Lu *et al.,* 2012	Human MSCs	AGEs inhibit proliferation of MSCs *in vitro*.	AGE-RAGE interaction inhibits SOD activity, resulting in increased ROS.
Shin & Peterson, 2012	Mouse MSCs	Prolonged diseases states impair the regenerative capacity of endogenous MSCs	↓ expression of proliferative, angiogenic and differentiation signalling factors contribute to MSC dysfunction
Fadini *et al.,* 2013	Human EPCs	BM stimulation with hrG-CSF results in increased circulating EPCs but only in non-diabetic subjects	G-CSF stimulates expression and activity of CD26/DPP4 resulting in degradation of SDF-1α.
van de Vyver *et al., 2016*	Mouse MSCs	Impaired wound closure was observed in MSCs from *ob/ob* mice	Increased inflammation causes dysregulation of IL-6/STAT3 signalling pathway which may be critical to MSC function
Zeng *et al.,* 2017	Rat bone marrow derived EPCs	AGEs induce EPC apoptosis. Lycopene improved EPC proliferation	Lycopene reverses effects of AGE-induced oxidation and thus improves EPC survival

Footnote: The table above displays some of the studies which have demonstrated the effect of inflammation and T2DM on endothelial progenitor and mesenchymal stem cell function. **Abbr.:** AGE – advanced glycation end products; BMMSC – bone marrow-derived mesenchymal stem cell; EPCs – endothelial progenitor cells; G-CSF- granulocyte – colony stimulating factor; IFG – impaired fasting glucose; IGT – impaired glucose tolerance; MSCs – mesenchymal stem cells; ob/ob mice – transgenic mouse model of obesity; RAGE – receptor for advanced glycated end products, ROS – reactive oxygen species; SOD – superoxide dismutase

The dysfunction of endogenous bone marrow MSCs in diabetic patients has limited the success of autologous MSC therapies for the treatment of co-morbidities in these patients. Adipose tissue-derived stem cells (ADSCs) do however have similar multi-functional properties to bone-marrow derived MSCs. Taken together with their abundant presence in adipose tissue and the relative ease of accessibility, ADSCs have thus become an attractive alternative therapeutic agent. However, the pathological microenvironment within adipose tissue has also been shown to modulate the behaviour of ADSCs (Pachón-Peña *et al.*, 2016).

2.2.1. *The effect of metabolic dysregulation and inflammation on adipose tissue resident stem cells.*

ADSCs are multipotent cells capable of differentiating into any tissue of mesodermal lineage (i.e. adipose, bone, cartilage, skeletal muscle, cardiac muscle, endothelial, hematopoietic, pancreatic, and epithelial cell types) (Baglioni *et al.*, 2009; Maharlooei *et al.*, 2011) and share the multifunctional and immunomodulatory properties with bone marrow-derived MSCs (Ivanova-Todorova *et al.* 2009). They can be isolated from subcutaneous as well as visceral adipose tissue (Baglioni *et al.*, 2009) and cultured under standard cell culture conditions (Zuk *et al.*, 2001).

The metabolic dysregulation of adipose tissue that lead to adipocyte hypertrophy, chronic adipose tissue inflammation and the formation of crown-like structures (excessive M1 pro-inflammatory macrophage infiltration) has however been shown to limit the self-renewal capacity of ADSCs (Martyniak & Masternak, 2017). ADSCs derived from diabetic patients are furthermore unable to differentiate into functional adipocytes *in vitro* (Barbagallo *et al.*, 2017), suggesting that metabolic and inflammatory changes within the localized tissue microenvironment impairs the function of ADSCs. It is however unclear at which stage of disease progression, ADSC function becomes compromised, since adipose tissue inflammation

is also thought to play a key role in governing the plasticity and differentiation of ADSCs (Badimon & Cubedo, 2017; Strong *et al.*, 2015).

It has been shown that low grade adipose tissue inflammation increases the size of the progenitor cell pool (Zhu *et al.*, 2016) and the migration rate of ADSCs (Pachón- Peña *et al.*, 2016). This beneficial effect is however lost under conditions of long term chronic tissue inflammation, since metabolic syndrome negatively impacts the therapeutic potential of ADSCs (Oliva-Olivera *et al.*, 2015). Oliva-Olivera *et al.* (2015) emphasized that the metabolic profile of patients should be taken into account when evaluating the reliability of ADSCs for therapeutic use. In their study, the authors demonstrated a decrease in the osteogenic potential of ADSCs in individuals with metabolic syndrome and that alterations occur in the transcriptional pattern of enzymes involved in cellular redox balance (Oliva-Olivera *et al.,* 2015), suggesting that slight changes within the gene expression profile of ADSCs may alter the therapeutic efficacy of these cells. This was confirmed in a study by Pachón-Peña *et al.* (2016) that compared the immunophenotypic profile of ADSCs derived from lean and obese individuals and investigated its association with ADSC plasticity. The authors indicated that in low oxygen tension conditions, increased proliferation and migration of ADSCs were only evident in cells derived from lean individuals. Furthermore, differences in proliferation, migration and differentiation capacity correlated with an altered immunophenotypic profile in ADSCs derived from obese individuals (Pachón-Peña *et al.,* 2016; Yamamoto *et al.*, 2013). Similarly, Ferrer-Lorente *et al.* (2014) demonstrated that ADSCs isolated from the subcutaneous adipose tissue of diabetic rats had reduced viability, proliferation and an impaired potential to differentiate into endothelial cells (ECs). Disruption of the delicately balanced inflammatory status of the adipose tissue can thus influence the multifunctional properties of ADSCs. It is however unclear whether systemic factors also contribute to the functional decline of ADSCs or if it is limited to changes in the localized tissue environment.

Chapter 3

Aims and Objectives

We *hypothesized* that the inflammatory changes in the systemic microenvironment observed during disease progression from obesity to type 2 diabetes mellitus (T2DM), can affect the viability, proliferation and migration capacity of adipose-derived mesenchymal stem cells (ADSCs). In order to test this hypothesis, we therefore (i) determined the inflammatory and metabolic profile of healthy lean-, healthy obese-, obese metabolic syndrome- and T2DM patients; (ii) used the patient-derived serum to treat a human ADSC cell line and assessed subsequent changes in ADSC functional capacity.

AIM 1

Participant (reproductive aged Black Xhosa females) recruitment and detailed assessment of the medical history, body composition and lifestyle of the participants.

Objectives

i) Collect serum (blood samples) from Black Xhosa reproductive aged females (*n=47*) who are either healthy lean, healthy obese, obese metabolic syndrome or obese type 2 diabetic.

ii) To have participants complete questionnaires in order to assess their medical history, nutritional and physical activity habits.

iii) To determine each participant's body composition using Dual X-ray Absorptiometry (DXA) and anthropometric measures.

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AIM 2

Determination of the inflammatory and metabolic profile of each participant.

Objectives

i)   Analyse serum for biomarkers of inflammation using multi-plex bead-array technology and ELISA assays to quantify the concentration of 8 cytokines (IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, IFN-γ, SDF-1α, CRP) in each serum sample.

ii)   Perform serum lipid panel analyses to quantify the concentration of total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL) and triglycerides in each serum sample.

AIM 3

Determination of the effect of each participant's serum on the multi-functional ability of an adipose tissue derived stem cell line.

Objectives

i)   To assess the effect of each participant's serum on MSC viability, proliferation rate, migration capacity by using various cell culture techniques.

ii)   To determine if correlations exist between the inflammatory biomarkers and metabolic profile of each participant and the effect that the serum has on the functional abilities of ADSCs.

Chapter 4

Methodology

This research study was conducted according to the ethical norms and principles for research established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health, South Africa).

Ethical approval (N15/07/066) to conduct this study, was attained from the Health Research Ethics Committee (HREC) of Stellenbosch University. HREC is registered with the South African Department of Health's National Health Research Ethics Council (NHREC) and with the US Office for human Research Protections (OHRP). Federal-Wide Assurance (FWA) numbers FWA00001372 IRB numbers IRB00005239 and IRB00005240. The HREC therefore complies with the South African National Health Act No. 61 2003 as it pertains to health research and the United States Code of Federal Regulation Title 45 Part 46.

Permission to conduct research at the Tygerberg hospital was granted by the Western Cape Department of Health (National Health Research Database Project: WC2016RP14858) in accordance with the Provincial Research Policy and Tygerberg Hospital Notice No 40/2009.

This non-interventional human study consisted of three phases that were performed over a period of 24 months. The flowchart below gives an overview of the study design that will be discussed in more detail throughout this chapter.
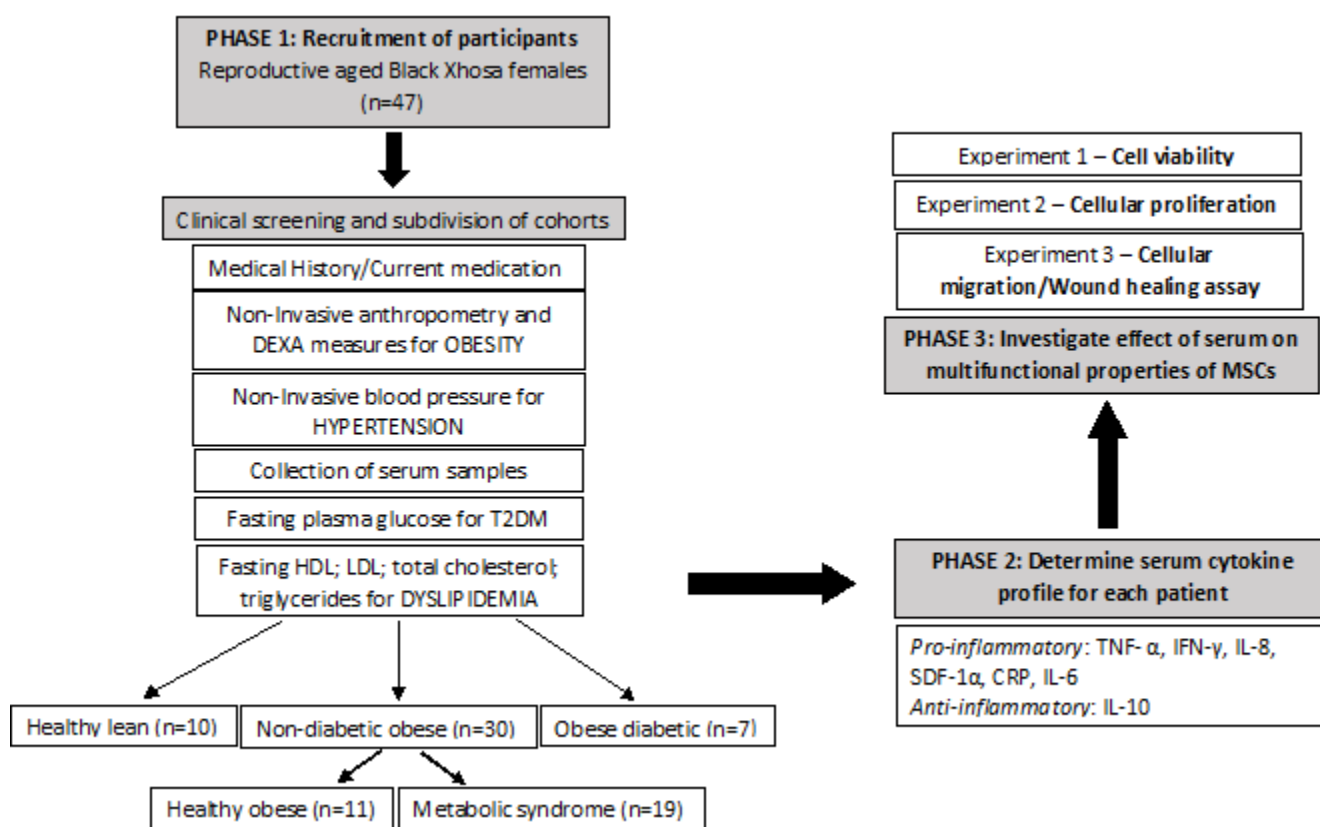
Figure 4.1. Overview of study design. Phase 1: Black Xhosa women were recruited into three groups; healthy lean, non-diabetic obese and previously diagnosed type 2 diabetes mellitus (T2DM) groups. The non-diabetic group was further subdivided into healthy obese and obese metabolic syndrome groups. Participants' metabolic profiles were determined and blood samples were collected. Phase 3: Participant derived serum was used to treat adipose tissue-derived mesenchymal stem cells (ADSCs) in order to investigate its effect on the multifunctional properties of ADSCs.

4.1. PHASE 1: Participant recruitment, clinical data collection and subdivision of cohorts

4.1.1. Participant recruitment

Reproductive aged (18 - 45 years), Black South African women of Xhosa ancestry (*n=47*) were recruited *via* flyers or word-of-mouth, from peri-urban areas surrounding the Tygerberg tertiary hospital in Cape Town, Western Cape. Participation in the study was completely voluntary and participants were required to sign an informed consent form (Appendix A, pg. 96) before inclusion in the study.

The following inclusion/exclusion criteria were used to recruit participants into specific groups:

- o **Healthy lean group** (*n=10*): *Inclusion criteria:* Black Xhosa women; aged 18 - 45 years; body mass index (BMI) ≤ 25 kg/m^2; normal fasting glucose level (< 5.6 mmol/L).

- o **Obese non-diabetic group** (*n=30*): *Inclusion criteria:* Black Xhosa women; aged 18 - 45 years; BMI ≥ 30 kg/m^2.

- o **Obese type 2 diabetic group** (*n=7*): *Inclusion criteria:* Black Xhosa women; aged 18 - 45 years; BMI ≥ 30 kg/m^2, previously diagnosed with type 2 diabetes mellitus.

Exclusion criteria

- o Men

- o Women who are pregnant or breastfeeding

- o Women who suffer from any inflammatory disorders and/or infectious disease including HIV

Note: Following the clinical screening of participants the obese non-diabetic group (*n=30*) were further subdivided into a healthy obese (*n=11*) and metabolic syndrome (*n=19*) group. Refer to Chapter 5 (pg. 61) for detailed description of metabolic criteria used for the subdivision of this cohort.

The target study population was chosen based on unpublished observational data from the Endocrinology ward at Tygerberg hospital suggesting an increased incidence of type 2 diabetes mellitus (T2DM) in Black South African women. This observation is supported by Goedecke et al., (2013) demonstrating a higher prevalence of insulin resistance in Black South African women, indicative of a higher risk for the development of T2DM.

4.1.1.1. Clinical screening

Participants arrived at the Tygerberg hospital Endocrinology ward (A10) after a fasting period of at least 4 hours. Each participant was interviewed and a medical history, nutritional and lifestyle questionnaire completed (Appendix A, pg. 105). These questionnaires were compiled in collaboration with a dietician (Mrs L v Wyk, Tygerberg Hospital, Western Cape Government). The purpose of these questionnaires was to characterize our study population as well as to give an indication of their medical history.

In addition to the questionnaires, the clinical screening included non-invasive anthropometrical and body compositions measures (section 4.1.1.2) to assess the extent of obesity, blood pressure measures (section 4.1.1.3) to screen for hypertension, fasting blood glucose measures (section 4.1.1.4) to screen for T2DM and serum lipid panel analysis (section 4.2.1) to screen for dyslipidaemia.

4.1.1.2. Anthropometry and body composition

The following non-invasive anthropometrical measures were performed:

o Body mass index (BMI): Each participant's weight (kg) (standard platform scale) and height (m) (portable stadiometer) were measured and their BMI calculated using the formula: BMI = total body weight (kg) / height squared (m^2).

o Waist-to-hip ratio: Participants stood erect, arms at their sides, feet together and abdomen relaxed. A standard non-elastic measuring tape was used to measure the widest circumference of the buttocks (hip circumference) (cm) and the narrowest part between the umbilicus and the xiphoid process (waist circumference) (cm). This information was used to calculate the waist-to-hip ratio using the formula: waist circumference (cm)/hip circumference (cm).

