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Assessment of the relationship between adiposity, Sirtuin 1 and miR-30a-5p in a mixed-ancestry population in Bellville South, Cape Town.

Thesis submitted in fulfilment of the requirements for the degree:

Master of Science: Biomedical Sciences, in the Faculty of Health and Wellness at the Cape Peninsula University of Technology. Bellville campus.

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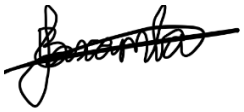
2024

Declaration

I, Babalwa Melissa Gaxamba, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.



Signed **Date**

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Dedication

"Lord, You are my God; I will exalt You and praise Your name, for in perfect faithfulness You have done wonderful things, things planned long ago."
Isaiah 25:1

To my beloved husband, Lwazi, and my three wonderful boys, Nathi, Thando, and Ndalo, thank you for your patience, understanding, and love. Each of you has given me strength and motivation to push through, and your unwavering support has allowed me the time and space to pursue this goal. I could not have done it without you by my side.

Abstract

The steadily increasing rise in obesity and metabolic disorders is a global problem that requires in depth understanding of the molecular mechanisms underlying adiposity. This study assessed the regulatory relationship between miR-30a-5p, sirtuin 1 (SIRT1) and adiposity in a mixed-ancestry population from Bellville South and Belhar, Cape Town, South Africa. The study analysed samples from 300 participants grouped into three body mass index (BMI) categories: normal-weight, overweight and obese. The groups were further classified by waist circumference (WC) as either normal or high. The study aimed to determine whether miR-30a-5p expression was affected by adiposity and its regulatory impact on SIRT1. SIRT1 concentration, *SIRT1* expression, and miR-30a-5p expression were measured from whole blood and serum samples using enzyme-linked immunosorbent assay (ELISA) and real-time quantitative polymerase chain reaction (RT-qPCR). Statistical analysis included Spearman's correlation to assess relationships between the variables, logistic regression models for differences between miR-30a-5p and *SIRT1* expression across BMI and WC categories. Finally, receiver operating characteristic (ROC) curves were generated and the area under the curve (AUC) analysed to determine whether either miR-30a-5p, *SIRT1* or both are predictors of elevated BMI or high WC. *p*-values less than 0.05 were considered statistically significant.

The study findings demonstrated that miR-30a-5p was significantly higher in the overweight and obese participants when compared to the normal weight participants which supports its role in adipogenesis. In contrast, SIRT1 concentration levels tended to decrease with obese participants, which aligns with the hypothesis of SIRT1 repression by miR-30a-5p. Interestingly, *SIRT1* expression tended to increase in obese and high WC when compared to normal-weight and normal WC. This suggests post-transcriptional regulation of *SIRT1* by miR-30a-5p. Comprehending the relationship between miR-30a-5p and SIRT1 in human adiposity provides novel insights into the molecular pathways involved in obesity development and points to miR-30a-5p as a potential biomarker and therapeutic target for metabolic regulation. The study findings contribute to the understanding of epigenetic regulation in obesity and suggest pathways for targeted intervention in populations with high obesity prevalence, such as South Africa's mixed-ancestry community.

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Terms and concepts

The following is an alphabetical list of definitions for the key concepts within the study:

3’ Untranslated region: The section of mRNA, at the 3’ end, that immediately follows the stop codon.

3T3-L1: A mouse cell line widely used for adipose tissue research.

Acute myocardial infarction: A blockage of blood flow to the heart muscle. Commonly referred to as a ‘heart attack’.

Adipocyte: A specialised cell in the human body that stores excess energy as fat. It is also called a fat cell.

Adipogenesis: The generation and development of adipocytes (fat cells) from immaturity to maturity in the human body.

Adipokine: This refers to cytokines produced by adipose tissue.

Adipose tissue: Connective tissue found both subcutaneously and viscerally that is composed mainly of adipocytes.

Adipose tissue macrophages: These are leucocytes of the innate immune system, found in adipose tissue, that modulate adipose inflammatory response.

Adiposity: The amount of adipose tissue in an individual. It is also used to describe an increase above the recommended body fat range.

Anthropometry: The measurement of the human body and its proportions.

C57BL/6: A laboratory mouse strain used in biomedical research.

Calorie: A unit of energy measurement.

Cardiovascular disease: Pathology in the heart or the blood vessels.

Cytokines: Small proteins produced by immune cells that play a significant role in cell signalling.

Deacetylase: An enzyme that removes acetyl groups from proteins.

Delta-like 4: A transmembrane ligand found in blood vessels.

Deoxyribonucleic acid: A molecule found in most living organisms that carries the genetic instructions specific for each organism.

Dicer: An enzyme that is crucial to late microRNA (see definition below) processing and cleaves precursor microRNA molecules to a more mature and shorter structure.

Drosha: An enzyme that is involved in the early stages of microRNA production and trims long premature microRNA to smaller precursor structures.

DiGeorge Syndrome Critical Region 8: A protein that partners with Drosha to recognise and select the premature microRNA for processing.

Epigenetics: The study of reversible, heritable genetic changes that affect cellular behaviour or function without altering the DNA sequence of the cell.

Forkhead box proteins: A family of transcription factors linked to cell cycle processes and apoptosis.

Global gross domestic product: The value of all goods and services produced over a specific time period within a country's borders.

Histone acetyltransferases: enzymes that catalyse the addition of acetyl groups to the lysine residues of histones and nonhistone proteins.

Histone deacetylases: enzymes that catalyse the removal of acetyl functional groups from the lysine residues of both histone and nonhistone proteins.

Lysine acetyltransferases: A newer term for histone acetyltransferases, increasingly used in literature, as it more accurately describes the substrate of the enzyme which are lysine residues in both histones and non-histone proteins.

Lysine deacetylases: A newer term for histone deacetylases, increasingly used in literature, as it more accurately describes the substrate of the enzyme which are lysine residues in both histones and non-histone proteins.

Metabolic syndrome: A group of medical conditions, including abdominal obesity, high fasting glucose, high blood pressure, high triglycerides, and low high-density lipoprotein cholesterol, that together increase the risk of an individual acquiring type 2 diabetes mellitus, stroke, and cardiovascular disease.

Murine: A scientific term for members of the Murinae subfamily which includes the common household mouse and rat.

MicroRNAs: Short non-coding RNA sequences involved in the regulation of biological processes by inhibiting gene expression.

Mixed-ancestry population: members of multiracial ethnic communities in Southern Africa who may have ancestry from more than one of the various populations inhabiting the region.

Nicotinamide Adenine Dinucleotide: A coenzyme found in all living cells that is necessary for various metabolic processes.

Non-communicable disease: A disease that cannot be transmitted from one person to another. NCDs are usually chronic.

Nucleotide: Organic nucleic acid building blocks that are made of a nitrogenous base, a pentose sugar, and a phosphate group.

Obesity: A medical condition resulting from the excessive accumulation of body fat, which is associated with various disease states and characterised by a body mass index of 30 kg/m^2 and above.

Overweight: An increase in body fat characterised by a BMI of between 25 – 29.9 kg/m^2

p53: A tumour suppressor protein.

Peroxisome proliferator-activated receptor-γ coactivator 1 alpha: A human protein that drives gene transcription.

Preadipocytes: Committed adipocyte precursor cells.

Peroxisome proliferator-activated receptor gamma: A nuclear receptor found in various human cell types.

Reverse transcription quantitative polymerase chain reaction: A method of PCR used when the template to be amplified is RNA. Reverse transcription is a step that converts the RNA to DNA for the PCR to commence.

Ribonucleic acid: A nucleic acid involved in protein synthesis.

Runt-related transcription factor 2: A transcription factor associated with osteoblast differentiation.

Sirtuin: A family of NAD+ dependent histone deacetylases that are crucial in metabolic pathway regulation.