Each participants' body composition was assessed using a Dual Energy X-ray Absorptiometry (DXA) scan (Hologic Discovery W, Serial Number 70215, Manufactured by Scientific Division of Hologic Inc, USA). The global DXA scan assessed the total bone mineral density (BMD), lean muscle mass as well as the percentage fat mass (%fat) and its distribution throughout specific body regions [left and right arms (Region 1); left and right legs (Region 2); trunk (Region 3); head (Region 4); android- (Region 5) and gynoid- (Region 6) regions] (Fig. 4.2).
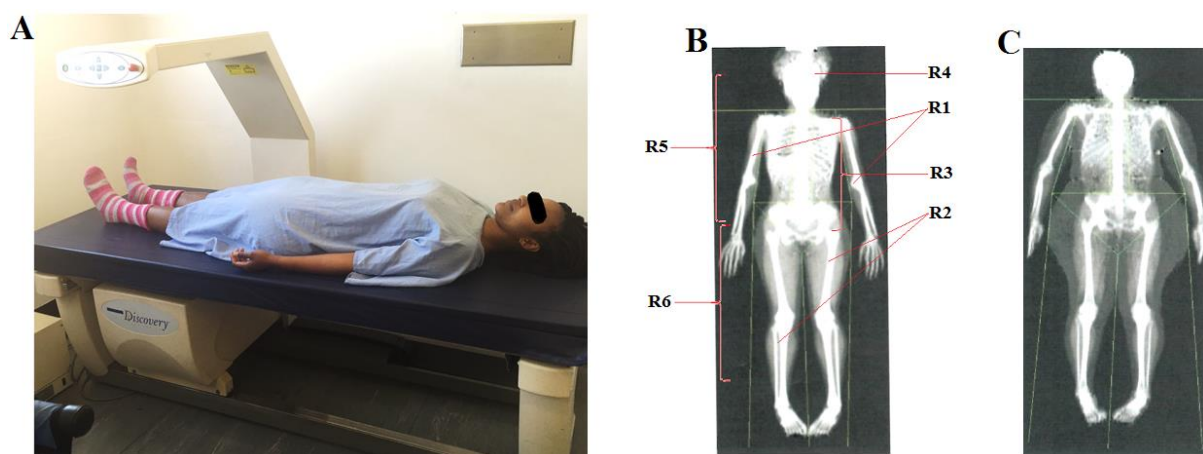
Figure 4.2. Body composition. A) Representative image of a study participant undergoing a DXA scan. **B-C)** Body composition comparison between a healthy lean (BMI ≤ 25) (**B**) and obese (BMI ≥ 30) (**C**) participant illustrating the different regions (R) on the DXA scanned images. R1 – Arms; R2 – Legs; R3 – Trunk; R4 – Head; R5 – Android region; R6 – Gynoid region. Specific care was taken to analyse the same regions on each participant's whole body scan.

4.1.1.3. Blood pressure

While seated in a resting position, systolic and diastolic blood pressure levels were measured using a standard blood pressure measuring cuff and stethoscope (Aneroid Sphygmomanometer, HI-CARE int.).

4.1.1.4. Fasting blood glucose

Fasting blood glucose levels were measured using a Contour plus glucometer system (BAYER, Germany). Each participant's finger was wiped with an alcohol swab to clear any dirt and debris before a small needle prick was made using a sterile lancet (Accu-chek; Roche, Germany). A drop of blood from the pricked finger was placed on a glucose measuring strip (Contour plus; BAYER, Germany) which was inserted into the glucometer. A reading between 3.9 - 5.6 mmol/L was considered normal. In the event of a reading > 5.6 mmol/L, the test would be repeated and the participant was asked to disclose any food/beverage consumption prior to data collection in order to confirm fasting status.

4.1.2. Blood sample collection and processing

Qualified phlebotomists collected blood samples from the antecubital vein of participants, using PrecisionGlide^{TM} Multi Sample Needles (BD Vacutainer®, USA). For each participant, blood was collected into one 10 mL Ethylenediaminetetraacetic acid (EDTA) vacutainer and three 5 mL serum separating (SS) tubes (SG Vac; BD, USA).

o Blood samples collected in the SS tubes were allowed to clot before centrifugation (Eppendorf 5804; Sigma-Aldrich, Germany) at 3000 RPM for 10 minutes. Serum was aliquoted into 1.5 mL microfuge tubes (500 μl serum per tube) and stored at -80 °C until subsequent analyses.

o Blood samples collected in the EDTA tubes were placed on ice immediately after collection, and used to determine each participant's differential white blood cell count (WBC) using histological analysis of blood smears as described below.

Four blood smears were prepared per participant: 1.5 to 2 μl of whole blood was pipetted onto each slide and an even smear created using a thin glass slide before leaving it to air dry for 1 to 2 minutes. The blood smears were then fixed in methanol for 30 seconds, and once again allowed to air dry for approximately a minute. Crystal violet stain (1:1000) (Sigma-Aldrich, Germany) was pipetted onto each slide (500 μl) and incubated at room temperature for 7 minutes. After staining, slides were dabbed onto paper towels in a vertical position, rinsed in distilled water to remove excess stain and allowed to dry before mounting with a coverslip using DPX mounting media (Saarchem; Merck, SA). The stained blood smears were viewed under a microscope (Primovert; Zeiss, Germany) at 40x magnification and five random images taken (Canon EOS 1000D camera) per slide (Fig. 4.3). Images were analysed using ImageJ software (v 6.0, NIH, USA) and the total count of red and white blood cells recorded. Each image was loaded onto the ImageJ software and a grid with an area of 0.54 mm^2 was overlaid onto the image. The total number of red blood cells (RBCs) were counted in 4 random squares on the grid for each image. The white blood cell (WBC) count for each participant were calculated as the average number of WBCs per 1000 RBCs. Different WBC subtypes were identified based on morphology according to a Basic Histology Text & Atlas (Junqueira & Carneiro, 2005).
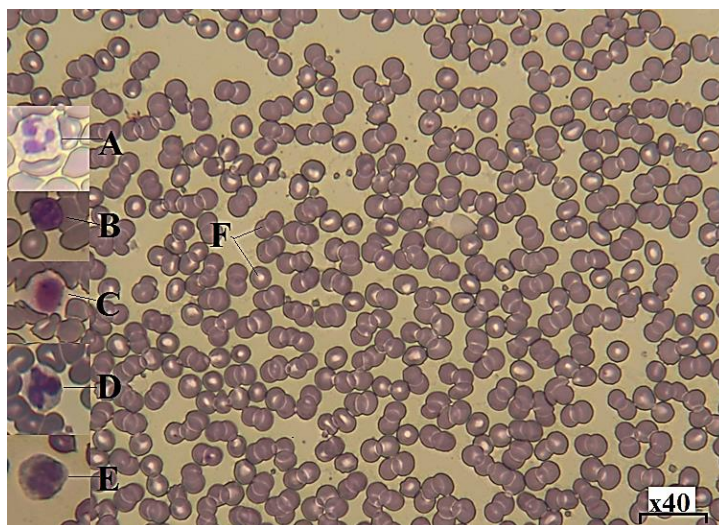
Figure 4.3. Representative image of a whole blood smear demonstrating the different blood cell types. **A)** Neutrophil. **B)** Basophil. **C)** Lymphocyte. **D)** Monocyte. **E)** Neutrophil. **F)** Red blood cell.

4.2. PHASE 2: Determination of each participants' metabolic and inflammatory profile.

4.2.1 Serum Lipid profile

Serum was analysed using an automated lipid panel test cartridge (*#10183107*, Alere Afinion[TM] Lipid Panel; Alere, USA) in combination with the Afinion AS100 Analyzer (Alere, USA) (Fig. 4.4).

Principle of the analysis: Total cholesterol (mmol/L) and triglyceride levels (mmol/L) are measured by an enzymatic colorimetric method. Esterified and free cholesterol gets enzymatically converted into cholest-4-en-3-one and hydrogen peroxide which is used by hydrogen peroxidase to couple phenol and 4-aminoantipyrin to a red quinine-imine dye. Triglycerides are enzymatically converted into glycerol by lipoprotein lipase. Glycerol is then further catalysed in 2 steps to di-hydroxy-acetone-phosphate and hydrogen peroxide. The hydrogen peroxide reacts with 4-aminophenazone and 4-chlorophenol through peroxidase to form a red dyestuff. The colour intensity of these reactions is directly proportional to the concentration of triglycerides and cholesterol in the sample.

To measure high density lipoprotein (HDL) (mmol/L); in a first reaction (R-1), anti-human apoliprotein B (apoB) antibody binds to apoB present on all lipoproteins except HDL (i.e. non-HDL) (R-1). The antibody protects non-HDL from being degraded by pegylated cholesterol metabolizing enzymes in the second reaction (R-2). In R-2, free and esterified cholesterol of HDL are converted into cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide is used by peroxidase to couple 4-aminoantipyrin to F-DAOS and forms a blue colour complex of free and esterified HDL cholesterol.

Low density lipoprotein (LDL) cholesterol is calculated using the Friedwald formula: LDL (mmol/L) = total cholesterol – HDL – triglycerides/2.2.
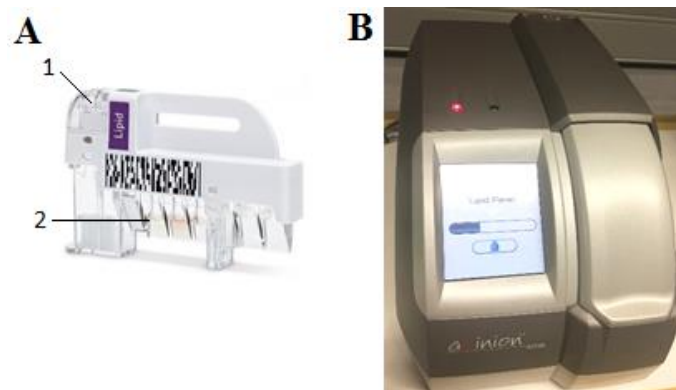
Figure 4.4. Alere Afinion Lipid Analyser. **A)** Lipid panel test cartridge with 1) capillary for sample loading and 2) reaction wells. **B)** Afinion AS100 Analyzer.

4.2.2 Cytokine analyses

4.2.2.1 Serum derived factor – 1 alpha (SDF-1 α)

A Human ELISA kit (*#0184080916*; Thermo Scientific, USA) was used to determine serum SDF-1α concentrations according to the manufacturers' instructions. *Principle of the assay:* samples are incubated with a pre-coated antibody (i.e. anti-SDF-1α). A streptavidin-HRP solution is added as the enzyme-linked antigen to detect presence of and bind to the antibody in the sample. Then a colourless substrate solution is added which produces a colour to indicate presence of antibody.

- ✓ Serum was thawed on ice for approximately three hours prior to experiment and all reagents (provided in the kit) were brought to room temperature (RT).

- ✓ Standards (serial dilutions: 0-6000 pg/mL) and serum samples were added to specific wells (in duplicates, i.e. *n=2*) in a pre-coated 96-well plate (100 μl per well) and incubated at RT for 2h 30 min on a platform shaker (Thermofisher, USA). Wells were washed four times with 300 μl 1X wash buffer per well, between steps.

- ✓ Contents were discarded and 100 μl Streptavidin-HRP solution was added in each well and the plate incubated for 45 min at RT, on a platform shaker.

- ✓ Contents were discarded and 100 μl TMB substrate solution (*#H032916*) was added per well and incubated for 30 min at RT on a platform shaker.

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- ✓ When colour change was apparent, the plate was removed from platform shaker and 50 µl Stop solution was added in each well.

- ✓ Absorbance was read at 450 nm (constant 37 °C) using a standard spectrophotometer (Multiskan GO 1.00.40, Thermo-Scientific Group, USA).

Results obtained from the spectrophotometer were exported into an Excel sheet and the linear regression model was used to generate a standard curve. The equation: $y = ax - c$, with $R^2 = 0.98$, was used to calculate the concentration of each sample.

4.2.2.2 C-Reactive Protein (CRP)

A sandwich CRP Human ELISA kit (*ab99995,* Abcam, UK) was used to analyse serum according to manufacturer's instructions. *Principle of the assay*: Samples are added to wells that are pre-coated with a specific antibody (i.e. anti-CRP). The CRP present in the samples binds to the wells by the immobilized antibody. A biotinylated antibody HRP-conjugated Streptavidin is added to the wells to detect presence of antibody in samples. Then a TMB substrate solution is added to the wells and colour develops in proportion to the amount of CRP bound.

- ✓ Samples were thawed on ice for 3 hours prior to use in the experiment and all reagents were brought to RT. Wells were washed four times with 300 µl 1X wash buffer per well, between steps.

- ✓ 100 µl of each standard (serial dilutions ranging from 0-25 000 pg/mL) and sample were pipetted into specific wells in the pre-coated 96-well plate and incubated overnight in a 4 °C cold room, on a platform shaker.

- ✓ The next morning, 100 µl of the 1X Biotinylated anti-Human CRP detector antibody was added in each well, after a washing. This was incubated for 1h at RT on a platform shaker.

- ✓ Solution was discarded and 100 µl 1X HRP-Streptavidin solution was added to each well and plate was incubated for 45 min at RT on a platform shaker.

- ✓ The solution was discarded and 100 µl of TMB One-Step substrate reagent was added to each well and incubated at RT for 30 min, on a platform shaker.

- ✓ Then 50 µl Stop Solution was added to each well and the absorbance read at 450 nm (Multiskan GO 1.00.40, THERMO-Scientific Group, USA). Results obtained from the reader were exported onto an excel sheet and a linear regression model was used to generate a standard curve. The equation $y = ax^2 - bx + c$, with $R^2 = 0.99$, was used to calculate the concentration of CRP in each serum sample.

4.2.2.3 Bio-Plex Pro^{TM} Human Cytokine assay: IL-2, IL-4, IL-6, IL-8, IL-10, IFN-γ, TNF-α, GM-CSF

A Bio-Plex Pro Human Cytokine 8-plex Assay kit (*#M50000007A*; Bio-Rad, USA) was used to assess the serum concentrations of interleukins; IL-2, IL-4, IL-6, IL-8, IL-10; interferon gamma (IFN-γ); tumour necrosis factor - alpha (TNF-α) and granulocyte macrophage - colony stimulating factor (GM-CSF). *Principle of the analysis:* The assay is a magnetic bead-based multiplex assay which uses an antibody directed against the desired targets that are covalently coupled to internally dyed beads. The assay was performed according to manufacturer's instructions using reagents supplied in the kit.

Serum samples were thawed on ice for approximately 2 hours prior to use in the assay and all reagents were brought to RT before use. The vial of assay standard stock was reconstituted with 500 µL Bio-Plex® sample diluent HB (Bio-Rad, USA). Table 4.1 displays the standard concentration ranges for each analyte.

Table 4.1. Normal physiological levels of group I human cytokines and the standard concentration ranges of the 8-plex cytokine assay

| Analyte | pg/mL | |
	Standard concentration range	Expected normal physiological range
GM-CSF	3.00 - 122	0.80 - 122
IFN-γ	7.00 - 124	0.60 - 124
IL-2	2.00 -90.0	0.60 - 124
IL-4	0.06 - 3.00	0.01 - 3.00
IL-6	0.50 - 9.00	0.02 - 9.00
IL-8	0.40 -116	0.08 - 116
IL-10	0.40 - 2.00	0.10 - 2.00
TNF-α	6.00 - 98.0	0.10 - 98.0

Footnote: Information derived from *Bio-Plex suspension array system tech note 6029.* Bio-Rad Laboratories, USA.

✓ The diluted assay beads were vortexed for 20 seconds and 50 μl were added in each well in the 96-well assay plate and incubated at RT for 5 min without agitation.

✓ Samples, standards and blanks were vortexed then 50 μl were added to specific wells. Plate was covered with foil and incubated at RT on platform shaker for 30 min.

✓ 25 μl detection antibody was added in each well and the plate incubated for another 30 min on platform shaker.

✓ 50 μl of 1X SA-PE were added to each well and plate incubated on platform shaker for 10 min.

✓ Assay beads were resuspended by adding 125 μl assay buffer in each well and plate was incubated on platform shaker for 30 seconds before analysis on the Luminex Bio-Plex 200 System (Bio-Rad; USA) (Fig. 4.5A).

Note: In between each of the above mentioned steps, the plate was washed twice with 100 μl 1X wash buffer per well, using an automated wash station (Bio-Plex[TM] Pro Wash Station; Bio-Rad, USA) (Fig. 4.5B).

Data were presented as the fluorescent intensity on the Bio-Plex Manager^{TM} software (Bio-Rad, USA). The observed concentration of each target molecule was calculated from its fluorescent intensity based on the 5PL regression standard curve generated for each target.
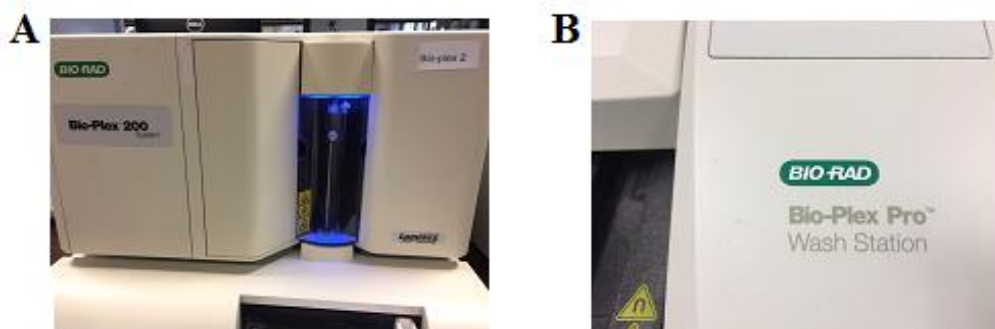
Figure 4.5. Bio-Rad Multiplex system. A) Luminex Bio-Plex 200 system. **B)** Bio-Plex Pro wash station.

4.3. PHASE 3: Investigating the effect of serum factors on the multifunctional properties of adipose-derived mesenchymal stem cells

All of the *in vitro* cell culture experiments (i.e. Phase 3 of the study) were performed using a primary human adipose-derived mesenchymal stem cell (hADSCs) line (Donor 26508, *#000034977*, Poeitics; Lonza, Switzerland). The initial 1 mL cryovial which was stored in liquid nitrogen, contained 1×10^6 immortalized hADSCs. The 1 mL ADSC cell stock (Passage 0) was thawed quickly in a 37 °C warm water bath and the cell number expanded for the initial passage at 1:20 dilution using cell culture dishes with 55 cm^2 surface growth area. Cells were maintained in standard cell growth medium (SGM) at 37 °C, 5% CO_2 and 90% humidity.

SGM consisted of 90% Dulbecco's Modified Eagle Medium (DMEM) with Ultraglutamine, containing 1% penicillin/streptomycin (pen/strep) (BioWhittaker; Lonza, Switzerland) and 10% Fetal Bovine Serum (FBS) (Biochrom, Germany). SGM was replaced every 4 days and cells were sub-cultured at approximately 85% confluence. For the initial passage, cells from 19 of the dishes were cryopreserved and cells from only one dish were further sub-cultured (passage 1).

Sub-culturing of cells:

✓ At 85% confluence, cells were washed twice with 3 mL phosphate buffered saline (PBS) (Refer to Appendix C for detailed preparation of PBS).

✓ 1 mL Trypsin (200mg/L Versene EDTA, *#BE171616*; Lonza, Belgium) was added in order to break down cell membrane integrity allowing for cells to detach from bottom of dish, then 2 mL SGM was added to stop Trypsin activity.

✓ The cell suspension was added to a clean 15 mL conical tube (Falcon; BD, USA) and centrifuged at 1000 RPM (Eppendorf Centrifuge 5804; Sigma-Aldrich, USA) for 5 minutes. The supernatant was discarded and cells were resuspended in 1 mL SGM.

✓ 20 μl cell suspension was collected for cell counting. The cell suspension was added into the haemocytometer chamber, and viewed under a microscope (Primovert; Zeiss, Germany) at 40x magnification. Refer to Appendix C for a detailed description of the cell counting. Cells were further plated in new 100 mm culture dishes (55 cm^2) containing 10 mL SGM, according to the recommended seeding density (Table 4.2) and incubated at 37 °C, 5% CO_2 and 90% humidity.

Freezing of cells:

Cells were washed with PBS, trypsinized and centrifuged as previously described, then resuspended in 1 mL freezing media. Freezing media contained 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany), 80% DMEM with Ultraglutamine (containing 1% pen/strep) and 10% FBS. Cells were stored in 1.5 mL cryogenic vials (Corning Inc., USA) at -80 °C overnight, then at -196 °C in liquid nitrogen. Refer to Table 4.2 for the seeding density.

Table 4.2. Recommended seeding densities according to culture insert diameters.

Corning dishes/Transwell Insert format	Approximate Growth Area (cm^2)	Seeding density (at 5 000 cells/cm^2)	Average Cell Yield
100 mm	55	$2.75 * 10^5$	$5.5 * 10^6$
96 well	0.143	$1.6 * 10^3$	$1.4 * 10^4$
24 well	0.33	$9.5 * 10^3$	$3.3 * 10^4$

4.3.1. Characterization of ADSC cell line using flow cytometry.

Human adipose derived stem cells (ADSCs) (Donor 26508, *#000034977*, Poietics) were purchased from Poietics (Lonza; Basel, Switzerland). The ADSCs were obtained as lipoaspirates from patients undergoing elective liposuction surgery procedures and after purification, the phenotype characterization (CD13+, CD29+, CD44+, CD73+, CD90+, CD105+, CD166+ and CD14-, CD31-, CD45-) as well as quality control checks (HIV-, hepatitis B/C-, mycoplasma-free, bacteria-free, fungi-free, yeast-free) were performed by the manufacturer (Poietics; Lonza, Basel, Switzerland). The identity of the ADSCs were confirmed in our laboratory by using the MACS Miltenyi Biotech human MSC phenotyping kit (*#130-095-198*; Miltenyi Biotech Inc., USA) and the FACS Calibur flow cytometer with CellQuest software (BD, USA). ADSCs (80% confluent, passage 7) were harvested through trypsinization and resuspended in PBS containing 20% FBS (Biochrom, Germany). Cell suspension at a concentration of $1x10^6$ cells were co-labelled with a cocktail of fluorochrome-conjugated monoclonal antibodies: Positive markers – CD90-FITC (clone DG3, isotype IgG1), CD73-APC (clone AD2, isotype IgG1), CD105-PE (clone 43AE1, isotype IgG1) and Negative markers - CD14-PerCP (clone TUK4, isotype IgG2a), CD20-PerCP (clone LT20.B4, isotype IgG1), CD34-PerCP (clone AC136, isotype IgG2a), CD45-PerCP (clone 5B1, isotype IgG2a) (Miltenyi Biotech Inc., Auburn, USA). Since a multicolour cytometric analysis was performed, fluorescent compensation settings were established through a compensation experiment and regions of positive and negative staining were determined through a fluorochrome minus one (FMO) experiment. An isotype control cocktail (IgG1-FITC/PE/APC/PerCP, clone IS5-21F5 and IgG2a-PerCP, clone S43.10) (Miltenyi Biotech Inc., Auburn, USA) was used as a negative control for gating purposes. Data analysis was performed using Flow Jo Vx (Treestar, Oregon, USA) software.

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Table 4.3. Acquisition settings on the FACS Calibur

	Detector	V	A	Mode
	FSC	E-1	4.75	Lin
	SSC	366	1.00	Lin
FITC	FL1	366		Log
PE	FL2	327		Log
PerCP	FL3	381		Log
APC	FL4	463		Log

Footnote: The above acquisition settings were applied for the flow cytometric analyses. **Abbr.**: V – voltage; A – amperes.

4.3.2. Treatment of ADSCs with participant derived serum

Participant-derived serum was sterile filtered prior to use in experiments. Using a glass syringe, serum was filtered through a sterile filter *(#14140003907*; Cellulose Acetate Membrane, GVS, England, UK) into a clean 15 mL conical tube (Falcon; BD, USA). This was then used in treatment medium consisting of 20% participant-derived serum and 80% DMEM with Ultraglutamine (containing 1% pen/strep). The treatment medium was used in subsequent experiments to investigate the effects of participant-derived serum ADSC function.

4.3.2.1. Cell proliferation assay

The 5-bromo-2'-deoxyuridine (BrdU) proliferation assay was used to measure the mitogenic response of cultured ADSCs following exposure to participant-derived serum for a period of 24-hours. All experiments were done in triplicate.

For the initial experiment, standard cell culture growth media containing different concentrations (%) of FBS were used to determine coefficient of variance (CV) in order to validate our method and the reproducibility thereof.
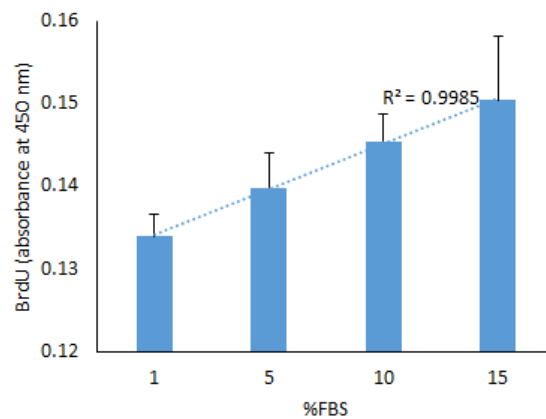
Figure 4.6. BrdU proliferation assay. Graph showing results of the BrdU cell proliferation assay at different concentrations of serum in the growth medium. CV = 4.99 %

ADSCs were cultured in a 96-well plate (refer to Table 4.2, pg. 53, for seeding density). SGM was added to make up a volume of 100 μL per well, these were incubated at 37 °C, 5% CO_2 and 90% humidity until ADSCs adhered to the bottom of the dish (approximately 5 hours). Once adhered, ADSCs were serum starved by removing all SGM from wells and adding 100 μL cell media containing 1% FBS, in each well, and incubating overnight. The following day, the 1% SGM was discarded and new media was added into specific duplicate wells at concentrations of 1%, 5%, 10% and 15% FBS, respectively. These were incubated for 24 hours.

A BrdU cell proliferation kit (*#11417600*, Roche; Switzerland) was utilised as per the manufacturer's instructions. At 20 hours of incubation, 100 μL BrdU labelling reagent was added in each well and incubated for a further 4 hours. During this labelling period, the BrdU becomes incorporated into the DNA of proliferating cells which are then detected by the anti-BrdU antibody. After 24 hours of incubation, media was removed and 200 μL FixDenat solution was added in each well and the plate was incubated for 30 minutes at room temperature without agitation. Solution was removed and 100 μL anti-BrdU-POD working solution was added to each well and the plate was then incubated for 90 minutes at RT. The antibody was removed and wells were washed three times with 300 μL washing solution. Then 100 μL Substrate solution was added in each well, the plate was covered with foil and incubated at RT for 12 minutes until colour developed. The plate was inserted into a spectrophotometer to

measure absorbance at 450 nm and 550 nm. Results obtained from the reader were exported onto an excel sheet and a graph was generated (Fig. 4.6).

Subsequent experiments were performed using cell media containing 20% participant serum as treatment.

4.3.2.2. Cell viability assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) *In Vitro* Toxicology assay kit (*#SLBM0752V*, Sigma-Aldrich; Germany) was used to measure cell viability. *Principle of assay*: The mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple formazan crystals which can be dissolved in acidified isopropanol. The absorbance of the resulting purple solution is measured spectrophotometrically. An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test material.

All experiments were repeated three times in duplicate wells. In the initial experiment, different concentrations (%) of FBS in standard cell culture media were used as treatment. This was in order to check the validity and reproducibility of our method.
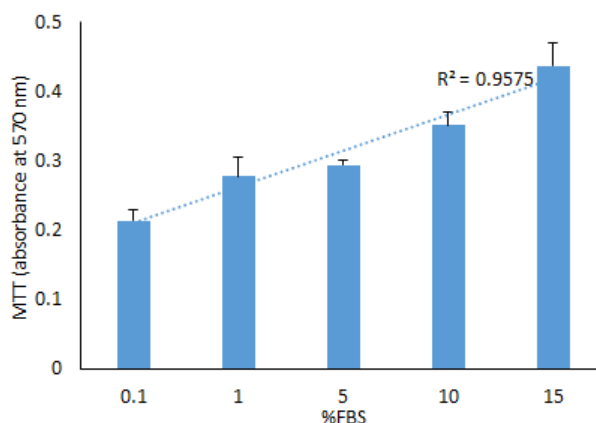
Figure 4.7. MTT *In Vitro* Toxicology assay. Graph showing results of the MTT *in vitro* Toxicology assay, using different concentrations of serum in the cell culture medium. Values obtained at the reference wavelength of 690 nm were subtracted from 570 nm to produce the values represented on graph, to correct for any background absorbance.

Cells were cultured in a 96-well plate (refer to Table 4.2, pg. 53 for seeding density). SGM was added in each well to a total volume of 100 μL. Plates were incubated at 37 °C, 5% CO_2 and 90% humidity until 100 % confluence (7 days on average, with media change after 4 days). At 100 % confluence, media was discarded and cell culture media containing 0.1%; 1%; 5%; 10% and 15% FBS were added to specific wells (100 μL) and plates were incubated for a further 24 hours.

A vial of 15 mg MTT (*#MKBR4419V*, Sigma-Aldrich, Germany) was reconstituted in 3 mL DMEM with Ultraglutamine (containing 1% pen/strep). After 20 hours of incubation, 10 μL reconstituted MTT reagent was added in each well and plates were incubated for further 4 hours, for the formazan crystals to form. After the incubation period (24 hours in total), 100 μL MTT solubilisation solution was added to each well and triturated with a pipette to dissolve the crystals. After approximately 5 minutes, the plate was inserted into a spectrophotometer to measure absorbance at wavelengths of 570 and 690 nm. Results from the spectrophotometer were exported into an excel sheet. Values obtained at 690 nm were subtracted from values at 570 nm (to reduce background absorbance).

Subsequent experiments were performed using cell media containing 20% participant derived serum as treatment.

4.3.2.3. Cellular migration assay

In order to investigate the effect of participant derived serum on the migration response of ADSCs, we performed a migration assay using 2 well μ-plates (ibidi, Germany). ADSCs were cultured at a seeding density of ~3.0 x 10^4 cells per well. 70 μL cell suspension was added to each of the wells/chambers of the μ-plate, the culture insert (Fig 4.8A) produced the *in vitro* wound (Fig. 4.8B) which is used to measure migration response. These were incubated at 37 °C, 5% CO_2 and 90% humidity overnight.
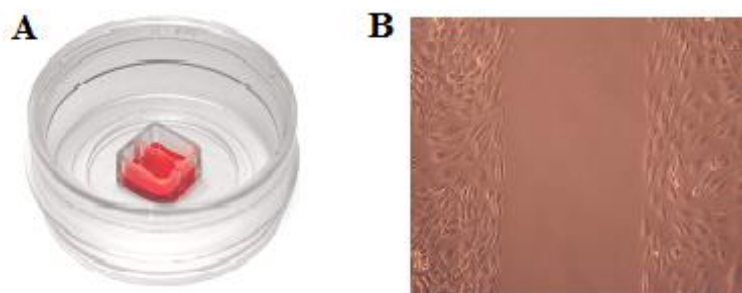
Figure 4.8. Migration assay. A) The ibidi 2 well µ-plate dish with culture insert intact. B) ADSCs as cultured in the µ-plate dish, after removal of culture insert, displaying the *in vitro* wound at 0 hours.

The following morning, cells were 100% confluent and adhered to bottom of dish. The culture insert was removed using sterile tweezers. SGM was added and three random images were taken (EOS600D Canon digital camera) of the *in vitro* wounded area, at 0, 4, 7 and 24 hours (Fig. 4.8B) using a light microscope (10x/0.25 PhP objective; Primovert, Zeiss, Germany). The cell free area was calculated by tracing along the border of the *in vitro* wound using Image J software (version 6.0, NIH, USA) and the surface area (μm^2) of the cell-free gap was determined. The percentage gap closure (% gap closure) was calculated using the following equation: [(area 0h –area nh) / (area 0h)] x100.

4.4. Statistical analyses

Data are presented as mean ± standard error (SE). Statistical analyses were performed using Statistica software (version 10, StatSoft, South Africa). One Way ANOVA with Tukey post hoc test was used to determine group effects. In cases where data was not normally distributed, non-parametric Kruskall Wallis ANOVA with Dunns multiple comparison test was used to determine differences between groups. For *in vitro* experimentation, the non-parametric Mann Whitney-U test was used to determine differences between treatment groups and standard culture conditions. Factorial ANOVA with Tukey post hoc test was used to determine the effect of group vs time. Case-wise Product-Moment correlation analysis was used to determine associations between serum cytokine concentrations and changes in the functional capacity of ADSCs. Level of significance was accepted at $p < 0.05$.

Chapter 5

Results

5.1. PHASE 1: Participant demographics

A total of forty-seven (*n=47*) reproductive aged (32 ± 1.1 years) (*mean ± SE*) females were included in the study. These participants were recruited to specifically fit into one of three groups as described in the inclusion/exclusion criteria (pg. 42). The initial participant grouping was as follows: 1) Ten (*n=10*) healthy lean (BMI ≤ 25 kg/m^2, age 25.6 ± 1.5 years), 2) thirty (*n=30*) obese non-diabetic (BMI ≥ 30 kg/m^2, age 32.3 ± 2.2 years) and 3) seven (*n=7*) previously diagnosed type 2 diabetic (T2DM) (BMI ≥ 30 kg/m^2, age 39.9 ± 1.1 years) patients. All participants had a similar socio-economic background and other than a higher rate of occasional alcohol consumption in the younger healthy lean participants (70%) compared to the other groups (obese 33.3% and T2DM 14.3%) (*p < 0.05*). No differences were observed in the nutritional or lifestyle habits of participants (Table 5.1.1 and Appendix B).

Table 5.1.1. Summary of participant characteristics as reported on the medical history, nutritional and lifestyle questionnaires.