Transcription factor: Proteins that control the process of DNA transcription to RNA.

Type 2 diabetes mellitus: A chronic condition, common in adults, where the body either produces insufficient insulin or the body is resistant to the produced insulin resulting in high blood sugar levels.

World Health Organisation: A United Nations agency responsible for international public health.

Abbreviations

3’ UTR	3’ Untranslated region
AMI	Acute myocardial infarction
AGO	Argonaute
ATMs	Adipose tissue macrophages
BMI	Body mass index
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
DGCR8	DiGeorge Syndrome Critical Region 8
ELISA	Enzyme linked immunosorbent assay
FOXOs	Forkhead box proteins
GDP	Global gross domestic product
HATs	Histone acetyltransferases
HDACs	Histone deacetylases
IFNγ	Interferon gamma
KATs	Lysine acetyltransferases
KDACs	Lysine deacetylases
MetS	Metabolic syndrome
miRNA	micro-RNA
NAD	Nicotinamide Adenine Dinucleotide
NCD	Non-communicable disease
nc-RNA	Non-coding RNA
Nt	Nucleotide
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha
PPARγ	Peroxisome proliferator-activated receptor gamma
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
RUNX2	Runt-related transcription factor 2

SIRT1	Sirtuin 1
SREBP-1c	Sterol regulatory element-binding protein 1c
TF	Transcription factor
T2DM	Type 2 diabetes mellitus
WC	Waist circumference
WHO	World Health Organisation

Chapter 1: Literature Review

1.1 Introduction

Adiposity and obesity are global issues that tripled in prevalence between 1975 and 2016, and are still rapidly increasing (WHO, 2021). It is estimated that 20% of the world's population will suffer from increased adiposity by 2025. Obesity is defined as an extreme accumulation of fat in adipocytes, which results in excessive weight gain (Blüher, 2019). Extreme weight gain is closely associated with insulin resistance, hyperglycaemia, low levels of high-density lipoprotein cholesterol (HDL cholesterol), increased fasting blood levels of triglycerides, and hypertension. A combination of obesity and any two of these associations is commonly diagnosed as a condition called metabolic syndrome (MetS). Metabolic syndrome is a documented predictor of cardiovascular disease (CVD), type 2 diabetes mellitus (T2DM), acute myocardial infarction (AMI), and stroke (James et al., 2004; Nilsson et al., 2019). Obesity complications and the resulting chronic diseases account for up to 70% of global morbidity and mortality and places considerable strain on the global economy (Blüher, 2019). Additionally, it is estimated that by 2035, the economic burden due to obesity will cost approximately 3% of the global gross domestic product (GDP) (World Obesity Federation, 2023).

In Africa, as a result of globalization, obesity is predicted to rise from 8 million to 27 million in men, and from 26 million to 74 million in women, between 2010 and 2030 (Barata Cavalcanti et al., 2022). Within Africa, South Africa is noted to have one of the highest prevalence of the disease (Sartorius et al., 2015). For instance, in South Africa, the number of individuals with a body mass index (BMI) ≥25 kg/m^2 has increased significantly between 2002 and 2016, from 56% to 68% in women and 29% to 31% in men (Boachie et al., 2022). Interestingly, obesity was highest among women, with the highest prevalence previously found amongst the mixed-ancestry population (26%), followed by the African population (20%) (Statistics South Africa, 2012). However, more recent findings show that though women still have the highest prevalence, there is now no significant difference in obesity prevalence between South African populations. Noteworthy however, is that within South Africa, the Western Cape was

found to have the highest rates of obesity both among men and women (Figures 1.1 and 1.2) (Statistics South Africa, 2016).

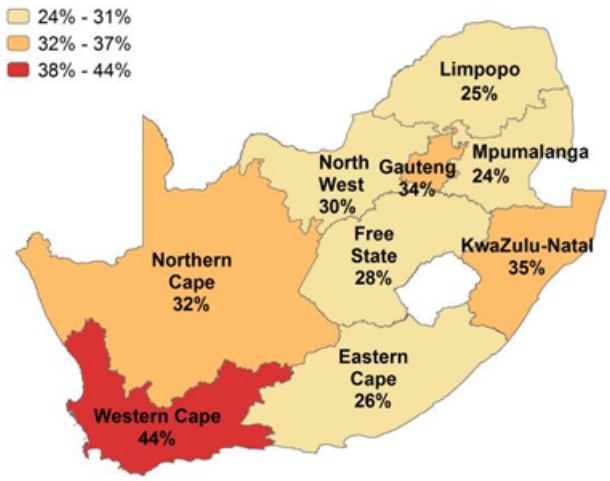

Figure 1.1: *South African men provincial prevalence of obesity* (Statistics South Africa, 2016).

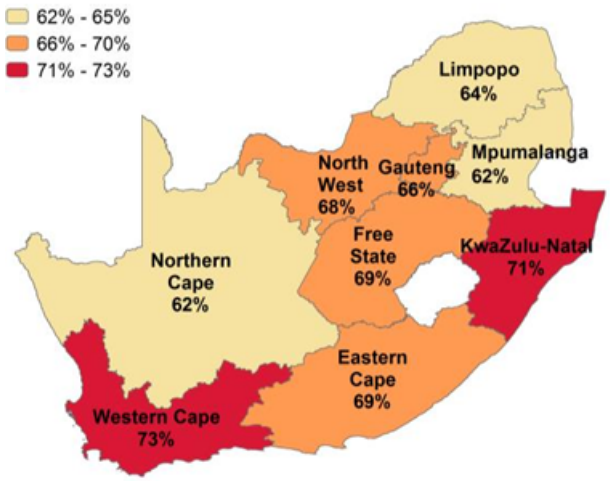

Figure 1.2: *South African women provincial prevalence of obesity* (Statistics South Africa, 2016).

To date, interventions and treatments of adiposity remain largely unsuccessful, which may be attributable to the misconception that increased weight gain is simply a result of excessive consumption of unhealthy foods with little to no exercise. Clinicians treating patients with adiposity have previously focused on recommending weight loss

regimes and treatment without assessing potential other exacerbations (van der Valk et al., 2019). However, emerging evidence shows that there is a complex variety of factors that influence the onset of obesity. These include underlying disease, hormone and genetic abnormalities, side effects from medicines, mental and socio-cultural factors among others. Overlooking these possible root causes is suspected to be directly proportional to the large failure of current adiposity treatments to achieve sustained weight loss (van der Valk et al., 2019; Endalifer & Diress, 2020). Excess energy, stored as body fat, is tightly controlled by several physiological measures, including cytokines, adipokines, hormones, proteins, and the adipose tissue that houses the fat. Disruptions, especially at gene expression level, in any of these controlling measures could, when coupled with a high caloric food diet and sedentary lifestyle, lead to an accumulation of fat and eventually obesity (Dhurandhar, 2022). This suggests that obesity management requires a deeper understanding, even at molecular level, of causative and contributing factors and mechanisms.

1.2 Adipocytes and adipose tissue

Energy from ingested food (calories) is used by the body in various metabolic processes, body temperature control and in physical activity (Woods et al., 1998). Any excess energy is stored for use in times of energy scarcity as seen in fasting/calorie restriction or starvation. These excess calories are stored as triglycerides, a form of lipid or fat, in specialized cells called adipocytes (Levine, 2005). Body fat stores are ideally kept constant over long intervals by strict mechanisms that ensure that energy intake, storage and expenditure remain balanced. When there is an imbalance between energy intake and expenditure, this leads to an excessive accumulation of fat in the adipocytes, at which point obesity occurs (Dhurandhar, 2022).