Characteristics	Healthy lean (n=10)	Obese (n=30)	T2DM (n=7)
Age	25.6 ± 1.5	32.3 ± 2.2*#	39.9 ± 1.1*
BMI (kg/m^2)	22.1 ± 2.7	37.1 ± 4.6	30.6 ± 3.6
Lifestyle			
Physical activity	2.3 ± 1.1	2.5 ± 0.7	2.86 ± 1.1
Sleeping pattern (> 6 hrs)	80%	83%	85%
Smoking	30%	12%*	0%*
Alcohol consumption	70%	33%*	14%*
Nutrition			
Meals per day	2.6 ± 0.5	2.5 ± 0.7	2.43 ± 0.5
Snacking in between	1.6 ± 1.2	1.03 ± 1.2	1.42 ± 1.2
Tea/coffee tsp sugar	1.9 ± 1.3	2.9 ± 1.4	1.7 ± 1.1
Medication/supplements			
Contraceptives	Y = 3	Y = 3	Y = 0
BP medication	-	ND = 2	ND = 1
		Lisinopril = 1	Accord glimepiride = 2
			Hydrochlorothiazide = 2
			Gliclazide = 1
Cholesterol	-	-	Simvastatin = 2
Diabetic treatment	-	-	Indo metformin = 6
			Glucophage =1
			Insulin injections = 2

Footnote: Data is presented as mean ± SE. *Physical activity* was rated according to a modified Borg scale: 1 = sedentary; 2 = light activity; 3 = moderate activity and 4 = vigorous activity. *Sleeping pattern* refers to the percentage of participants within each group who regularly sleep more than 6 hours at night. *Smoking* and *alcohol consumption* refers to the percentage of participants in each group who reported the occasional use of these substances. *Meals* and *snacking* refer to the average amount of times in a day in which participants consumed full meals or snacks. *Medication: "=" refers to the number of participants in that particular group who reported the use of medication.* **Statistical analyses**: One way ANOVA with Tukey's posthoc test. *$p < 0.05$ indicates significant difference between a specific group compared to healthy lean group. #$p < 0.05$ indicates significant difference between groups. **Abbr.:** BP – blood pressure; ND – not disclosed; Y – yes.

5.2. PHASE 2: Metabolic and inflammatory profile of participants

Following assessment of each participant's metabolic risk factors (Table 5.2.1), the non-diabetic obese participants (BMI $\geq$ 30 kg/m^2) (*n=30*) were further subdivided into healthy obese (*n=11*) (< 3 metabolic risk factors) and metabolic syndrome (*n=19*) ($\geq$ 3 metabolic risk factors) groups. The criteria used for identifying individuals with metabolic syndrome is described in Table 5.2.1 and complied with the recommendations of the International Diabetes Federation (IDF, 2005).

Table 5.2.1. Metabolic risk factors associated with metabolic syndrome.

Risk factor	Normal physiological values
Fasting blood glucose (mmol/L)	$\leq$ 5.60
Systolic BP (mmHg)	$\leq$ 130
Diastolic BP (mmHg)	$\leq$ 85
Triglycerides (mmol/L)	1.70 - 2.25
HDL (mmol/L)	1.04 - 1.55
LDL (mmol/L)	2.59 - 3.34
Total cholesterol (mmol/L)	$\leq$ 6.20
Waist-to-hip ratio	$\leq$ 0.85

Footnote: Expected normal physiological values that pose no metabolic risk. Participants were classified as having metabolic syndrome if (in addition to BMI $\geq$ 30kg/m^2) more than three (>3) of the above metabolic risk factors were abnormal. Criteria used according to the International Diabetes Federation's recommendations (IDF, 2005). **Abbr.:** BP – blood pressure; HDL – high density lipoprotein; LDL – low density lipoprotein.

Following subdivision of the obese participants based on their metabolic risk, indication of dyslipidaemia, the body composition of participants and their inflammatory cytokine profiles were compared.

5.2.1. Metabolic profile

The metabolic profile of healthy lean (*n=10*), healthy obese (*n=11*), obese metabolic syndrome (*n=19*) and T2DM (*n=7*) participants are represented by Figures 5.2.1 and 5.2.2 (pg. 64). As expected, T2DM participants had significantly elevated fasting blood glucose levels (8.8 ± 3.8

mmol/L) ($p < 0.05$) compared to the other groups whom were all within normal physiological range (< 5.6 mmol/L) (Fig. 5.2.1A). Although the BMI of all the obese participants (healthy obese, metabolic syndrome and T2DM) were significantly higher than that of the healthy lean group (22.1 ± 0.84 kg/m^2), the T2DM participants had a lower BMI (30.6 ± 1.4 kg/m^2) compared to the metabolic syndrome participants (37.8 ± 1.0 kg/m^2) (Fig. 5.2.1B). The waist-to-hip-ratio was however abnormal (> 0.85) in both the metabolic syndrome and T2DM groups (Fig. 5.2.1C). None of the participants had severe hypertension, although blood pressure levels were slightly higher in the metabolic syndrome group (systolic 127.7 ± 16.5 mmHg; diastolic 83 ± 7.9 mmHg) ($p < 0.05$) compared to that of healthy individuals (Fig. 5.2.1D).

Participants' serum was furthermore analysed for total cholesterol, triglyceride, low density lipoprotein (LDL) and high density lipoprotein (HDL) levels in order to assess for signs of dyslipidaemia. Healthy lean participants tended to have lower total cholesterol (3.81 ± 0.12 mmol/L), whilst a tendency was observed for increasing levels at the different metabolic stages (healthy obese 4.1 ± 0.24 mmol/L; metabolic syndrome 4.5 ± 0.28 mmol/L; T2DM 4.7 ± 0.35 mmol/L) (Fig. 5.2.2). Participants in the T2DM group had significantly higher triglyceride levels (1.56 ± 0.66 mmol/L) ($p < 0.01$) compared to the healthy lean (0.58 ± 0.04 mmol/L), healthy obese (0.65 ± 0.05 mmol/L) and metabolic syndrome (0.79 ± 0.04 mmol/L) groups. Significantly higher levels of LDL were observed in the metabolic syndrome group (3.1 ± 0.9 mmol/L) ($p < 0.05$). There were no significant differences in the levels of HDL between the different groups (Fig. 5.2.2). Taken together, these results confirmed that our groups are representative of different metabolic stages typically expected in disease progression from healthy to obese metabolic syndrome and ultimately T2DM. Thus validating our model for subsequent *in vitro* experiments utilizing the participant-derived serum.
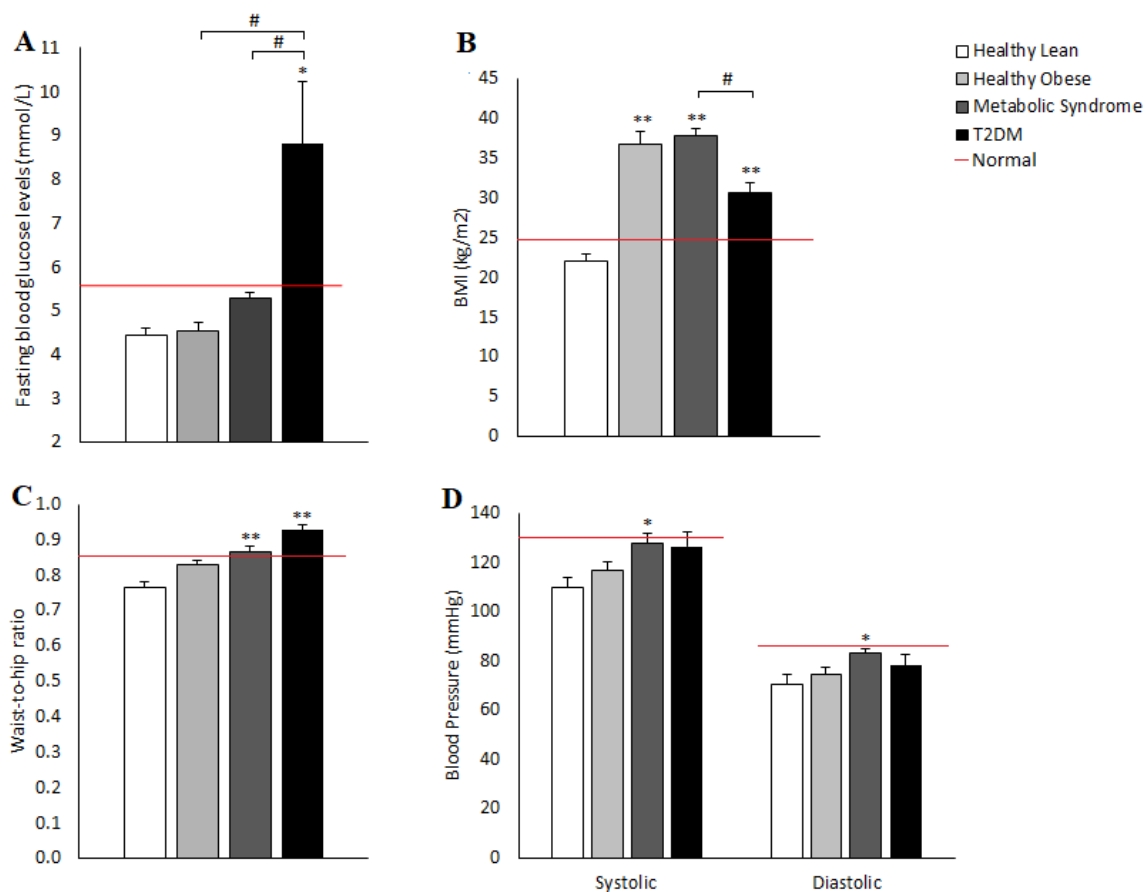
Figure 5.2.1. Metabolic markers. Graphic representation of the average **A)** fasting blood glucose levels **B)** BMI, **C)** waist-to-hip ratio and **D)** blood pressure levels in each group. **Statistical analyses:** One way ANOVA with Tukey's post hoc test. *$p < 0.05$; **$p < 0.01$ indicate significant difference between a specific group compared to healthy lean group. # $p < 0.01$ indicates significant differences between groups. The red line refers to normal healthy physiological values.
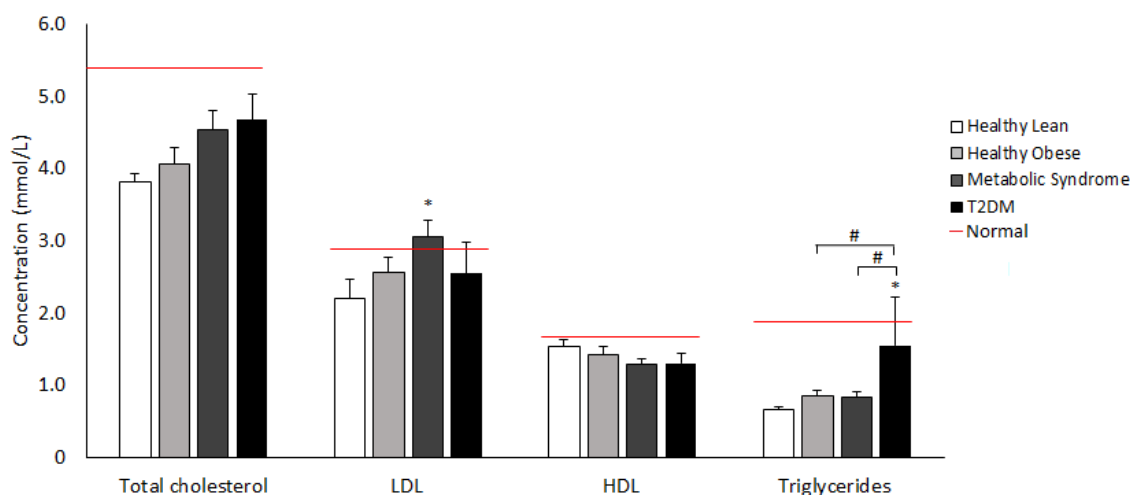
Figure 5.2.2. Lipid profile. Results of the lipid panel analysis showing total serum levels of cholesterol, LDL, HDL and triglyceride levels of participants in the different groups. **Statistical analysis**: One way ANOVA with Tukey post hoc test. *$p < 0.05$ indicates a significant difference between a specific group compared to healthy lean control group. #$p < 0.01$ indicates significant differences between groups. **Abbr.**: HDL – high density lipoprotein; LDL – low density lipoprotein; T2DM – type 2 diabetes mellitus. The red line refers to normal healthy physiological values.

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5.2.2. Body composition and bone mineral density

The body composition of each participant, with a specific focus on the distribution of fat throughout different body regions, were assessed and is presented in Table 5.2.2 as well as Figures 5.2.4 and 5.2.5. As expected, the healthy obese (49.3 ± 0.84 %) *(mean ± SE) (p < 0.001)* and metabolic syndrome (50.1 ± 0.73 %) *(p < 0.01)* groups had a significantly greater percentage of total body fat compared to the healthy lean group (37.5 ± 2.1 %). In contrast to the BMI data for the T2DM group (section 5.2.1, Fig. 5.2.1B), the percentage of total body fat in the T2DM group (40.3 ± 2.4 %) did not differ from the healthy lean group. The only significant differences in fat distribution between the healthy lean and the T2DM groups were observed in the gynoid region (healthy lean 44.0 ± 1.7 %; T2DM 42.0 ± 3.6 %) *(p < 0.05)*.

The adipose indices did however indicate a higher android-to-gynoid, as well as trunk-to-leg fat ratio in the T2DM (1.0 ± 0.04 & 1.1 ± 0.04), healthy obese (0.98 ± 0.02 & 0.93 ± 0.03) and metabolic syndrome (0.99 ± 0.02 & 0.97 ± 0.02) groups compared to the healthy lean group (0.82 ± 0.04 & 0.77 ± 0.04) *(p < 0.01)*. The metabolic syndrome and T2DM groups also displayed a significantly higher *(p < 0.05)* trunk-to-limb fat mass ratio compared to healthy lean participants that was not evident in the healthy obese group (Fig 5.2.4). Taken together, the whole body DXA scan demonstrated a normal body composition for healthy lean participants, whereas excessive body fat for healthy obese participants was predominantly in the subcutaneous regions (pear-shape body) and predominantly in the visceral regions (apple-shaped body) for the metabolic syndrome and T2DM groups.

In addition to adipose indices, the DXA scan also provided information regarding each participant's bone mineral density (BMD). There were no significant differences in BMD observed between the different groups. However, the metabolic syndrome group showed significantly higher T-score (1.1 ± 1.4) and Z-scores (1.4 ± 1.3) *(p < 0.05)* compared to the healthy lean group (0.04 ± 1.2 & 0.05 ± 1.1) (Fig. 5.2.5).

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Table 5.2.2. Summary of body fat distribution throughout specific body regions.

Body region	Healthy lean (n=10)	Healthy obese (n=11)	Metabolic syndrome (n=19)	T2DM (n=7)
L Arm	38.7 ± 3.5	50.4 ± 1.5*#	52.3 ± 1.3*#	41.5 ± 2.1
R Arm	37.0 ± 3.6	50.8 ± 1.5*#	51.6 ± 1.3*#	42.0 ± 2.5
Trunk	34.4 ± 2.8	49.2 ± 1.3***#	50.7 ± 0.9***#	40.8 ± 2.2
L Leg	44.1 ± 1.8	53.1 ± 0.9*#	52.6 ± 0.6*#	42.5 ± 3.4
R Leg	44.0 ± 1.6	52.7 ± 0.9*#	52.2 ± 0.7*#	42.0 ± 3.4
Android	36.3 ± 3.2	51.9 ± 1.1***#	51.5 ± 1.3***#	41.6 ± 2.9
Gynoid	44.0 ± 1.7	53.0 ± 0.9***#	52.2 ± 0.6***#	42.0 ± 3.6*
Total	37.6 ± 2.1	49.3 ± 0.8***#	50.1 ± 0.7***#	40.3 ± 2.4

Footnote: DXA scan results showing total percentage fat in the left (L) and right (R) arms, trunk, left (L) and right (R) legs, android and gynoid regions as well as the total percentage of fat in the body. **Statistical analysis:** Data is presented as mean ± SE. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ indicates significant differences between a specific group compared to the healthy lean group. #$p < 0.05$ indicates significant difference between a specific group and the T2DM group.
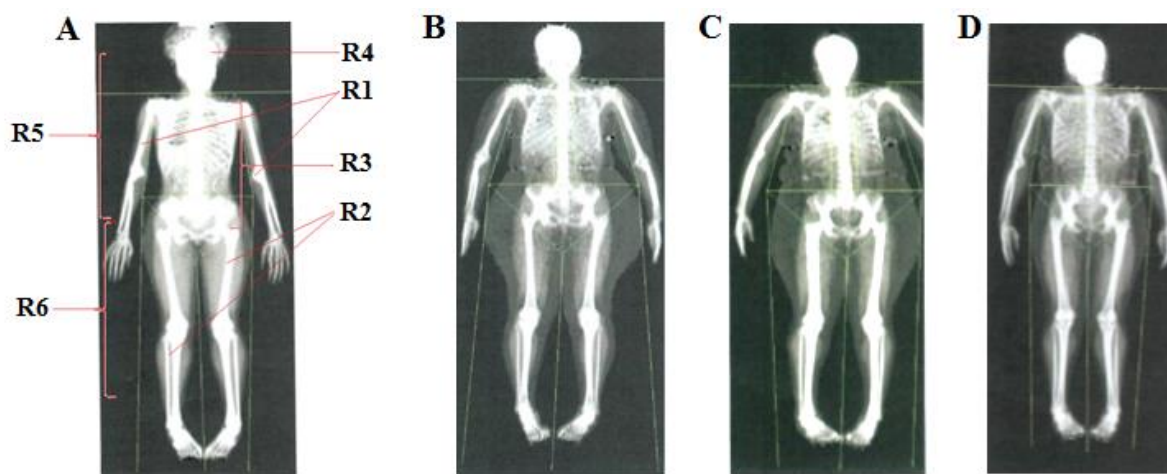
Figure 5.2.3. Dual energy x-ray absorptiometry (DXA) scans. Representative images of participants in the different groups. **A)** Healthy lean participant. **B)** Healthy obese participant. **C)** Metabolic syndrome participant. **D)** T2DM participant. **Abbr.:** R – region; R1 – left and right arms, R2 – left and right legs, R3 – trunk, R4 – head, R5 – android, R6 – gynoid.
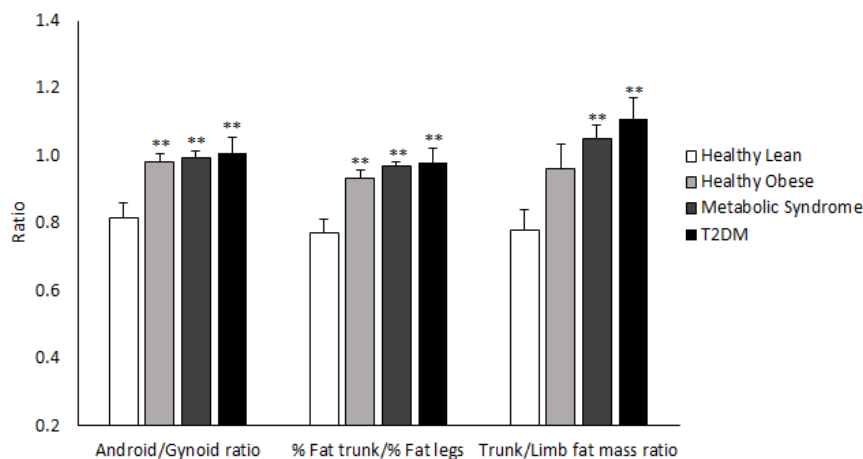
Figure 5.2.4. Adipose indices. DXA scan results of the ratio of fat in the android versus the gynoid regions; the percentage of fat in the trunk region versus percentage of fat in the leg region and trunk fat versus limb fat. **Statistical analysis**: One way ANOVA with Tukey post hoc test. **p < 0.01 indicates significant difference between a specific group compared to healthy lean group.
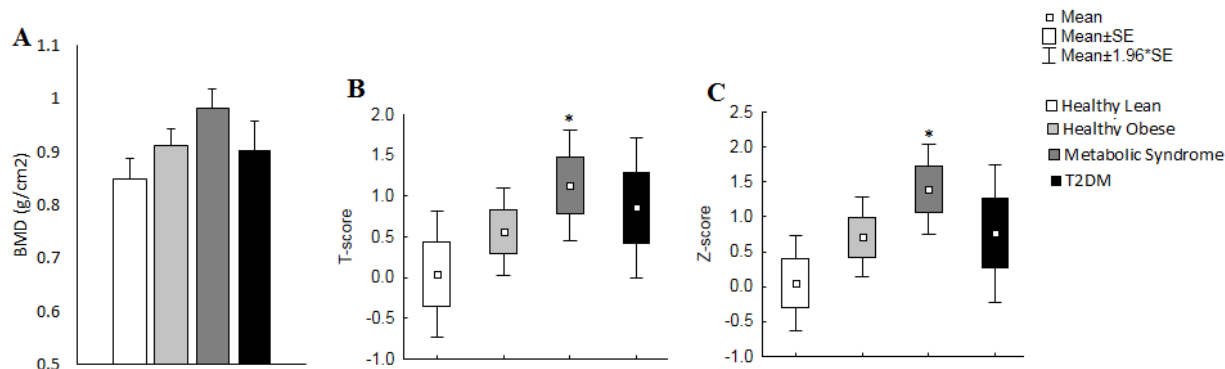
Figure 5.2.5. Bone mineral density. T-score refers to BMD values obtained from individual participants when compared to that of an average woman in their thirties. Z-score refers to BMD values obtained from individuals in the study, compared to that of women within their age range. T- and Z-scores $\geq$ -1.0 are considered normal for healthy individuals. **Statistical analysis**: One way ANOVA with Tukey post hoc tests. *p < 0.05 indicates significant difference between a specific group compared to the healthy lean control group. **Abbr.:** BMD – bone mineral density; cm^2 – centimetre squared; g – grams.

5.2.3. Inflammatory profile

The serum circulating levels of pro- (CRP, IL-6, IL-8, TNF-α, IFN-γ, SDF-1α) and anti-inflammatory (IL-10) cytokines were determined for each participant as indication of their overall inflammatory status. Individual variability was high and data are therefore presented as box-and-whisker plots in Figures 5.2.6 and 5.2.7 (pg. 68 - 69).

Significantly higher levels of the C-reactive protein (CRP) were observed in the metabolic syndrome group (50.87 ± 24.2 pg/mL) (*mean ± SE*) (*p < 0.05*) compared to the healthy lean group (6.08 ± 5.03 pg/mL), indicating inflammation in this group. The healthy obese group

(29.8 ± 8.78 pg/mL) also showed a tendency towards higher CRP levels, although this was not significant (*p=0.07*) (Figure 5.2.6A). Due to individual variability, no significant differences were observed in the circulating levels of pro-inflammatory cytokines interleukins – IL-6, IL-8, tumour necrosis factor - alpha (TNF-α) and interferon – gamma (IFN-γ), between the different groups (Fig. 5.2.6 B-E). Serum levels of SDF-1α were variable, especially in the metabolic syndrome (113.7 ± 26.6 pg/mL) and T2DM (139.3 ± 45.7 pg/mL) groups compared to the healthy lean (60.55 ± 4.3 pg/mL) and healthy obese (71.86 ± 4.86) groups, but these were not significant (Fig. 5.2.6B).
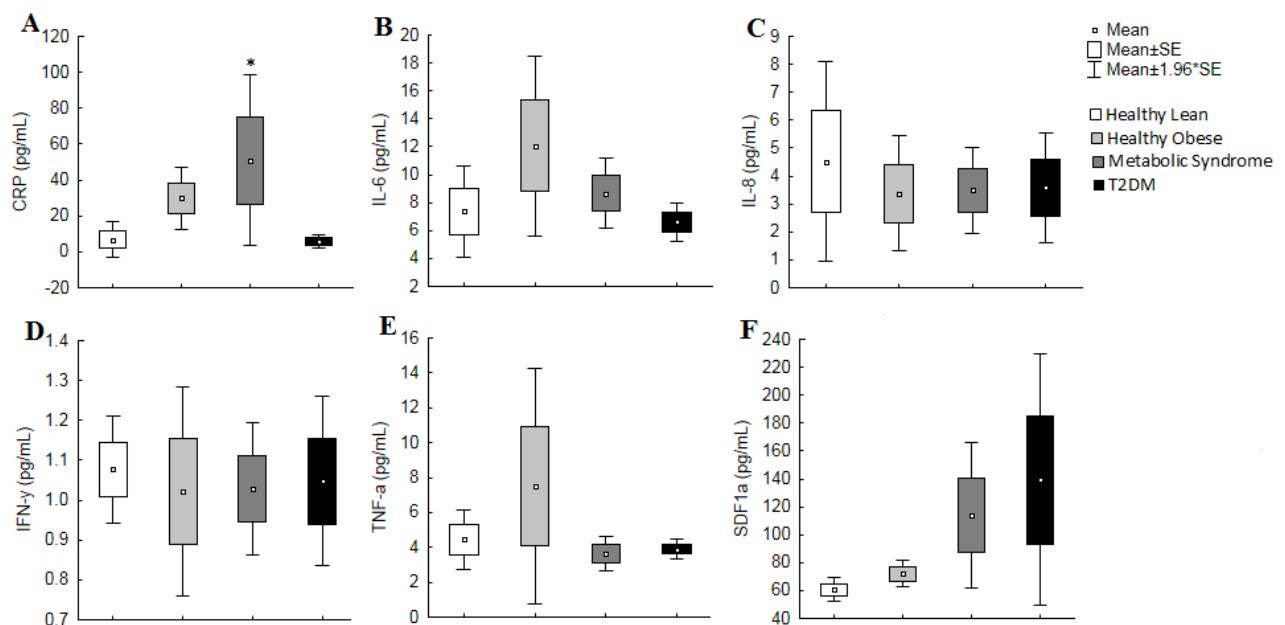
Figure 5.2.6. Pro-inflammatory cytokines. A) CRP **B)** IL-6 **C)** IL-8 **D)** IFN-γ **E)** TNF- α and F) SDF1- α levels as determined using the Bio-Plex Pro™ human cytokine and ELISA assays. **Statistical analyses**: One-way ANOVA with Tukey's post hoc test and Non-parametric Kruskall Wallis test with Dunns multiple comparison post hoc tests. *p < 0.05 indicates significant difference between a specific group compared to healthy lean group. #p < 0.05 indicates significant difference between groups. **Abbr.:** CRP – C-reactive protein; IFN- γ – interferon gamma; IL – interleukin; SDF-1 α – stromal derived factor-1 alpha; TNF- tumour necrosis factor.

Circulating levels of the anti-inflammatory cytokine IL-10 were significantly lower in the T2DM participants $(0.42 \pm 0.69$ pg/mL$)$ $(mean \pm SE)$ $(p < 0.05)$ compared to the healthy lean $(3.13 \pm 0.33$ pg/mL$)$, healthy obese $(7.10 \pm 4.1$ pg/mL$)$ and metabolic syndrome $(2.93 \pm 0.45$ pg/mL$)$ groups.
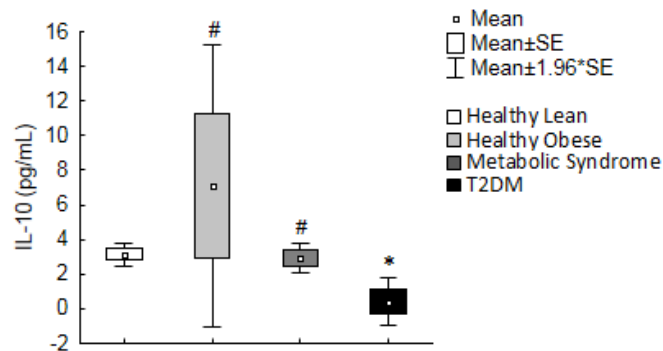
Figure 5.2.7. Serum cytokine levels of IL-10. Statistical analysis: One-way ANOVA with Tukey post hoc test. *p < 0.05 indicates significant difference between specific group compared to healthy lean group. #p < 0.05 indicates significant difference between groups.

5.3. PHASE 3: The effect of participant derived serum on adipose tissue derived mesenchymal stem cell function

5.3.1. ADSC characterization

The identity of the human adipose tissue derived mesenchymal stem cells (ADSCs) used in subsequent cell culture experiments was confirmed using flow cytometry. ADSCs (P7) *(n=3)* positively expressed the mesenchymal stem cell surface markers CD90+ (> 99 %), CD105+ (> 99 %) and CD73+ (> 98 %), whereas only < 41 % of the cell population expressed either CD34+ or CD45+ markers (Fig. 5.3.1J-L).
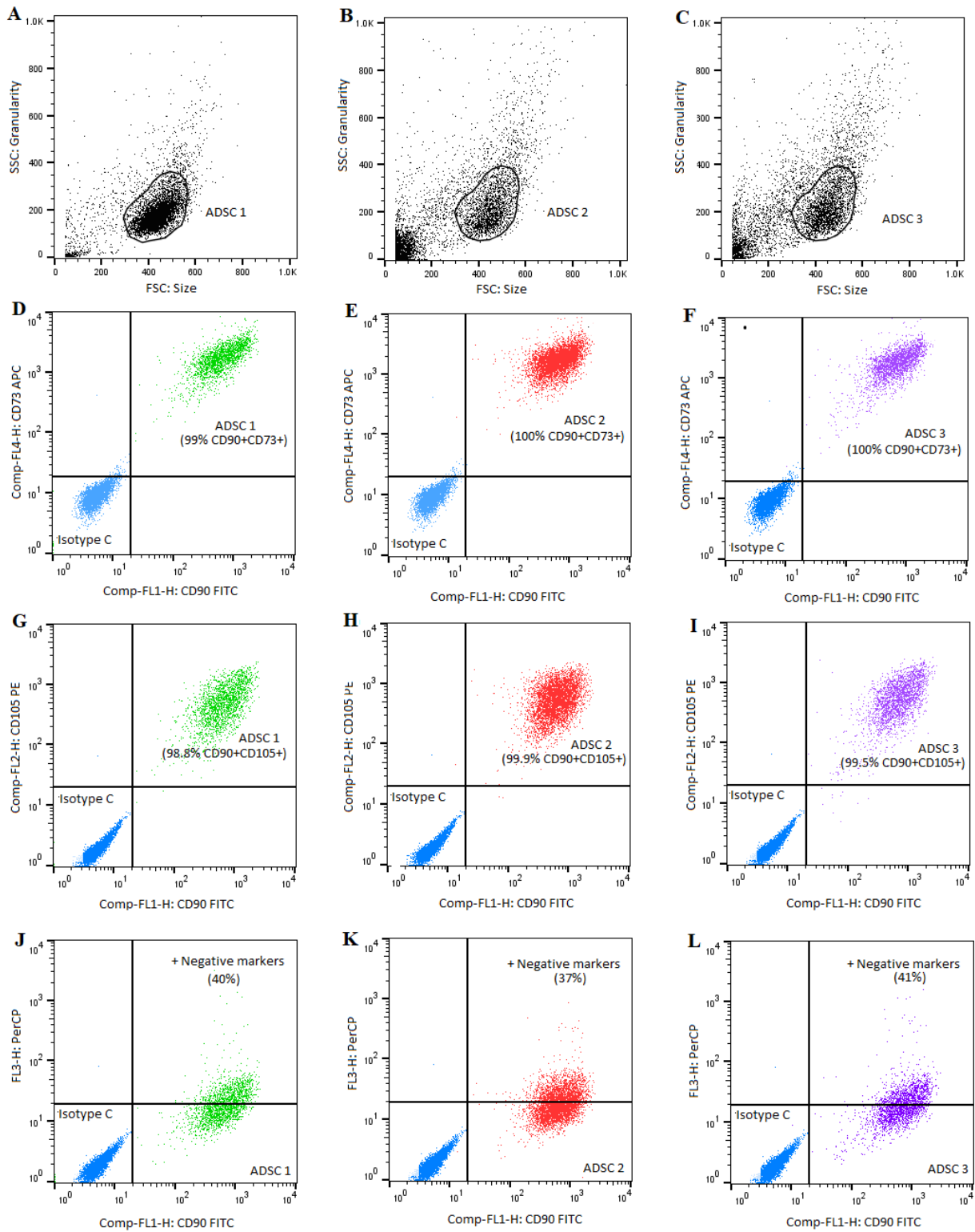
Figure 5.3.1. ADSC characterization. A-C) forward and side scatter dot plots displaying the cell population from samples ADSC 1, 2 and 3. **D-F)** dot plots for surface markers CD90+ and CD73+ and the isotype cell population as a negative control (ADSC 1 -3). **G-H)** dot plots for CD90+ and CD105+. **J-L)** dot plots of CD90+ and PercP stain for negative populations.

5.3.2. Cell viability

In order to assess the effect of participant derived serum on the viability of ADSCs, the MTT *in vitro* toxicology assay was performed. There were no significant differences observed in cell viability between the different groups, compared to treatment with standard growth medium (SGM) (Fig. 5.3.2). These results suggested that participants' serum could sustain ADSCs in culture, to a similar extent as the SGM.
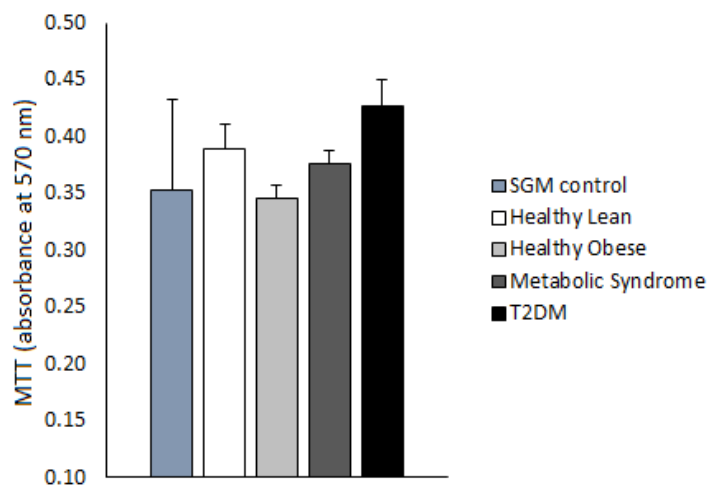
Figure 5.3.2. Cell viability. Average absorbance levels in the different groups, indicating the measure of cell viability as detected using MTT. **Abbr.:** SGM – standard growth medium, T2DM – type 2 diabetes mellitus.