Adipose tissue is a loose connective tissue made up of mostly adipocytes and to a lesser extent immune cells, blood vessels and nerve cells. Adipose tissue is regarded as a complex organ that has an endocrine function, that is, it secretes various proteins collectively called adipokines, that regulate various physiological processes. Primarily, adipose tissue is the energy storing organ of the body and is located under the skin where it is termed subcutaneous or parietal fat, while those around the organs are termed visceral fat (Rosen & Spiegelman, 2006; Kershaw & Flier, 2004). Adipose tissue size is regulated by an adipocyte process called adipogenesis, which is the

commitment of a precursor cell to the adipose cell line, proliferation of the committed preadipocyte, the cessation of the proliferation and then differentiation of the preadipocytes to mature fat cells (K. Zhang et al., 2020; Kershaw & Flier, 2004). Most of the mature adipocytes contain a single vacuole which is where the fat is stored, with very little cytoplasm and a flattened nucleus that is pushed by the fat droplet to the periphery of the cell (Tanowitz et al., 2011).

The newly differentiated adipocytes are small and avidly absorb fatty acids and bind to glycerol, thus converting them to triglycerides for storage. As more fatty acids are absorbed, the adipocytes increase in size to accommodate the growing fat droplet. As the fat cells expand, new fat cells are also produced to help store the free fatty acids in circulation and maintain energy homeostasis (Fajas, 2009). This is usually balanced by simultaneous energy expenditure which results in triglyceride breakdown to provide expandable energy for the body and halts the synthesis of new adipocytes (Rosen & MacDougald, 2006). In adiposity, excessive fatty acid absorption leads to increased triglyceride formation and results in high levels of intracellular lipids. This primarily causes abnormal adipocyte hypertrophy and can also overpromote cell hyperplasia, both of which significantly increase adiposity (De Ferranti & Mozaffarian, 2008).

1.3 Complications associated with adiposity

Adiposity is a global epidemic that has been rapidly on the rise since 1975. The current projections of continued increase span to 2030, where an estimated 20% of the world's population will be obese (WHO, 2021). The African continent also shows a steady increase in adiposity prevalence, although the rates are still lower when compared to the other continents. Of significance, is the variation among the African countries with countries like South Africa having much higher-than-average adiposity rates due to factors like globalisation and urbanization (World Obesity Federation, 2016; Barata Cavalcanti et al., 2022).

The high prevalence of obesity is problematic due to adiposity being a documented risk factor for numerous chronic, non-communicable diseases (NCDs) that contribute to more than 50% of the world's morbidity and mortality rate (Blüher, 2019). The potential of adiposity to lead to chronic illnesses is attributed to the excessive lipid

accumulation in the fat cells that causes adipocyte hypertrophy. This progressive adipocyte growth, as seen in Figure 1.3, ultimately results in adipocyte dysfunction (Ibrahim, 2010). When the enlarged adipocytes are filled to capacity (~3 µg lipid/cell), they can no longer expand or accommodate the excess fat and begin to deposit lipids in external, non-adipose tissue that is not equipped to process fat (Danforth E., 2000; Gray & Vidal-Puig, 2007). The extracellular fat deposition stimulates the surrounding immune cells, termed adipose tissue macrophages (ATMs) to produce pro-inflammatory cytokines, resulting in low grade inflammation. The combination of inflammation and external fat spillage is the gateway to the development of NCDs such as T2DM, CVD, certain cancers, and stroke amongst others (Blüher, 2019; Koh et al., 2018; Miranda et al., 2018).

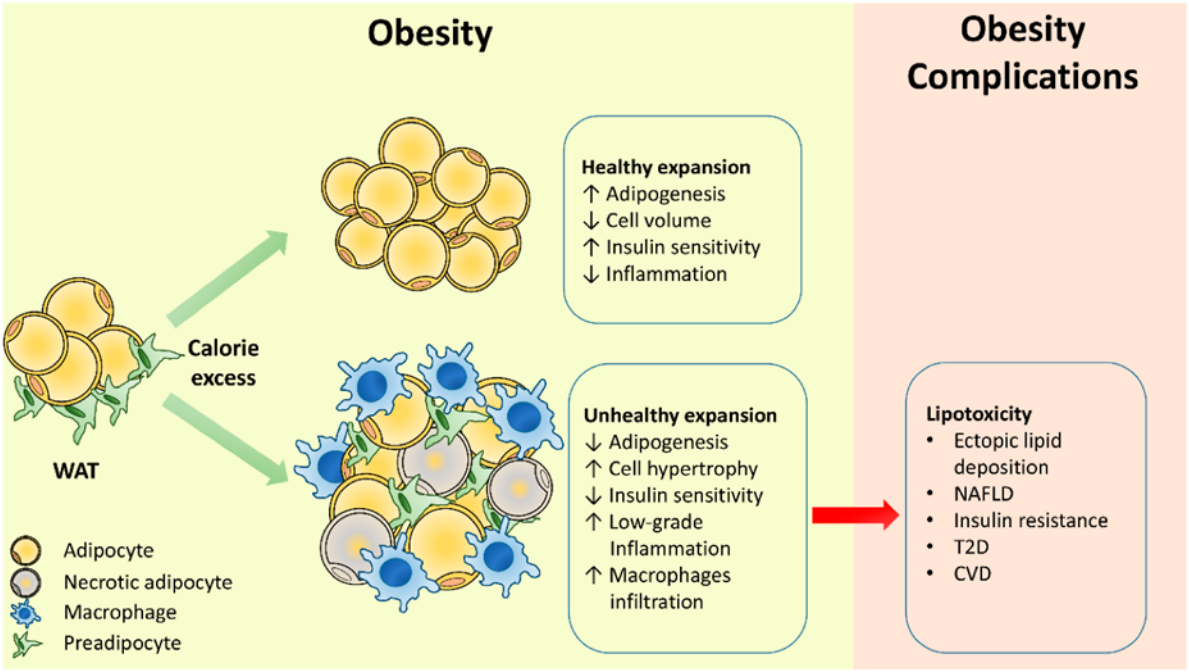

Figure 1.3: *Normal adipose tissue function vs adipose dysfunction* (Longo et al., 2019).

Due to the morbid consequences of obesity, it is imperative to diagnose and manage it as quickly as possible. Adiposity diagnosis is usually achieved by anthropometry. The most used anthropometric method of measuring obesity is BMI. This is derived from measuring an individual's weight in kilograms divided by their height in metres squared (kg/m^2) (Deurenberg & Yap, 1999). In 2010, the World Health Organisation (WHO) recommended the following BMI reference range categories: <18.5 kg/m^2

underweight, 18.5 – 24.9 kg/m^2 recommended weight range, 25 – 29.9 kg/m^2 overweight or pre-obese, and >/=30 kg/m^2 obese (WHO, 2010). BMI is considered to be an ideal measure, for population settings rather than individual measurements, due to it being inexpensive, universal, and non-invasive. It has been mentioned however, that because BMI is a direct measure of weight rather than body fat, there are limitations to consider (Romero-Corral et al., 2008). For example, a very muscular individual can have a high BMI due to their muscle mass and interpreting the raised BMI as obesity would be erroneous. Body fat is also known to increase with age while muscle mass decreases. This change may not affect the individual's height or weight; thus, it may not change the person's BMI which may misrepresent the individual's obesity status (Rothman, 2008). As a result, other body measurements like waist circumference (WC) and skin fold measurements have become increasingly favourable to measure obesity in conjunction with BMI (De Onis & Habicht, 1996; Ruban et al., 2019). WC measurement in particular is an attractive accompaniment for BMI as it assesses abdominal fat which is strongly linked to the development of NCDs (Chaoyang et al., 2007). However, the criteria for WC cutoffs can vary depending on the population under study. For instance, the WHO recommends WC cutoffs of <90 cm for men and <80 cm for women as within the normal range, with measurements of ≥90 cm for men and ≥80 cm for women indicating central adiposity, a key indicator of health risks associated with excess abdominal fat (WHO, 2010).