5.3.3. Cell proliferation

The BrdU cell proliferation assay was used to determine the effect of participant derived serum on ADSC proliferation capacity. A significant increase in the proliferation rate was observed in ADSCs treated with healthy lean participants' serum (0.160 ± 0.01) $(p<0.05)$ $(mean \pm SE)$ compared to SGM control (0.145 ± 0.01). This effect was not observed in the obese and T2DM groups suggesting the mitogenic effect of patient-derived serum is lost under conditions of obesity.

Additionally, there was a significant correlation observed between the mitogenic response of ADSCs and circulating levels of IL-6 in the healthy lean $(n=10$, r=0.912, $p < 0.01)$ and metabolic syndrome $(n=19$, r=0.616, $p < 0.01)$ groups (Fig 5.3.3B).

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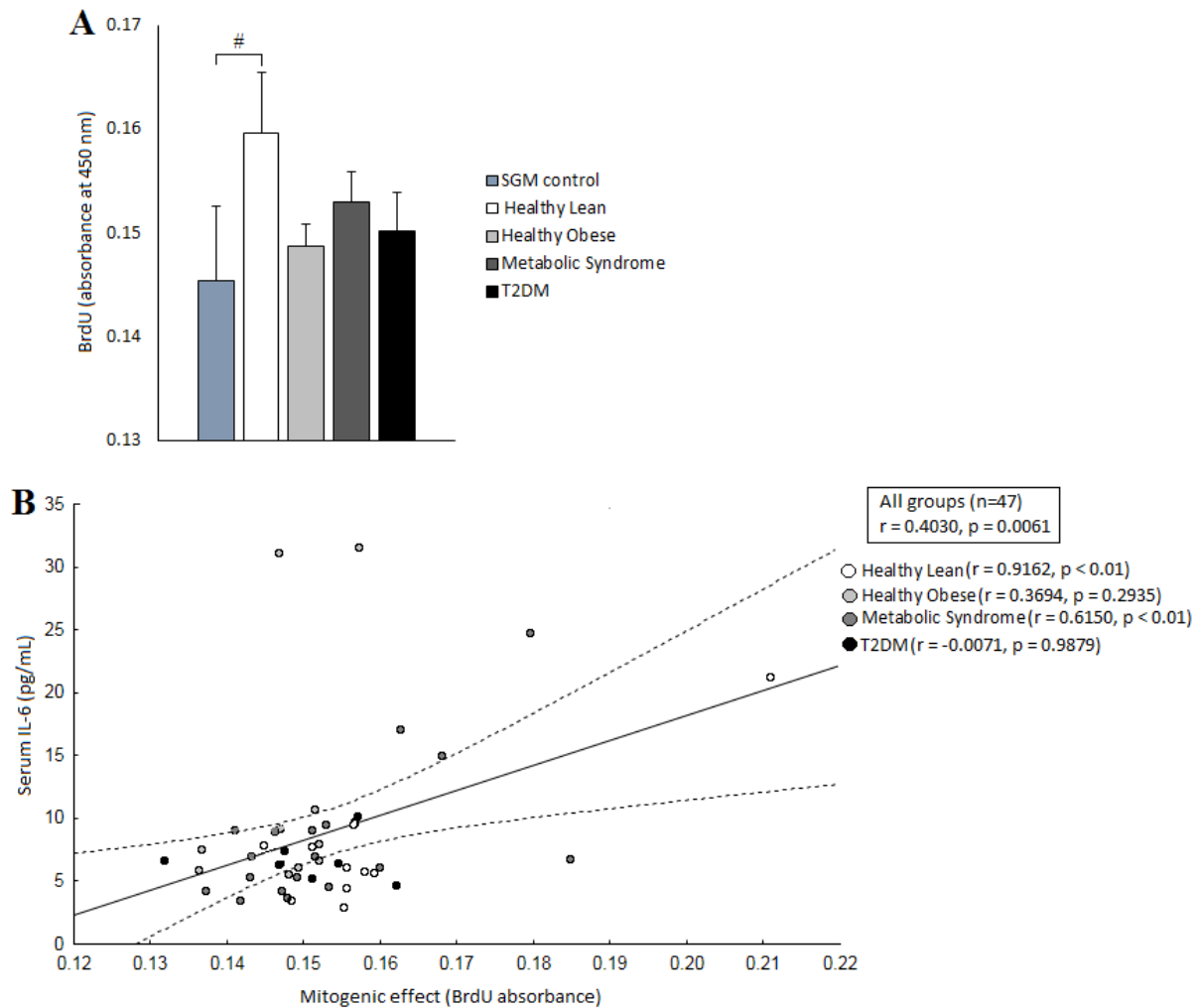
Figure 5.3.3. Effect of participant derived serum on ADSC proliferation. **A)** Average absorbance values in the healthy lean, healthy obese, metabolic syndrome and T2DM groups, as a measure of the BrdU detected during proliferation of cells. **B)** Relationship between circulating IL-6 and the proliferation of ADSCs. **Statistical analysis:** One way ANOVA with Tukey and Mann-Whitney U post hoc tests and Case-wise Product-Moment correlation analysis. #$p < 0.05$ indicates significant difference between specific groups compared to SGM control. **Abbr.:** ADSCs – adipose tissue-derived mesenchymal stem cells; SGM – standard growth medium.

5.3.4. Cell migration

The effect of participant-derived serum on the migration of ADSCs was determined through a migration assay. The rate at which cells migrate into an *in vitro* wound was assessed over a 24 hour period. Replacement of 10% FBS (SGM) with 20% participant derived serum (refer to pg. 55) resulted in the reduced migration rate of ADSCs regardless of metabolic profile (Fig. 5.3.4). ADSC treatment with SGM resulted in 27.0 ± 3.7 % *(mean ± SE)* closure of the cell free gap after 7 hours, whilst a cell free gap closure of only 11.9 ± 1.5 % with healthy lean participants' serum; 7.9 ± 1.9 % (healthy obese); 10.0 ± 0.9 % (metabolic syndrome) and 10.1

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± 1.3 % (T2DM) was evident at the same time point (Fig. 5.3.4A). A significant correlation was observed between circulating levels of IL-8 and the migration rate of ADSCs (n=19, r = 0.558, *p < 0.05*), particularly in the metabolic syndrome group (Fig. 5.3.4B).
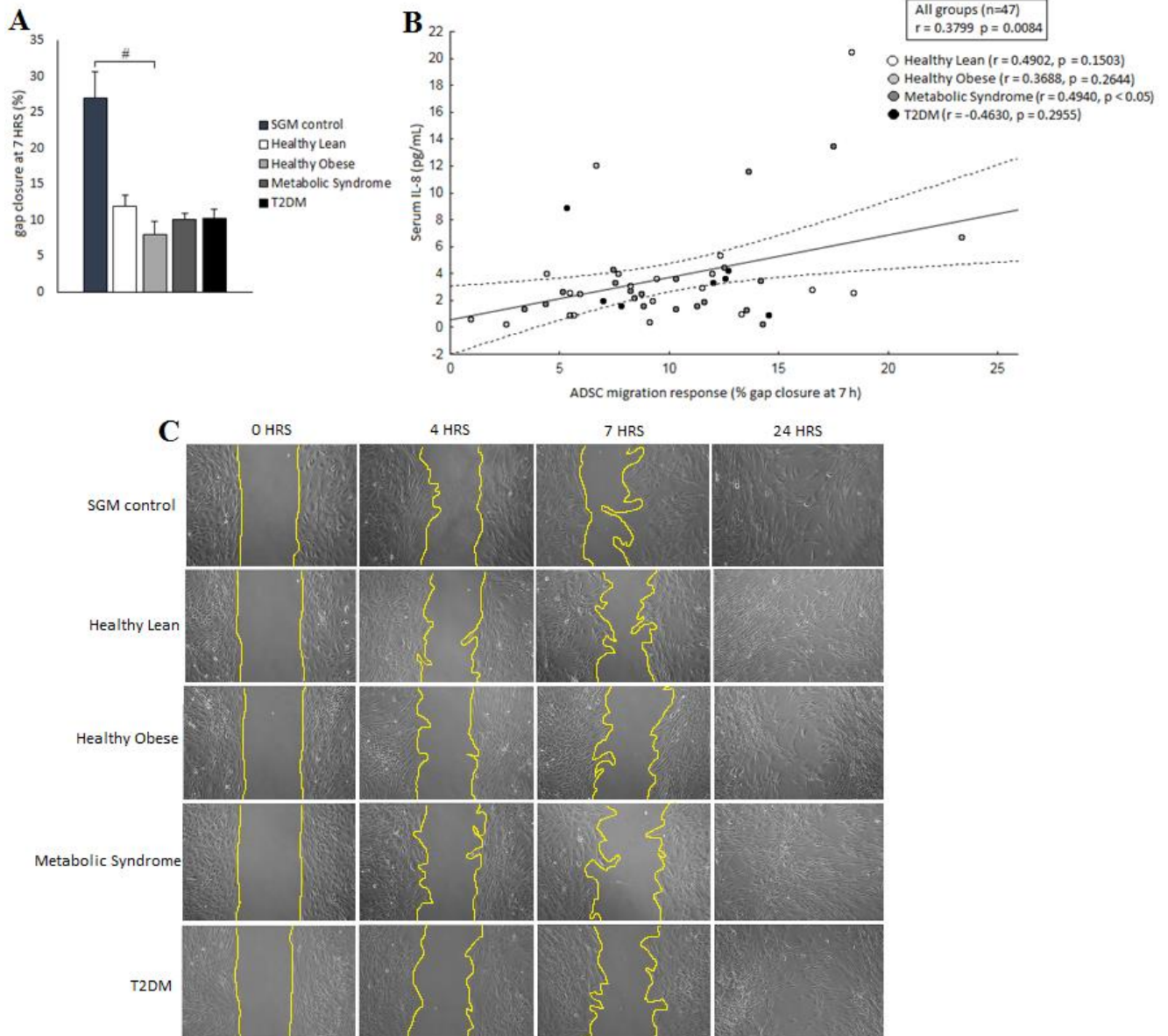
Figure 5.3.4. Effect of participant derived serum on ADSC migration. A) Percentage area covered by cells after 7 hours. **B)** Relationship between serum circulating IL-8 levels and the migration of ADSCs. **C)** Graphic representation of the *in vitro* wound area, demonstrating migration of ADSCs into the wound area over 24 hours. **Statistical analysis:** One way ANOVA with Tukey's post hoc test. #p < 0.05 indicates significant difference in specific group compared to SGM control. **Abbr.:** ADSCs – adipose tissue-derived mesenchymal stem cells; SGM – standard growth medium.

Chapter 6

Discussion

The detrimental effects of extreme obesity are evident in the growing burden of obesity-associated non-communicable diseases worldwide (Dalal *et al.*, 2011). The high prevalence of obesity in Black South African women (Micklesfield *et al.*, 2013) places them at a high risk of developing the metabolic syndrome and obesity-associated inflammatory disorders such as T2DM, which when poorly managed, present with numerous co-morbidities (Arababadi *et al.*, 2010; Dinh *et al.*, 2012; Sun *et al.*, 2016). Adipose tissue-derived mesenchymal stem cells (ADSCs) have become the focus of numerous investigations (Zhang et al. 2015; Kaisang et al. 2017; Wang et al. 2015) for the treatment of diabetes-associated co-morbidities, due to their immunomodulatory properties; their ability to differentiate into multiple tissue types as well as their sufficient quantities for effective therapy (Ivanova-Todorova *et al.*, 2009). However, autologous stem cell therapies have proven unsuccessful. We hypothesised that the inflammatory microenvironment observed in obesity and T2DM contributes to the functional decline of stem /progenitor cells. The exact mechanism and stage during disease progression when this occurs is not yet well understood. In this study, we demonstrated for the first time that the obesity-associated disruption in the systemic inflammatory balance, may have a negative effect on ADSC response, regardless of metabolic profile.

Participants in this study were characterized according to their metabolic profile, as well as results of the health, lifestyle and nutritional questionnaires. Overall, our study population had similar lifestyle and nutritional habits, regardless of BMI. This may be attributed to the fact that all participants were from similar socio-economic backgrounds (Dubowitz *et al.*, 2008). The significantly younger age of women in the healthy lean group potentially explains the high

reports of occasional consumption of alcohol and smoking in this group (Peltzer & Pengpid, 2016; Yi *et al.*, 2017). Furthermore, the women in the obese groups were significantly older, and this was consistent with studies that have reported that older Black women from lower socio-economic backgrounds still perceive being overweight as a sign of wealth and good health (Draper *et al.*, 2016; Gaston *et al.*, 2011; Mvo *et al.*, 1999; Okop *et al.*, 2016; Okoro *et al.*, 2014; von Lengerke *et al.*, 2012). The significantly older age of women in the T2DM group was furthermore consistent with studies that have suggested that T2DM develops with increasing age (Shai *et al.*, 2006; Frank *et al.*, 2013). This was apparent in that the non-diabetic obese participants, who despite their large BMI and excess body fat, were all normoglycaemic, with only four (*n=4/30*) participants reporting the use of hypertension medication.

Body composition results confirmed the apple shape body type hypothesis in the metabolic syndrome and T2DM groups, as observed in the excess abdominal fat; significantly large waist-to-hip ratio; trunk-to-leg and android-to-gynoid fat ratios. It has previously been shown that excess abdominal fat (i.e. the apple shape body type) is a great risk factor for the development of metabolic syndrome and ultimately T2DM (Hayes *et al.*, 2013; Goedecke *et al.*, 2009; Peppa *et al.*, 2013). The significantly less total body fat in T2DM participants compared to the non-diabetic obese groups, suggested weight loss in these women. This may be due to metformin treatment which has been shown to induce weight loss in diabetic patients independent of diet and exercise (Kheirandish *et al.*, 2017; Riediger *et al.*, 2017), with no changes in waist circumference (Bray *et al.*, 2012). Moreover, weight loss in T2DM can result from the inability of fat and muscle cells to take up glucose, due to insulin resistance, which then leads to the excess glycogen breakdown thus resulting in muscle and fat breakdown.

As expected, the T2DM group had significantly higher fasting glucose levels, which suggested poor glycaemic control in these participants. Recent studies have suggested that individuals from lower socio-economic backgrounds tend to not adhere to their recommended treatment,

due to lack of information and understanding with regards to their condition (Kokozska, 2017; Mendehall & Norris 2015). Increased inflammation was evident in the obese metabolic syndrome group as observed with the significantly higher circulating levels of CRP. Interestingly, participants in this group had normal average fasting blood glucose levels, higher low density lipoprotein (LDL), and a tendency towards lower high density lipoprotein (HDL) levels. This suggests that the metabolic syndrome participants were normoglycaemic despite the elevated inflammation, and dyslipidaemia.

Due to the high individual variability within the different groups, no differences were observed in circulating pro-inflammatory cytokines (IL-6, IL-8, IFN-γ and TNF-α). However, the lower levels of CRP and IL-6 observed in the T2DM group were consistent with reports of reduced inflammation due to metformin treatment. Studies have suggested that metformin exerts its anti-inflammatory effects through an AMPK-dependent mechanism (Huang *et al.,* 2009; Liu *et al.,* 2017; Schuiveling *et al*., 2017), by inhibiting signalling factors responsible for the activation of pro-inflammatory cytokines. Circulating levels of stromal derived factor 1 - alpha (SDF1- α), a potent inducer of the mobilization of MSCs from bone marrow into circulation, were not significantly different in our study population. However, as shown in previous studies, there was a tendency towards elevated levels of this chemokine in the T2DM group (Derakshan *et al*., 2012). The significantly low levels of IL-10 in the serum of T2DM participants was consistent with previous studies which showed low levels of IL-10 in T2DM patients (Acharya *et al*., 2015; Al-Daghri *et al*., 2015). This could furthermore explain the high fasting glucose levels observed in these participants, since IL-10 has been shown to improve pancreatic β-cell function in response to glucose (Pennline *et al*., 1994).

Treatment of ADSCs with the healthy lean participants' serum, demonstrated a mitogenic effect. However, this effect was lost in the healthy obese, metabolic syndrome and T2DM groups. Furthermore, a significant correlation was observed in the proliferation of ADSCs and

circulating IL-6 levels, particularly in the healthy lean and metabolic syndrome groups, which suggested a role for IL-6 in the maintenance of stem cells as previously reported (Pricola *et al.*, 2009; van de Vyver *et al.*, 2016). Studies have however shown that IL-6 has no proliferative effect on progenitor cells on its own, but it functions synergistically with multiple other cytokines such as oncostatin M (OSM), leukemia inhibitor factor (LIF), stem cell factor (SCF) and granulocyte stimulating factor (G-CSF) (Jacobsen *et al.*, 1994; Lee *et al.*, 2006; Miura *et al.*, 1993) to promote cell growth. A reduction in the migration rate of ADSCs was observed when standard growth medium treatment was replaced with participants' serum, including that of healthy lean participants. Furthermore, there was a correlation between circulating levels of IL-8 and the migration rate of ADSCs. This was consistent with studies that suggest IL-8 independently stimulates migration of MSCs (Braune *et al.*, 2017). Taken together, our study demonstrated the association between altered stem cell functionality and a distorted inflammatory profile.

Chapter 7

Conclusion/Future Perspective

One of the earliest evidence of successful stem cell therapy was by Rojas *et al.* (2005) where they made use of bone marrow-derived mesenchymal stem cells (BMSCs) to counteract the effect of bleomycin induced lung injury in mice models. Since then, there have been numerous reports of successful stem cell therapies in the treatment of diabetes associated complications using adipose tissue-derived stem cells (ADSCs). ADSCs have been shown to improve pancreatic β-cell function and suppress the dendritic cell differentiation in mice models of diabetes, thus improving glucose uptake and inhibiting the release of proinflammatory cytokines (Cao *et al.*, 2015; Hao *et al.*, 2013; Ivanova-Todorova *et al.*, 2009; Yeung *et al.*, 2012). However, autologous stem cell therapies using diabetic patients' stem cells have proven unsuccessful (Shin & Peterson, 2012). In T2DM the multifunctional property of ADSC is impaired (Rezaie *et al.*, 2017; Shin & Peterson, 2012). The mechanism and stage at which this impairment occurs in MSCs is not yet well understood.

In our study, we investigated this effect of obesity-associated T2DM on the viability, proliferation and migration capacity of ADSCs, using serum derived from healthy lean, healthy obese, metabolic syndrome and T2DM women. We demonstrated for the first time, that the disruption in the inflammatory balance as a result of obesity, regardless of metabolic syndrome, is detrimental to MSC function. Thus, future research should focus on the disease progression leading to T2DM, as well as the inflammatory cytokines involved (such as IL-6, IL-8 and SDF-1α) in order to take preventative measures to curtail MSC impairment.

Chapter 8

Limitations

Participants' responses to the health, lifestyle and nutritional questionnaires were all self-reported which leaves room for misinformation as a way to stay guarded and to give answers which they believe are what the investigator wants to hear. The small sample size in this study was limiting in that the individual variability in the cytokine levels made it difficult to attain statistical significance in some of the cytokines investigated. This was a cross sectional study and thus, we could not follow up with participants to observe how long term changes in their metabolic and inflammatory profile affects mesenchymal stem cell function, and thus disease progression was only investigated at a single time point. Furthermore, the study did not measure fasting insulin levels which would have provided further insight into results obtained from the metabolic syndrome participants who had normal fasting glucose levels and yet displayed increased inflammation.

Finally, the adipose tissue-derived mesenchymal stem cell line used in the study was an immortalized cell line which could possibly skew results since these cells have been manipulated for continuous proliferation.

Chapter 9

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Chapter 10

Appendices

Appendix A: Health, lifestyle and nutritional questionnaires

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT: Stem cell impairment associated with type 2 diabetes mellitus: Investigating the effect of obesity-associated systemic inflammation on mesenchymal stem cell behaviour in three different cohorts of patients.

REFERENCE NUMBER:

PRINCIPAL INVESTIGATOR: Dr M van de Vyver

ADDRESS: Room 3050, Clinical building, Department of Medicine, Stellenbosch University.

CONTACT NUMBER: **021-938 9263**

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the **Health Research Ethics Committee at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

☐ The World Health Organization indicated that almost half (41%) of South Africa's women are overweight or obese. These obese women have a high risk to develop type 2 diabetes mellitus. It is also known that the amount and distribution of body fat (hip, waist, legs) is not the same in women with black African, Caucasian (white European) or mixed ancestry. These differences in fat distribution may also result in differences in the blood levels of inflammation biomarkers. This is important, because one of the "side effects" of obesity-linked type 2 diabetes, is that the stem cells that are important for wound healing in your body, cannot function as they should. This study will investigate if there is a link between the blood biomarker levels in women with specific ethnicities and the ability of stem cells to help the healing of wounds.

☐ Altogether 90 women that are between 18-45 years old will take part in the study (see exclusion criteria below). Women from each ethnic population that are healthy and not overweight will be in the control group (n=30), women who are obese but not diabetic will be in the non-diabetic obese group (n=60) and obese women with type 2 diabetes (n=60) will be in the other group.

☐ Exclusion criteria (may not take part in study): Males/Females that are older than 45 or younger than 18 years; women who are pregnant, or have any infectious diseases or any long-term illness other than obesity-associated type 2 diabetes mellitus. If you do not know your

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HIV status, you have the option to be tested and if the test is negative you can take part in the study, otherwise you do not have to get tested for HIV and do not have to take part in the study.

☐ The study will be done at the Endocrinology Ward A10, Tygerberg Hospital and at the Department of Medicine, Stellenbosch University.

☐ To see if you can take part in the study, you will be asked to fill out a short lifestyle questionnaire (attached at the end of this leaflet) and we will take some body size measurements and a qualified clinician will draw blood from your arm.

☐ Your weight, height and the size of your hip and waist will be measured so that we can calculate your body mass index (BMI). We will also do a type of x-ray scan (DEXA) of your whole body that will give us information on where in your body the fat is located. This will be done by a qualified research nurse and a DEXA scan specialist. It should not cause you any discomfort.

☐ The blood sample that will be drawn by a qualified research nurse will consist of a single needle prick to your arm where a small amount (25mL) of blood will be collected into 4 tubes. Two of the tubes will be sent away to Pathcare laboratories, where it will be analysed for markers of type 2 diabetes (HbA1c and glucose) and your cholesterol levels (HDL, LDL, triglycerides, cholesterol) will be tested. The other 2 tubes will be used for later experiments on stem cells in a laboratory.

☐ Participants will be subdivided into one of the above mentioned groups. 1) **Obese type 2 diabetic patients** (n=20 from each ethic group: Black African, Mixed ancestry and Caucasian). 2**) Lean healthy control participants** (n=10 from each ethnic group: Black African, Mixed ancestry and Caucasian) and 3) **Non-diabetic obese participants** (n=20 from each ethic group: Black African, Mixed ancestry and Caucasian). No further testing or sample collection will be needed. The second part of the research will be done in a laboratory, where

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the blood samples that were collected will be analysed for the levels of inflammatory biomarkers and will then be used to treat stem cells.

☐   The effect that blood samples collected from control, non-diabetic obese and type 2 diabetes participants with different ethnicities has on the functions of stem cells will be tested. This is important for future patients, since a better understanding of why stem cells "stop working" during type 2 diabetes will help us to identify biomarkers that are "bad" and may contribute to the future development of new medicine that are specific for African populations.

☐   You will not receive any new medication or new treatment if you take part in this study. All the samples that are collected will be stored in a freezer for 5 years after which it will be discarded. No other testing will be done and your blood samples will not be used for anything else.

☐   All the information and data that are collected in this study will be strictly confidential and will not be shared with anyone other than the researchers involved in the study. You will remain completely anonymous and your name will not appear on any of the samples or anywhere other than the consent form.

Why have you been invited to participate?

☐   You are invited to participate if you are a type 2 diabetic patient visiting the weekly clinic at Tygerberg Hospital or if you are a healthy lean women or non-diabetic obese women that can fall within the control group. The control group is essential in order to have a "normal" response to compare the data of the type 2 diabetes group with.

What will your responsibilities be?

☐ You will only be expected to visit the Endocrinology Ward A10 Tygerberg hospital in one day when all the measurements will be taken. If you are interested in receiving feedback from the blood samples that were taken, you will be asked to visit the Endocrinology ward a second time, where a medical doctor or research nurse will explain the results to you. Otherwise, you can ask that we phone you with the feedback.

Will you benefit from taking part in this research?

☐ Your direct benefit from participating in the study will be the feedback you receive from the cholesterol tests and life style questionnaire. The research clinician will explain to you what it means and if necessary you will be referred to a medical specialist for follow-up. You will receive R150 for your time and effort spent to take part in this study.

☐ The research will better our understanding of the basic regulation of stem cells and the potential of these cells to enhance wound healing. The outcome of the research will enable more in depth studies to be performed looking at mechanisms and downstream effects of inflammatory biomarkers on stem cells that were identified as "bad". A better understanding of these processes can contribute to the future development of anti-inflammatory drugs in order to improve personalized treatment strategies within the African context.

Are there any risks involved in your taking part in this research?

☐ There are no serious risks involved in taking part in this research. You may feel some discomfort and slight pain when the blood sample is taken by the nurse.

If you do not agree to take part, what alternatives do you have?

☐   Participation is totally voluntary and you will not be penalized or disadvantaged in any way if you decide not to take part.

Who will have access to your medical records?

☐   All the information collected will be treated as confidential and your identity will be protected. Only the medical doctor, nurse and researcher will have access to your medical records and only with your prior permission. If the data is used in a publication all participants will remain anonymous.

What will happen in the unlikely event of some form injury occurring as a direct result of your taking part in this research study?

☐   In such an unlikely event of serious injury as a direct result of the study protocol, the Division of Endocrinology, Stellenbosch University will offer compensation to the participant for any medical fees or other related costs involved for the participant.

Will you be paid to take part in this study and are there any costs involved?

There will be no costs involved for you to take part in this study and you will receive R150 for your time and effort.

Is there anything else that you should know or do?

☐   You can contact Dr M van de Vyver at Tel: 021-938 9263 if you have any further queries or encounter any problems.

☐   You can contact the Health Research Ethics Committee at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by the study researcher and clinicians.

☐   You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I …………………………………..…………. agree to take part in a research study entitled (insert title of study).

I declare that:

•   I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.

•   I have had a chance to ask questions and all my questions have been adequately answered.

•   I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.

•   I may choose to leave the study at any time and will not be penalised or prejudiced in any way.

•   I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (place) ....…........…………….. on (date) …………...……….. 2016.

Signature of participant   **Signature of witness**

Declaration by investigator

I (name) …………………………………………..……… declare that:

•   I explained the information in this document to …………………………………..

•   I encouraged him/her to ask questions and took adequate time to answer them.

•   I am satisfied that he/she adequately understands all aspects of the research, as discussed above

•   I did/did not use an interpreter. (If an interpreter is used then the interpreter must sign the declaration below.)

Signed at (place) ....…….......……………….. on (date) …………...……….. 2016.

Signature of investigator   **Signature of witness**

Declaration by interpreter

I (name) …………………………………………..……… declare that:

•   I assisted the investigator (name) ……………………………………. to explain the information   in   this   document   to   (name   of   participant) ………………..………………………….. using the language medium of Afrikaans/Xhosa.

•   We encouraged him/her to ask questions and took adequate time to answer them.

•   I conveyed a factually correct version of what was related to me.

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• I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (place) ....…........…………….. on (date) …………...……………..

Signature of interpreter   **Signature of witness**

MEDICAL HISTORY AND LIFESTYLE QUESTIONNAIRE

NURSES DOCUMENTATION AND CHECK LIST FOR THE LIFESTYLE AND MEDICAL HISTORY QUESTIONNAIRE

This document is constructed to ensure that all the relevant information is collected from the patients who consent to freely participate in the study (Protocol #N15/07/066).

All information provided is private and confidential and must be maintained as such. This information cannot be shared with any other third parties and can only be accessed by the medical practitioners and investigators directly involved in this study.

Please ensure that all questions are completed on behalf of the patients and that all the information provided is accurate. In case of any uncertainty, please ask for assistance from the primary investigator (Dr. M van de Vyver, Office phone 021-938-9263).

OVERALL SUMMARY CHECK LIST

Please check once performed

☐	Does the patient fully understand what participation will entail and has she given consent to participate in this study
☐	Ensure that the patient has completed, signed and dated the Lifestyle and Medical Questionnaire
☐	Section 1 of this document has been completed
☐	Section 2 of this document has been completed
☐	Section 3 of this document has been completed
☐	Four vials of blood have been drawn, labelled and stored correctly.

☐	A DEXA scan was conducted, within the last year, on the patient. If not please refer the patient accordingly.
☐	Ensure that this document is attached to the questionnaire completed by the patient

I, _____, acknowledge the accurate collection and completion of the above stated tasks.

HSPCS number: _____

Signature: _____

Date: _____

SECTION 1: BASIC PATIENT DETAILS

Patient number: _____

Date of Birth: _____

Ethnicity: ☐ African   ☐ Caucasian   ☐ Mixed Race Ancestry

☐Other; if other, please specify: _____

Gender: ☐ Female   ☐ Male

Does the patient have children?   ☐ Yes ☐ No

SECTION 2: MEDICAL HISTORY OF PATIENT

This section is to be completed after the patient has given written consent. The information should be obtained from the patients' clinical file and accurately transposed. If possible, this section should be completed in the presence of the patient to ensure accuracy.

Current Health States

Please select one of the following statues that best describes the patient's current flu and/or cold status (this includes conditions such as pneumonia and bronchitis)

☐ The patient does not currently have a cold or flu

☐ The patient does currently have a cold or flu

Is the patient currently pregnant?   ☐ Yes ☐ No

HIV status

What is your HIV status?   ☐ Positive   ☐ Negative   ☐ Unknown

If your HIV status is unknown, would you like to be referred in order to find out?

☐ Yes ☐ No

TB status

What is your current TB status?   ☐ Positive   ☐ Negative   ☐ Unknown

If your TB status is unknown,

Do you have any of the following conditions listed below? ☐ Yes ☐ No

A bad cough that lasts 3 weeks or longer

Pain in the chest

Coughing up blood or phlegm

If your TB status is unknown, would you like to be referred in order to find out?

☐ Yes ☐ No

Other infectious diseases

Does the patient suffer from any other infectious disease, other than those described above?

☐ Yes ☐ No

If yes, please specify: _____

Current Medications

Please list all prescription medications (drug name and dosage) that the patient is currently taking. This information should include all medications taken up to three months prior to this current assessment:

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

Please consult the patient and list any self-prescribed medications, dietary supplements, or vitamins they are currently taking. This list should also include any similar items that have been up to three months prior to this assessment:

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

Disease of or related to inflammation

Does the patient have any broken bones or flesh wounds or injuries?   ☐ Yes ☐ No

Has the patient

Had a heart attack before?   ☐ Yes ☐ No

Had a stroke before?   ☐ Yes ☐ No

Been diagnosed with arthritis?   ☐ Yes ☐ No

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Been diagnosed with any auto-immune disease? □ Yes □ No

If yes, please specify: _____

Been diagnosed with asthma? □ Yes □ No

Been diagnosed with cancer? □ Yes □ No

Metabolic syndromes

Has the patient

Been diagnosed as having undergone menopause? □ Yes □ No

Been diagnosed with any hormonal syndrome? □ Yes □ No

If yes, please specify: _____

Been diagnosed with type 1 diabetes mellitus? □ Yes □ No

Been diagnosed with type 2 diabetes mellitus? □ Yes □ No

If yes, when was this first diagnosed? _____

Been diagnosed with gestational diabetes mellitus? □ Yes □ No

If yes, when was this diagnosed? _____

SECTION 3: BIOLOGICAL PARAMETERS OF THE PATIENT

Height:

Weight:

Hip Size:

Waist size:

Has the patient been for a dual energy x-ray absorptiometry (DEXA) scan within the last year, with no change in relation to current BMI?

☐ Yes ☐ No

If no, please refer accordingly

If the patient was previously diagnosed with type 2 diabetes mellitus?

    The date of diagnosis: _____

Blood triglyceride levels _____

Fasting blood HDL levels _____

Fasting blood LDL levels _____

Fasting blood total cholesterol levels_____

Fasting blood glucose levels _____

Blood HbA1c levels _____

Systolic blood pressure _____

Diastolic blood pressure _____

Current parameters to be assessed for the patient, if previously undiagnosed for type 2 diabetes mellitus:

Blood triglyceride levels _____

Fasting blood HDL levels _____

Fasting blood LDL levels _____

Fasting blood total cholesterol levels_____

Fasting blood glucose levels _____

Blood HbA1c levels _____

Systolic blood pressure _____

Diastolic blood pressure _____

PATIENT LIFESTYLE AND MEDICAL HISTORY QUESTIONNAIRE

This document forms part of the XXX study and covers a range of lifestyle and medical related questions which you are asked to completed. You are asked to please answer all the questions to the best of your ability, ensuring that all information provided is accurate. If you are unsure of what is being asked, please enquire for further assistance and explanation.

All information that you provide is private and confidential and will be maintained as such. This information will not be shared with any other third parties and can only be accessed by investigators directly involved in this study.

BASIC PATIENT DETAILS

Patient number: _____ (For office use only)

Date of Birth: _____

Ethnicity:

☐ African ☐ Caucasian ☐ Mixed Race Ancestry

☐Other; if other, please specify: _____

Gender: ☐ Female ☐ Male

Do you have children? ☐ Yes ☐ No

LIFESTYLE SECTION

Occupation, transportation and physical activity

Are you employed? ☐ Yes ☐ No

If yes, how much physical activity is required by your job?

☐ None (spend most of the day sitting)

☐ Mild activity (walking and lifting light objects)

☐ Highly active (physically demanding)

From the list below please select the option which best describes your main mode of transportation?