Following adiposity diagnosis, the next crucial step is therapeutic intervention. Therapies for increased adiposity range from non-invasive to surgical. Non-invasive treatments include diet therapy which focuses on calorie restriction. If diet therapy is unsuccessful then pharmacological treatments that reduce the body's ability to absorb nutrients and/or decrease appetite thus lowering food intake are undertaken. The last resort when non-surgical methods fail is surgical intervention, collectively called bariatric surgery (Ruban et al., 2019). The continued rise in the obesity pandemic suggests that these interventions may not be ideal as they may be too generalised. In human health and disease studies it has become apparent that using community averages for population traits may obscure the differences present between unique populations. Multiple factors such as lifestyle, genetics and environment can affect various populations within the same community differently (Kaiser et al., 2024; Abettan, 2016). This must be accounted for when tackling diagnostic and therapeutic

interventions for worldwide issues like obesity. Tailoring such individualised therapy strategies requires in-depth understanding of all mechanisms, including the epigenetic, that are linked to weight regulation (Peng et al., 2014).

1.4 Epigenetics

Research shows that the development of various diseases is influenced by permanent changes to genetic make-up which affects gene function. These changes, known as mutations, include DNA nucleotide sequence deletions, substitutions, and insertions (Zhang & Gerstein, 2003; Lu et al., 2012; Karlsson et al., 2019; Golden & Kessler, 2020; Jin et al., 2019). More recently, it has additionally surfaced that factors like human lifestyle choices and environment can also effect changes in gene function. These changes differ from mutations in that they are not permanent and do not alter the DNA sequence of the affected gene. The study of these reversible changes that affect gene expression is called epigenetics (Wang et al., 2018).

Epigenetic mechanisms are extremely important as they are associated with modulation of protein synthesis and cell function regulation. Dysregulation of mechanisms that play such a crucial role could lead to abnormal cell function and ultimately, pathology (L. Zhang et al., 2020). Studied methods of epigenetic gene regulation include DNA methylation, histone modification and non-coding RNA (ncRNA) mediated regulation, see Figure 1.4 (Wang et al., 2018; Zhang et al., 2020).

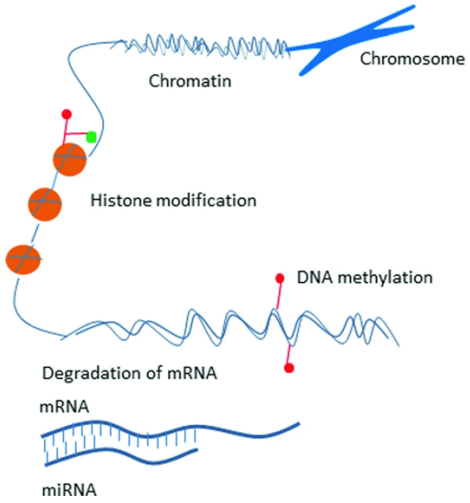

Figure 1.4: *An overview of epigenetic mechanisms* (Zhang et al., 2020).

DNA methylation involves the addition of methyl groups to cytosine residues in the

DNA. This action either recruits gene repressor proteins or inhibits transcription factor (TF) binding, resulting in gene repression (Moore et al., 2013). Histone modifications focus on the addition or removal of chemical moieties on the histone proteins of the nucleosome. This post-transcriptional protein modification includes acetylation, phosphorylation, SUMOylation and ubiquitination processes (Zhang et al., 2021). Histone acetylation, leads to neutralization of the positive charge of the histone protein which loosens the negatively charged chromatin wound around it. This chromatin loosening allows for TFs, polymerases, and other proteins to bind and gene transcription to occur. Conversely, deacetylation removes an acetyl moiety from the histone, thus increasing the overall positive charge and tightening the chromatin-histone interaction resulting in the inhibition of gene transcription (Shvedunova & Akhtar, 2022). The third most common epigenetic mechanism is short, ncRNA, specifically microRNA (miRNA), mediated regulation. miRNAs are RNA molecules that are approximately 22 nucleotides in length and do not carry a code for protein synthesis (Lu & Rothenberg, 2018). Their mode of action is silencing, post-transcriptionally, messenger RNA (mRNA) by binding to complementary sequences found on the target mRNA. This coupling of miRNA to mRNA results in either mRNA degradation or translational inhibition, thus the mRNA does not get translated into protein and is “silenced” (Gu & Kay, 2010).

Due to the continually rising nature of the adiposity epidemic, the associated morbidity and mortality, there is urgent need to understand the epigenetic mechanisms that modulate gene activity significant to adipose tissue function. Understanding these molecular mechanisms may lead to the development of novel therapies for increased adiposity.

1.5 Histone deacetylation

Histone deacetylation is a highly reversible process catalysed by enzymes termed either histone deacetylases (HDACs) or lysine deacetylases (KDACs) (Li et al., 2019). These enzymes are responsible for removal of acetyl groups from the lysine residues in histone and non-histone proteins. In histone proteins, this action results in the tightening of DNA chromatin around the histone due to the recovery of the positive charge of the histone. The increased attraction between the histone and negatively

charged DNA forms a dense structure which is not easily permeable to transcription factors and related proteins. This limits gene transcription and is the HDAC mechanism of gene regulation (Park & Kim, 2020). In direct contrast, histone acetyltransferases (HATs) or lysine acetyltransferases (KATs), add negatively charged acetyl constituents to the histone, repelling the similarly charged DNA and effectively exposing the chromatin to TFs and allowing gene transcription (Seto & Yoshida, 2014; Li et al., 2019). Maintaining balanced HDAC and HAT function is ideal for optimal cell function as a disruption in the balance results in abnormal gene expression which leads to epigenetic diseases (Vahid et al., 2015).

In humans, HDACs are grouped into four classes based on their genetic similarities. Class I houses four zinc-dependant proteins called HDAC 1, 2, 3 and 8. Class II comprises HDACs 4, 5, 6, 7, 9, and 10. Class IV has the single protein, HDAC 11. Of increased significance to this study are sirtuin proteins which are categorized in Class III and are named sirtuin 1-7 (SIRT1-7) (Wang et al., 2020; Shvedunova & Akhtar, 2022). Sirtuin protein activity is completely nicotinamide adenine dinucleotide (NAD+) dependent as it is an important coenzyme for maintaining energy balance and class III HDACs tend to be referred to as energy-sensing enzymes (Chang & Guarente, 2014). A significant number of studies have identified sirtuins, to play a significant role in processes that have a major impact in disease development including excessive adiposity. Thus, determination of relationships between sirtuin activity and obesity development may be crucial to novel therapeutic target discovery (Chalkiadaki & Guarente, 2012).

1.5.1 Sirtuins

Sirtuin enzymes, through either reversible deacetylation or ADP-ribosylation of target cellular proteins, regulate various cellular processes including DNA repair, apoptosis, cell metabolism, glucose output, insulin sensitivity, and cell differentiation among others (Nakagawa & Guarente, 2011; Houtkooper et al., 2012). This post-transcriptional regulatory activity of sirtuins is associated with increased cellular lifespan, cellular senescence delay, and disease prevention. This has resulted in sirtuins being identified as important therapeutic targets in various disease processes, including T2DM, cancer, diseases of ageing, and Alzheimer's disease (North & Verdin,

2004; Lee et al., 2019; Broussy et al., 2020). SIRT bind to the molecule NAD+ as well as the lysine residue of the target protein as their substrates. The deacetylation of the target protein is completely dependent on NAD+ to occur, hence sirtuins are often referred to as NAD+ dependent deacetylases. NAD+ is found in the cellular cytoplasm, nucleus, and mitochondria, and is an important coenzyme for multiple metabolic processes, including maintaining energy balance. NAD+ is a major acceptor of hydride ions, resulting in its reduction to NADH (nicotinamide adenine dinucleotide plus hydrogen). NADH donates its gained electron to adenosine triphosphate (ATP) formation. Caloric restriction, for example, results in an increase in NAD+ and, consequently, SIRT activation. This triggers a cascade of events that ultimately result in the cell regaining energy homeostasis. The sirtuin activity regulation by NAD+ fluctuations cause them to detect metabolism and energy balance disturbances and consequently coordinate stress responses that gear the cell for survival and prolong cellular lifespan (Sauve et al., 2006; Bosch-Presegué & Vaquero, 2015; Covarrubias et al., 2021; Houtkooper et al., 2012; North & Verdin, 2004; Sinclair, 2005).

Sirtuins are found in organisms ranging from bacteria to mammals. Sirtuin genes are highly conserved, meaning they have remained relatively unchanged throughout evolution (Polito et al., 2010). The seven mammalian sirtuins are found in cellular locations specific to their cellular function. For example, SIRT1, SIRT6, and SIRT7 are nuclear proteins and deacetylate intracellular signalling proteins, such as DNA histones, and control gene expression. SIRT3, SIRT4, and SIRT5 are found in the mitochondria and control energy metabolism. SIRT2 is the only sirtuin that functions outside of its cellular location. SIRT2 is found in the cytoplasm but regulates the cell cycle, which is a nuclear process (Lee et al., 2019).

SIRT1, the most studied of the sirtuin proteins, is documented to regulate fat metabolism and inhibit adipogenesis. This places SIRT1 in a crucial position in adiposity management and warrants deeper investigation into the enzyme's regulatory activity and factors that affect its production and function.

1.5.2 Sirtuin 1

SIRT1 is found ubiquitously in the body including the brain, kidney, muscle, liver and

adipose tissues (Clark et al.,2012). SIRT1's complete dependency on NAD+ allows it sensitivity to energy fluctuations in the cell and this capacitates SIRT1 to target proteins involved in energy regulation in direct response to the cell's energy requirements (Picard et al., 2004). Specifically, when SIRT1 senses low caloric intake it promotes gluconeogenesis in the liver which triggers triglyceride breakdown in adipose tissue and mobilises energy. Simultaneously, SIRT1 promotes insulin secretion which stimulates the uptake of energy from the blood into the energy depleted tissues thus restoring metabolic homeostasis and for this reason, SIRT 1 is termed a master metabolic regulator. The deacetylase facilitates these activities by its regulatory function on various transcription factors (TF) including p53, Forkhead box proteins (FOXOs), peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1α), and peroxisome proliferator-activated receptor gamma (PPARγ), amongst others which are either involved in the maintenance of metabolic balance or cellular stress response (Schug & Li, 2011; Kemper et al., 2013).

Maintenance of balanced glycaemic levels is one of the key hepatic functions (Sauve et al., 2006). When fasting is initiated, the liver balances the decreasing glycaemic levels by activating gluconeogenesis. If fasting is prolonged, then gluconeogenesis is coupled with fatty acid breakdown to keep up with increased systemic demand. In various studies on murine hepatocytes, it was found that this second phase of energy homeostasis is regulated by SIRT1. The sirtuin removes an acetyl group from PGC-1α and FOXO1. Deacetylation of these TFs promotes longer, enhanced expression of gluconeogenic genes as well as activates genes that promote fatty acid mobilization and oxidation. SIRT1 was also found to regulate STAT3 which inhibits its gluconeogenic gene suppression function (Rodgers et al., 2005; Chalkiadaki & Guarente, 2012; George, 2006).

SIRT1 is also associated with the deacetylation of the transcription regulator p53. When acetylated, p53 regulates critical cell processes like DNA repair, apoptosis, and cellular replication in response to stress signals. Conversely, the deacetylation of p53 by SIRT1 was observed to repress apoptotic regulation and encourage cellular survival in the face of DNA damage in a study using human derived p53 and murine SIRT1 *in vitro* (North & Verdin, 2004; Vaziri et al., 2001).

In adipose tissue, SIRT1 targets a nuclear receptor protein called PPARγ that controls the expression of genes involved in the induction of adipocyte differentiation, lipid storage and insulin sensitization. SIRT1, by PPARγ deacetylation and thus deactivation, inhibits adipocyte formation from preadipocytes and also inhibits the uptake of fatty acids by existing adipocytes allowing the body access to fatty acids that are converted to energy and thus restores energy homeostasis (Han et al., 2010; Yang et al., 2006; Picard et al., 2004).

SIRT1 is reported to be downregulated in physiological states like obesity and ageing. This was demonstrated in a study on obese women, women with T2DM, and women with normal weight, where Song and co-workers noted that SIRT1 was markedly decreased in the obese group when compared to the normal weight participants. They concluded that an excessive increase in fat has a negative impact on SIRT1 activity (Song et al., 2013). Since SIRT1 delays cellular senescence and plays an important role in fat breakdown and lowering adiposity, it may be favourable to identify key role-players in the pathways that regulate SIRT1 expression in these pathologies (Kwon et al., 2017; Braidy et al., 2011; Donato et al., 2011). Understanding the relationship between SIRT1 and these role-players could lead to therapeutic breakthrough in managing diseases like obesity. For example, it is well-documented that NAD+ is decreased in obesity, and because SIRT1 activity is dependent on NAD+, this subsequently negatively affects SIRT1 expression. As SIRT1 upregulation may potentially reduce obesity it may be worthy to identify other modulators of SIRT1 activity in adiposity. Manipulating those modulators could be the breakthrough needed to make an impact on the increasing obesity prevalence (Choi et al., 2013; Yamaguchi & Yoshino, 2017). miRNA have well-reported regulatory activity over 30% of protein-coding genes, with *SIRT1* mRNA falling within the miRNA regulatory spectrum. As SIRT1 plays a crucial role in energy homeostasis and adipogenesis mediation, it is worthy to investigate miRNA that influence its activity, especially in adiposity. (Buler et al., 2016; Yamakuchi, 2012; Li et al., 2009).

1.6 MicroRNAs

Human genome studies have revealed that 98% of the DNA sequences that transcribe into RNA, do not encode proteins (Yan & Bu, 2021). These RNA transcripts, termed

ncRNA, are documented to be involved in multiple biological processes including gene expression regulation. ncRNA are broadly categorized into two main groups according to their size, namely long ncRNA and short ncRNA (Yang et al., 2016). Of interest to this study is the subset of short ncRNA known as miRNA.