☐ I mostly make use of a motor vehicle (car, bus, taxi)

☐ I mostly make use of a bicycle

☐ I mostly walk

☐ Other, in this case please explain _____

Food

How many meals do you eat a day? ☐ 1 or less ☐ 1 ☐ 2 ☐ 3 ☐ 4 or more

From the list below please select the option which matches you're eating pattern most accurately

☐ I eat no meat, fish or dairy products

☐ I eat no meat

☐ I eat a mixture of foods, with very little fruit and vegetables

☐ I eat a mixture of foods, including fruit and vegetables

☐ I eat a mixture of foods, including chips, take away foods and sweets

☐ I eat a special medical diet, in this case please explain _____

When   last   did   you   eat   or   drink   anything?

_____

Smoking

Do you smoke? ☐ Yes ☐ No

If yes, How many cigarettes do you smoke per day? _____

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How old were you when you started smoking? _____

If no, have you

☐ Never smoked

☐ Previously smoked but have not smoked for less than one year

☐ Previously smoked but not smoked for more than one year

Alcohol

From the list below please choose the statement below which best described your average alcohol drinking habits in a week. Please remember that 1 unit of alcohol is defined as 1 of glass wine, ½ pint of beer or a single measure of spirit.

☐ I never drink alcohol

☐ I drink between 1 to 3 units per week

☐ I drink between 4 and 7 units per week

☐ I drink more than 7 units per week

Recreational drugs

From the list below please choose the statement below which best described your recreational drug usage habits. Please remember that this does this include medicines prescribed by a medical professional, supplements or vitamins.

☐ I never use recreational drugs

☐ I use recreational drugs infrequently

☐ I use recreational drugs frequently

MEDICAL HISTORY SECTION

For this section you are asked to provide consent that your medical information may be obtained from your clinical file at Tygerberg Academic hospital.

Please tick this box ☐, sign and date below to indicate that you have given consent that your medical information may be accessed for the completion of this section of the questionnaire.

Signature: _____

Date: _____

DIETARY QUESTIONNAIRE

Type of diet:

☐A healthy meal plan   ☐Diabetic diet   ☐Vegetarian   ☐Banting / LCHF

☐OTHER:_____

Eating Frequency

Full Meals (not snacks) per day?   ☐1 to 2 times   ☐3 times   ☐3 to 4 times

Eating between meals?   ☐Not usually   ☐Once or twice   ☐More than 3 times

QUALITY OF MEALS & SNACKS

Healthy meals per week	*Healthy snacks*

☐on most days ☐only on some days	☐on most days ☐only on some days
Not-so-Healthy meals per week (e.g. take-aways): ☐seldom ☐often ☐most days of week	*Luxuries* ☐seldom ☐often ☐most days of week

Usual intake of Fruit: ☐seldom   ☐often   ☐daily   (Portion/s _____ ☐daily ☐weekly ☐monthly)

Main meal of day mostly consists of (choose only 1)

☐ ± ½ plate starches (including starchy vegetables) with ≤¼ plate of non-starchy vegetables & ±¼ plate meat

☐ ± ½ plate of non-starchy vegetables with ≤¼ starches & ±¼ plate meat

☐   ± ½ plate of meat (including mince, chicken, fish, etc.) with ±¼ plate of non-starchy vegetables & ±¼ starches

☐ ± equal portions of meat, starches and non-starchy vegetables

☐ ± mainly starches with moderate meat portions; no non-starchy vegetables

Fat intake:

The use of oil / margarine / fat in preparation of food: ☐seldom   ☐often   ☐daily

Usual amounts: ☐small   ☐moderate   ☐large/plenty

Type of margarine: ☐tub   ☐blocks   ☐low fat   Name:_____

Estimated amount of margarine per month: ☐250g   ☐500g   x_____ per month

Type of oil: ☐sunflower   ☐canola ☐olive   ☐omega 3&6 ☐other:_____
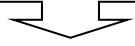
Estimated amount of oil per month: size of bottle: ☐750ml ☐ 1,5L ☐ 2L x _____ per month

Size of household for whom oil/margarine is used: _____ Adults _____ Children

Liquid Intake (for purpose of assessing sugar intake via liquids; excluding alcoholic beverages)

Mostly water: little to no cool drinks & no juice: YES☐ NO☐

Tea / coffee: mostly without sugar YES☐ NO☐

> If NO, complete

	Explore on usual volume (& frequency)
Juices &/ Normal cool drinks (JIVE/COKE/etc.) ☐seldom ☐often ☐daily	
Sport / Energy drinks &/or Flavoured waters / Iced tea ☐seldom ☐often ☐daily	
Tea/Coffee with sugar ☐seldom ☐often ☐daily	Tsp sugar / cup:_____ Cups per day:_____

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PATIENT DATA

BASIC PATIENT DETAILS

Patient #:	
D.O.B	
Ethnicity:	
Gender:	*FEMALE*
Age:	*18-45 years*

Patient #: GROUP

INFORMATION OBTAINED FROM PATIENT CLINIC FILE:

	YES/NO	Date	Comments
Previous diagnosis for type 1 diabetes?			
Previous diagnosis for gestational diabetes?			
Previous diagnosis for type 2 diabetes?			

		Units	Date of measurement	Criteria for inclusion
	tryclycerides			*>1.7mmol/L*
	HDL			*<1.3mmol/L*
	LDL			
Biomarkers	total cholesterol			
	blood glucose			
	HbA1c			*>48mmol/L (6.5%)*
	blood pressure			*Systolic >130mmHg; diastolic >85mmHg*

Note: If measures are more than 3 months old; it needs to be repeated on the new samples collected.

DEXA Scan:

	YES/NO	Date
DEXA Scan within previous 6 months?		
If NO, refer for new scan		
If YES, data obtained?		

Comments:

Current health status:

	YES/NO/Unkown	Details
Does the patient have cold/flu symptoms?		
Is the patient pregnant?		
Does the patient have children?		
Has the patient undergone menopouse?		
HIV status?		
TB status?		
Any infectious diseases?		
Diseases related to inflammation:		
Broken bones/ flesh wound?		
heart attack?		
stroke?		
arthritis?		
auto-immune disease?		
asthma?		
cancer?		

MEDICATION: (include all meds in the last 3 months)

	Drug	Dosage	Date prescribed / duration of use
Prescribed medication:			
Self-prescribed /over the counter supplements:			

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NEW MEASUREMENTS TAKEN AT TIME OF PATIENT VISIT TO A10:   *Date* _____

weight _____
height _____
hip _____
waist _____

Calculated BMI _____ *>30 kg/m2*

SAMPLE COLLECTION AND STORAGE:
2x SST (yellow) serum tubes
2x EDTA (purple) plasma tubes

Comments:

Physical activity:

Indicate 1 - 4

1 _____ Sedentary (spend most of the day sitting)
2 _____ Light activity (general household chores, lifting light objects, heart rate not raised)
3 _____ Moderate activity (walking/cycling at least 10 minutes a day, planned exercise of at least 10 min., heart rate slightly raised)
4 _____ Vigorous activity (walking/cycling for more than 1 hr, lifting heavy objects, heart rate increased on most dasy of week)

Smoking:

Do you smoke?   *IF YES*

_____ Cigarettes per day
_____ Age when patient started smoking

IF NO

_____ Never smoked
_____ Stopped smoking less than a year ago
_____ Stopped smoking more than a year ago

Alcohol:

Most accurately describe drinking habits?

_____ Never drink alcohol
_____ Ocassional drinker
_____ between 1 and 3 units per week
_____ between 4 and 7 units per week
_____ more than 7 units per week

Recreational Drugs:

Most accurately describe recreational drug use?

_____ Never
_____ Use recreational drugs infrequently
_____ Regular use of recreational drugs

Sleeping Pattern:

Average hours of sleep

_____ usually less than 6 hours
_____ usually more than 6 hours

Do you work night shifts?

_____ YES/NO
Frequency _____ About once a week
_____ More than 3 times a week

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Appendix B: Participant characteristics

Participant No.	Age	Physical Activity	Sleeping Patterns	Smoking	Alcohol Consumption	Full Meals per Day	Snacking btwn meals	Meal composition	Luxuries	Fruit intake (weekly)	Use of Oil	Type of Oil	Type of Butter	Tea/Coffee
Lifestyle								**Nutritional information**						
Control Group														
1	30	1	1	Y	N	2	2	1/3 veg, 1/3 starch, 1/3 meat	Seldom	4	often, moderate	Olive	Flora, LF	None
2	31	1	0	N	Y	2	0	1/2 meat, 1/4 starch, 1/4 veg.	Seldom	5	often, small	Canola & Olive	Stork, LF	seldom, no sugar
3	20	1	1	Y	Y	3	2	1/2 meat, 1/4 starch, 1/4 veg.	Seldom	2	seldom, moderate	Sunflower	Rama	1 daily, 4 sugars
4	28	3	0	N	Y	2	2	1/3 veg, 1/3 starch, 1/3 meat	Seldom	10	seldom, moderate	Canola & Olive	Flora, LF	2-3 daily, 1-2 sugars
5	22	4	1	N	Y	3	2	1/2 veg, 1/4 meat, 1/4 starch	Often	7	daily, moderate	Sunflower	Stork	1 daily, 3 sugars
6	31	3	1	Y	Y	2	0	1/3 veg, 1/3 starch, 1/3 meat	Often	3	often, moderate	Canola	Blossom, LF	3 daily, 2 sugars
7	29	2	1	N	N	3	0	1/3 veg, 1/3 starch, 1/3 meat	Seldom	10	daily, moderate	Sunflower	Rama	seldom, 2 sugars
8	23	2	1	N	N	3	3	1/3 veg, 1/3 starch, 1/3 meat	Seldom	10	seldom, small	Olive	Blossom, LF	2 daily, 2 sugars
9	23	3	1	N	Y	3	3	1/2 veg, 1/4 meat, 1/4 starch	Seldom	2	seldom, small	Canola	Flora, LF	1 daily, 3 sugars
10	19	3	1	N	Y	3	2	1/2 veg, 1/4 meat, 1/4 starch	Seldom	10	seldom, small	Canola	None	seldom, 1 sugar
Obese Group														
11	26	2	0	N	N	3	2	1/2 meat, 1/4 starch, 1/4 veg.	Seldom	10	daily, moderate	Sunflower	Sunshine, LF	1 daily, 3 sugars
12	38	3	1	N	N	2	0	1/3 veg, 1/3 starch, 1/3 meat	Seldom	3	daily, moderate	Sunflower	Rama	1 daily, 2 sugars
13	30	3	1	N	N	3	2	1/3 veg, 1/3 starch, 1/3 meat	Seldom	10	often, small	omega 3&6	Flora, LF	1 daily, 2 sugars

Continues on next page

14	23	2	1	N	Y	3	2	1/2 starches, 1/4 meat, 1/4 veg.	Often	7	daily, moderate	Sunflower	Rama	1 daily, 6 sugars
15	36	3	1	N	N	3	2	1/3 veg, 1/3 starch, 1/3 meat	Seldom	7	daily, moderate	Sunflower	Sunshine, LF	3 daily, 3 sugars
16	34	2	1	N	N	2	3	1/2 starch, 1/4 meat, 1/4 veg.	Often	2	often, moderate	Sunflower	Rama	1 daily, 4 sugars
17	37	1	1	N	N	2	3	1/2 meat, 1/4 starch, 1/4 veg.	Often	2	daily, moderate	Sunflower	Rama	3 daily, 2 sugars
18	41	2	1	Y	Y	2	2	1/3 veg, 1/3 starch, 1/3 meat	Seldom	7	daily, small	Sunflower	Rama	3 daily: 3 sugars
19	21	2	0	N	Y	2	0	1/2 starch, 1/4 meat, 1/4 veg.	Seldom	7	often, small	Sunflower	Sunshine	often
20	23	3	1	N	N	4	0	1/2 veg, 1/4 meat, 1/4 starch	Seldom	2	often, moderate	Canola		seldom
21	43	3	0	N	N	2	2	1/2 starch, 1/4 meat, 1/4 veg.	Seldom	3	daile,moderate	Sunflower	Rama	2 daily: 2 sugars
22	23	3	1	N	N	3	0	1/2 starch, 1/4 meat, 1/4 veg.	Seldom	3	daily, moderate	Sunflower	Rama	3 daily, 3 sugars
23	23	3	1	N	N	2	2	1/3 veg, 1/3 starch, 1/3 meat	Seldom	14	often, small	Olive	Stork	3 daily, 1 sugar
24	43	3	1	N	N	3	1	1/2 starch, 1/4 meat, 1/4 veg.	Seldom	1	daily, moderate	Sunflower	Sunshine, LF	1 daily, 5 sugars
25	43	3	1	N	N	3	0	1/2 meat, 1/4 starch, 1/4 veg.	Often	2	seldom, moderate	Sunflower	Stork	2 daily, 4 sugars
26	35	3	1	N	N	2	0	1/3 veg, 1/3 starch, 1/3 meat	Seldom	7	daily, moderate	Sunflower	Sunshine D	2daily, 3 sugars
27	19	1	1	N	Y	2	1	1/3 veg, 1/3 starch, 1/3 meat	Seldom	0,5	daily, moderate	Sunflower	None	1 daily, 5 sugars
28	27	3	1	N	N	2	3	1/2 starch, 1/4 meat, 1/4 veg.	Often	14	daily, moderate	Sunflower	Sunshine D	1 daily: 3 sugars
29	24	2	1	Y	Y	2	0	mainly starches, moderate meat and no vegetables	Often	2	seldom, plenty	Canola	-	seldom
30	40	4	0	N	N	3	0	banting diet/LCHF	Seldom			Coconut/Olive oil		1 daily: 0 sugars
31	45	2	1	N	N	4	0	1/2 veg, 1/4 meat, 1/4 starch	Seldom	2	daily, moderate	Sunflower	Rama	1 daily: 2 sugars
32	26	2	1	N	Y	3	3	1/2 starch, 1/4 meat, 1/4 veg.	Often	7	daily, moderate	Sunflower	Sunshine D	seldom
33	30	2	1	Y	Y	2	0	1/2 starch, 1/4 meat, 1/4 veg.	often	7	often, moderate			
34	44	1	1	N	Y	2	1	1/3 veg, 1/3 starch, 1/3 meat	Often		daily, moderate	Sunflower		2 daily: 2 sugars

35	34	3	0	N	N	3	2	1/3 veg, 1/3 starch, 1/3 meat	Seldom	7	daily, plenty	Sunflower	Sunshine, LF	None
36	44	3	1	N	N	2	0	1/3 veg, 1/3 starch, 1/3 meat	Seldom	2	seldom, small	Sunflower	Rama	2 daily, 2 sugars
37	40	2	1	N	N	3	0	1/3 veg, 1/3 starch, 1/3 meat	Seldom	2	daily, small	Sunflower	Rama	2 daily, 3 sugars
38	29	3	1	N	Y	2	0	1/2 meat, 1/4 starch, 1/4 veg.	Seldom	3	daily, plenty	Sunflower	Sunshine, LF	1 daily, 5 sugars
39	40	3	1	N	N	3	0	1/3 veg, 1/3 starch, 1/3 meat	Seldom	10	seldom, small	Sunflower	Stork	3 daily: 2 sugars
40	31	3	1	N	Y	1	0	1/2 veg, 1/4 meat, 1/4 starch	Seldom	7	seldom, small	Sunflower	Flora, LF	None
T2DM Group														
41	39	3	0	N	Y	3	2	1/2 veg, 1/4 meat, 1/4 starch	Seldom	7	seldom, small	Sunflower	Rama	seldom, 1 sugar
42	38	4	1	N	N	2	2	1/3 veg, 1/3 starch, 1/3 meat	Seldom	7	daily, moderate	Sunflower	Rama	2 daily, 2 sugars
43	43	3	1	N	N	2	3	1/3 veg, 1/3 starch, 1/3 meat	Seldom	14	daily, moderate	Sunflower	Flora, LF	1 daily, 3 sugars
44	35	3	1	N	N	3	0	1/3 veg, 1/3 starch, 1/3 meat	Seldom	10	seldom, moderate	Sunflower	Rama	seldom, no sugar
45	42	4	1	N	N	2	3	1/3 veg, 1/3 starch, 1/3 meat	Often	3	daily, plenty	Sunflower	Rama	2 daily: 2 sugars (rooibos)
46	39	1	1	N	N	2	0	1/3 veg, 1/3 starch, 1/3 meat	Seldom	4	daily, moderate	Sunflower	Rama	1 daily: 3 sugars
47	43	2	1	N	N	3	0	1/3 veg, 1/3 starch, 1/3 meat	Seldom	2	daily, moderate	Sunflower	Blossom, LF	seldom, 1 sugar

Footnote: Raw data from participant completed nutrition and lifestyle questionnaires.

Appendix C: Cell culture consumables

1.   Phosphate buffer saline (PBS)

8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 were added to 800 mL of distilled water and pH set at 7.4 then filled up to 1 L with distilled water.

2.   Freezing Media

10 % dimethyl sulphurous oxide (DMSO), 80% DMEM (with 1% penicillin/streptomycin), 10% FBS