Blood circulating miRNAs were discovered in 2008 and later their presence in other biological fluids were documented (Makarova et al., 2016). This discovery led to studies that revealed that miRNAs synthesized in the cells are released in circulation to reach extracellular targets. In the nucleus, miRNAs are usually transcribed from DNA by RNA polymerase II, and less commonly by RNA polymerase III, into a large, approximately 600 nucleotide (nt) long, precursor molecules called primary miRNA (pri-miRNA). pri-miRNA has a hairpin structure due to possessing a double stranded 33 base-pair stem, a terminal loop. The unlooped end of the double stranded stem is attached to single-stranded segments, as demonstrated in Figure 1.5 (Liu et al., 2008). Next, a microprocessor complex consisting of an RNAse III endonuclease called Drosha and a double stranded RNA binding cofactor named DiGeorge syndrome critical region 8 gene (DGCR8) which binds and cleaves the pri-miRNA into approximately 70 nt long stem-loop precursors called pre-miRNA (Felekkis et al., 2010). pre-miRNA is shuttled out of the nucleus by Exportin 5 into the cytoplasm. Once in the cytoplasm, the precursor is further processed by a second endoribonuclease enzyme called Dicer which cleaves the loop structure of the pre-miRNA. This gives rise to a short double-stranded molecule called a miRNA duplex (MacFarlane & Murphy, 2010). An Argonaute (AGO) protein associates with the duplex, leading to unwinding and release of one of the miRNA strands. The released strand, called the passenger, is degraded while the remaining strand is referred to as the guide strand. Mature miRNAs are annotated with the suffix -5p or -3p depending on which of the miRNA duplex strands was selected as the guide strand (Griffiths-Jones et al., 2006). Various proteins associate with the guide miRNA strand/AGO protein molecule to form an RNA-induced silencing complex (RISC) (Arroyo et al., 2011). It is within this RISC molecule that miRNA perform their gene silencing function. RISC recognises target mRNA with the first eight bases at the guide miRNA's 5' end, called the seed sequence. This seed sequence is always completely complementary to bases found on the target gene's binding site. RISC, via the miRNA, binds to the recognised target at the mRNA's 3' untranslated region. Consequently, the short length of the seed

sequence causes a single miRNA to match with various genes which allows it an ability to target hundreds of genes from related and unrelated physiologic pathways. After the seed region binds with the target binding site, the rest of the miRNA will either have complete or partial complementarity with a target. The nature of the miRNA/mRNA interaction decides which fate the mRNA receives. When the miRNA is perfectly complementary to the mRNA, the gene then becomes silenced by degradation. However, if the miRNA is only partially matched to the mRNA, which is the most common occurrence, then translation is inhibited without degradation (Matsuyama & Suzuki, 2020; O'Brien et al., 2018; Bushati & Cohen, 2007). The ability of the seed sequence to bind with various targets, even without complete complementarity in the rest of the miRNA, means that one miRNA can regulate multiple cellular processes, including cell growth, apoptosis, differentiation, migration, and metabolism. Consequently, when miRNAs become dysregulated, a range of biological processes can be simultaneously affected. Abnormal expression of specific miRNAs has been associated with numerous chronic conditions, including cancers and potentially obesity (Jansson & Lund, 2012; Matsuyama & Suzuki, 2020).

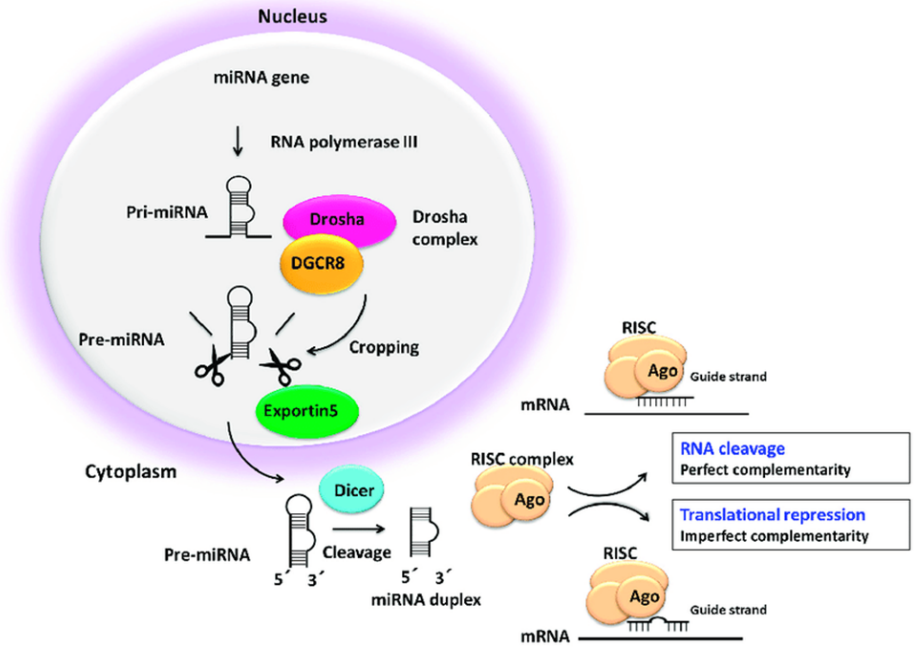

Figure 1.5: *Diagram highlighting the process of miRNA biosynthesis* (Misiewicz-Krzeminska et al., 2019).

Furthermore, it was found that changes in levels of circulating miRNAs are linked to

pathological states. This makes miRNA ideal biomarkers for numerous diseases including adiposity, leading to the need to identify the miRNA involved in pathways that regulate obesity development (O'Brien et al., 2018; Weber et al., 2010). In a study comparing obese and lean human participants, it was noted by Ortega and colleagues that a significant number of human miRNAs, which are upregulated in adipogenesis become downregulated in obesity and vice versa. This suggests that miRNAs that have a regulatory role in fat cell maturation may also have a role in the development of obesity (Ortega et al., 2010). Thus, it is necessary to identify the miRNAs displaying this inverse relationship between adipogenesis and obesity and to identify their target genes as this may yield helpful information on obesity development mechanisms and future therapeutic target options.

1.6.1 miR-30a-5p

Due to the important role played by miRNAs in various cellular processes, it follows that, studies of adipocyte miRNA expression, especially during adipocyte development, may uncover important information that helps with better understanding obesity mechanisms (Koh et al., 2018). In the past decade, miR-30a-5p has been extensively studied and found to be centrally implicated, both positively and negatively, in the differentiation of various cells, in malignant processes, and crucial in both inflammation modulation and adipogenesis (Zhang et al., 2016; Wang et al., 2013; He et al., 2015; Baraniskin et al., 2012; Youssef et al., 2020).

A study on mice showed evidence that miR-30a-5p modulates inflammation in adipose tissue by targeting a pro-inflammatory cytokine inducer called Delta-like 4. This activity inhibits the ATMs from producing inflammatory cytokines and prevents adipose tissue inflammation. In this same study it was found that a high fat diet suppresses miR-30a-5p expression, which results in Delta-like 4 upregulation and the consequent inflammation that is characteristic of obesity (Miranda et al., 2018). In a subsequent study by (Koh et al., 2018), it was demonstrated that when miR-30a is overexpressed, in both mice and human cell lines, it limits the pro-inflammatory and adipocyte differentiation opposing function of interferon gamma (IFNγ) and thus minimizes the inflammation process that leads to adipocytic insulin resistance. In IFNγ inhibition induced by miR-30a overexpression, adipocyte differentiation is also favoured. miR-

30a inhibits IFNγ by targeting the signal transducer and activator of transcription 1 (STAT1) protein. This was also confirmed by Cox and co-workers who determined that diminished IFNγ induced by STAT1 knockout results in inhibited inflammation, even in obese adipocytes. This hinders insulin resistance even without correcting the obese status of the cells (Cox et al., 2020). These studies reveal miR-30a-5p as an attractive therapeutic option for insulin sensitivity promotion in obesity.

Different studies on both C57BL/6 mice and human adipose tissue-derived stem cells (hMADS), revealed that miR-30a-5p expression is elevated during adipogenesis, and the high levels are maintained throughout adipocyte differentiation. This finding demonstrates the involvement of the miRNA in promoting adipogenesis. Conversely, miR-30a-5p suppression results in complete inhibition of adipogenesis, highlighting the significance of miR-30a-5p in adipocyte development (Hilton et al., 2013; Hsieh et al., 2015; Zaragosi et al., 2011). One method of the miRNA's adipogenesis regulatory function was suggested to be promotion of PPARγ activation by inhibiting runt-related transcription factor 2 (RUNX2). This transcription factor promotes chrondrocytic differentiation from the multipotential stem cell that adipocytes are developed from. Chondrocyte development from the stem cell inhibits adipocyte formation which means that RUNX2 inhibits adipogenesis (Enomoto et al., 2004; Fajas, 2009).

A few other studies have confirmed that miR-30a-5p targets SIRT1 in various other pathologies. In a study on rats with cardiac failure, Wu and colleagues concluded that inhibiting miR-30a-5p upregulates SIRT1 thus promoting improved cardiac function. They further confirmed the existence of complementary sequences between the 3' UTR of *SIRT1* mRNA and miR-30a-5p (Wu et al., 2022). Another study found that miR-30a-5p is upregulated during the progression of Alzheimer's disease in mice. Target testing identified SIRT1 to be a target of miR-30a-5p. Further testing in the study showed that miR-30a-5p overexpression silenced SIRT1 production while miR-30-5p inhibition upregulated SIRT1 expression in the Alzheimer's disease affected mice (Sun et al., 2022). The pattern in these studies shows evidence of a regulatory relationship between miR-30a-5p and SIRT1 across a number of varied pathologies in murine populations. This encourages investigating whether the relationship occurs in obesogenic pathways as well.

Cui and colleagues found that miR-30a-5p increased remarkably during adipocyte maturation (Cui et al., 2018). This miRNA is also believed to promote the production of fat in existing adipocytes while suppressing new fat cell synthesis, a process that when dysregulated can lead to increased adiposity. Interestingly, while miR-30a-5p had a notable increase, SIRT1 was found to show a dramatic decrease. These findings conclusively showed that miR-30a-5p is able to promote adipogenesis due to its silencing effect on SIRT1. SIRT1 suppression results in increased levels of PPARγ, which is a known inducer of adipogenesis. The conclusions were drawn from the findings of a study on murine cell line 3T3-L1, where the murine cells were overexposed to miR-30a-5p and the miRNA regulated *SIRT1* post-transcriptionally, resulting in low levels of SIRT1 and consequently, increased adipogenesis (Cui et al., 2018).

The key question that remains to be clarified is whether a similar relationship exists between obesity, SIRT1, and miR-30a-5p in human samples. This study is the first to investigate the relationship between these parameters, in humans from each BMI category (normal weight, overweight and obese) and WC (normal and high), using whole blood and serum samples from a mixed-ancestry population in Bellville South and Belhar, Cape Town.

Chapter 2: Methodology

2.1 Statement of research problem

Previously, adiposity was considered a simple consequence of overeating and lack of exercise. However, current studies confirm it to be more accurately caused by a complex interplay between certain factors, including epigenetic modification of metabolic compounds leading to dysregulation of various biological pathways (Williams et al., 2015; Safaei et al., 2021). One example of this phenomenon is the dysregulation of miRNAs, involved in fat metabolism and storage due to a high caloric diet. This may result in the silencing of genes involved in energy metabolism and storage and could subsequently cause adiposity. Therefore, studies profiling, quantifying, and comparing various miRNAs and proteins in populations with varying BMI and WC measurements are necessary to build on the current available knowledge in order to understand the mechanisms of adiposity development.

2.2 Research questions

This research study sought to address the following:

1) Are there differences in the expression of miR-30a-5p across the BMI (normal, overweight, and obese) and WC (normal and high WC) categories of the mixed-ancestry population in Bellville South and Belhar?
2) Does a relationship exist between adiposity, SIRT1, and miR-30a-5p in the mixed-ancestry population of Bellville South and Belhar?

2.3 Research aim

The aim of the study was to investigate the relationship between adiposity, SIRT1 and miR-30a-5p in a mixed-ancestry population in Bellville South and Belhar, Cape Town.

2.4 Study population

The South African population is highly diverse, with 81.4% identified as African descent, 8.2% as mixed-ancestry, 7.3% as Caucasian, and 2.7% as Indian/Asian (Statistics South Africa, 2022). For this study, we will focus on the mixed-ancestry population as it is the second biggest population group in South Africa, which has a dearth of information regarding adiposity and the link between SIRT1 and miR-30a-

5p.

The mixed-ancestry population is comprised of approximately 32-43% Khoisan, 20-36% Bantu-speaking African, 21-28% European, and 9-11% Asian ancestry, highlighting its unique genetic heritage (De Wit et al., 2010; Matsha et al., 2013).While mixed-ancestry individuals live throughout South Africa, the majority are concentrated in the Western Cape, a legacy of apartheid's Group Areas Act of 1950, which segregated ethnic groups into specific regions. Areas like Bellville and Belhar were designated for those classified as mixed-ancestry, and today, these communities remain largely composed of individuals of mixed-ancestry descent, making them an important focus for research (Matsha et al., 2013; Massey & Gunter, 2020; Maharaj, 2020).

2.4.1 Inclusion criteria

- Individuals residing in the Bellville South and Belhar community.
- Individuals between 20 and 79 years of age.
- Mixed-ancestry ethnicity.

2.4.2 Exclusion criteria

- Individuals who were bedridden or acutely ill.
- Women that were pregnant at the time of the study taking place.

2.4.3 Sample Selection

This study is a case-control study that compared the SIRT1 and miR-30a-5p levels across the BMI categories and WC. For BMI, although there are four categories, namely: underweight, normal weight, overweight, and obese, only three categories were assessed as part of this study. The three categories assessed were normal weight, overweight, and obese. The reason for excluding underweight participants is due to insufficient samples from underweight participants in the dataset. Therefore, the final sample size for this study was 300, with each group having equal numbers in each category, i.e., 100 normal weight, 100 overweight and 100 obese participants. Regarding WC, two categories were defined, namely, normal <90cm and ≥90cm high WC. This criterion was based on analysis by Matsha et al 2013.

2.5 Research objectives

In order to achieve the above aim and address the research questions, the following objectives formed the basis of this study:

a. To quantify the whole blood levels of miR-30a-5p and *SIRT1* mRNA by quantitative polymerase chain reaction (qPCR) and the serum SIRT1 protein levels by enzyme linked immunosorbent assay (ELISA) in mixed-ancestry individuals residing in Bellville South and Belhar using the criteria for:
    i. BMI (World Health Organization (WHO), 2024):
        1. 18.5 – 24.9 kg/m^2 – Normal weight
        2. 25 – 29.9 kg/m^2 – Overweight
        3. >/=30 kg/m^2 – Obese
    ii. WC (Matsha et al., 2013)
        1. WC < 90cm – Normal
        2. WC ≥ 90cm- High WC

b. To compare the expression of *SIRT1* mRNA, SIRT1 concentration levels and quantified miR-30a-5p between the BMI and WC categories.

c. To determine whether relationships exist between the following variables:
    i. Adiposity and SIRT1.
    ii. Adiposity and miR-30a-5p.
    iii. miR-30a-5p and SIRT1.
    iv. Adiposity, miR-30a-5p and SIRT1.

2.6 Hypothesis

We hypothesized that:

- in overweight and obesity, miR-30a-5p has a repressive effect on SIRT1
- in high WC, miR-30a-5p has a repressive effect on SIRT1

2.7 Data collection

The study samples were retrieved from the Vascular Metabolic Health (VMH) study, where recruiters approached each dwelling in the Belville South and Belhar suburbs

and invited residents who fulfilled the inclusion criteria to participate in the survey. Participants who met the inclusion criteria were invited to the designated research site where trained personnel collected both clinical and biochemical data. Interviews were conducted using a standardized questionnaire, which included the WHO StepWise approach. Anthropometric measurements, such as WC and hip circumference, were taken three times using a non-elastic measuring tape, following standardized techniques recommended by the WHO. These measurements were averaged for the purposes of the study. Height was measured to the nearest centimetre (cm) using a stadiometer, while weight was measured using the Omron Body Composition Monitor (BF511: Omron, Japan). BMI was calculated by dividing the weight in kilograms (kg) by the height in meters (m) squared (kg/m²). Blood pressure measurements were taken three times at 3-minute (min) intervals using the Omron M6 Comfort-preformed Cuff Blood Pressure Monitor. The lowest systolic blood pressure (SBP) and corresponding diastolic blood pressure (DBP) readings were used for analysis. Additionally, blood samples were collected by trained nurses after participants fasted overnight. Fasting and 2-hour (h) post samples were drawn for various tests including an oral glucose fasting tolerance test (OGTT) to assess diabetes mellitus.

This study involved the analysis of numerous biochemicals that are key markers of tissue and organ health status including liver enzymes, metabolic profile, and a lipid profile among others. Plasma glucose levels were assessed using the hexokinase method on the Cobas 6000 platform (Roche Diagnostics, Cape Town, SA), while HbA1c levels were determined through high-performance liquid chromatography (HPLC) using the Bio-Rad Variant Turbo (Bio-Rad, Hercules, California, USA). Plasma insulin concentrations were measured by a paramagnetic particle chemiluminescent immunoassay. The lipid profile, which included high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and total cholesterol (TC), was analysed using enzymatic methods on a Beckman AU analyser (Beckman Coulter, California, USA). Triglyceride levels were measured using the glycerol phosphate oxidase (GPO-POD) assay. Ultrasensitive C-reactive protein (CRP) was assessed through a high-sensitivity immunoassay, while serum creatinine levels were evaluated using the kinetic Jaffe method on the Beckman AU analyser. All of these biochemical tests were performed at PathCare, an ISO 15189 accredited laboratory service in Cape Town, South Africa (PathCare, Reference Laboratory, Cape Town, SA).

2.8 Laboratory methodology

2.8.1 Total RNA Extraction

For total RNA isolation, including miRNA, 3 mL of blood was drawn into Tempus Blood RNA Tubes (Thermo Fisher Scientific, Johannesburg, SA) following standard laboratory procedures. Immediately after collection, the tubes were gently shaken for 10 seconds (s) to ensure thorough mixing with the stabilizing reagent, which is crucial for maintaining RNA integrity. The tubes were then stored at −80 °C until needed for further processing. Proper storage and handling are essential to prevent degradation of RNA and to ensure the stabilization of the gene expression profile.

The total RNA was extracted using the MagMAX™ for Stabilized Blood Tubes RNA Isolation Kit (Thermo Fisher Scientific). The frozen whole blood samples were thawed and then diluted with Tempus 1x PBS. The crude RNA was pelleted by vortex and centrifugation and then washed with Tempus Pre-Digestion Wash. Then the pellet was treated with protease and TURBO™ DNase to remove DNA and any other proteins. The following step involved addition of binding beads and then several washes with washing solutions included in the kit. The beads were then allowed to dry, and the RNA was extricated from them using elution buffer. After collecting the RNA from the beads, its purity and integrity was assessed using a nanodrop (NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer, Thermo Fisher). Samples with a concentration of >15 ng/ml and an optical density ratio of A260/A280 > 1.8, were accepted for the study. The RNA was then preserved by freezing at -80 °C until conversion to cDNA.

2.8.2 cDNA conversion

Conversion of the previously extracted total RNA to cDNA was achieved using the TaqMan Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific), which has a protocol that is able to simultaneously detect both miRNA and mRNA from whole blood. . The kit uses three steps to convert RNA to cDNA namely: 1) poly(A) tailing, 2) adapter ligation and, 3) reverse transcription. In the first step poly(A) polymerase catalyses the addition of a 3'-adenosine tail to the RNA. This involves placing 2 µl of RNA into individual wells of a MicroAmp™ Optical 96-well reaction plate and adding 3

µl of poly(A) tailing reagent mix to each well. The plate was sealed, mixed, centrifuged and incubated in a QuantStudio™ 7 Flex Real-Time PCR System (Thermo Fisher Scientific) set to perform polyadenylation for 45 min at 37 °C, then a stop reaction at 65 °C for 10 min and finally an infinite hold at 4 °C. Following incubation, ligation of adapters to the 5' ends of the RNA with poly(A) tails began. This step involved adding 10 µl of prepared adapter ligation reaction mix to each well containing the poly(A) tailing reaction product then sealing, mixing and centrifugation. The plate was then incubated on the QuantStudio™ 7 Flex under the following settings: ligation for 60 min at 16 °C, then an infinite hold at 4 °C. The ultimate step of miRNA conversion to cDNA comprised binding the 3' poly(A) tails of the RNA to a universal RT primer by adding 15 µl of prepared reaction mix to each well containing the adapter ligation products. For the mRNAs within the sample, the RT reaction was facilitated by a universal RT primer and random hexamers. The plate was then sealed, mixed, centrifuged and incubated at 42 °C for 15 min to complete the reverse transcription. This was followed by a stop reaction for 5 min at 85 °C and then an infinite hold at 4 °C. The product of this reaction was then stored at -80 °C until needed for miR-30a-5p and SIRT 1 mRNA expression analyses.

2.8.3 RT-qPCR

RT-qPCR was the method employed to quantify the expression levels of miR-30a-5p and *SIRT1*. This technique allows for the sensitive and precise amplification of RNA, following its conversion to cDNA. The procedure was optimized to assess the respective gene expression of miR-30a-5p and *SIRT1* and provide valuable insights into their relative levels in the patient whole blood samples for both the BMI and WC patient categories.

2.8.3.1 miRNA 30a-5p

For the miRNA qPCR, converted cDNA sample was aliquoted and diluted 1:10 for optimal quantitative analysis, while the remaining cDNA was kept frozen at -80 °C. miRNA expression levels were then evaluated on the 1:10 cDNA dilutions, using pre-designed TaqMan Advanced miRNA Assay primers for the miR-30a-5p. The miR-Amp reaction was performed using the TaqMan™ Advanced miRNA cDNA Synthesis Kit

(Thermo Fisher Scientific). Sufficient miR-Amp Reaction Mix was prepared based on the number of reactions required, combining 2X miR-Amp Master Mix, 20X miR-Amp Primer Mix, and RNase-free water. Each reaction was assembled by adding 5 µL of the reverse transcription product (the cDNA) to 45 µL of the prepared miR-Amp Reaction Mix in fresh reaction tubes. The tubes were briefly vortexed, centrifuged, pipetted into a 96-well plate and loaded into the QuantStudio™ 7 Flex for thermal cycling. The miR-Amp protocol consisted of an initial enzyme activation step at 95 °C for 5 min, followed by 14 cycles of 95 °C for 3 s and 60 °C for 30 s, and concluded with a stop reaction step at 99 °C for 10 min before being held at 4 °C.

To quantify miR-30a-5p expression, data was normalized to the endogenous control, miR-16-5p. Cycle threshold (CT) values were obtained from the amplification curves. Data was grouped by BMI categories and WC, with the normal weight category serving as the control group. Relative expression levels were calculated as follows:

$$\Delta\mathrm{CT}_{sample} = CT_{target\ miRNA} - Average\ CT_{group\ miR16-5p}$$

With $2^{-\Delta CT}$ being the measure of miR-30a-5p for each sample within a BMI category, compared to the average amount of the housekeeping gene, miR16-5p, for that category.

And,

$$\Delta\Delta\mathrm{CT} = \Delta\mathrm{CT}_{target\ miRNA} - \Delta\mathrm{CT}_{mean\ normal\ group}$$

With $2^{-\Delta\Delta CT}$ representing the change in miR-30a-5p expression between each sample in the overweight, obese, central adiposity groups when compared to the average expression in the normal group.

2.8.3.2 SIRT1

SIRT1 mRNA was quantified using qPCR according to the Biorad SsoAdvanced™ Universal SYBR Green Supermix protocol (Bio-Rad). This required the preparation of master mix containing 5 µl SYBR Green, 1 µl forward primer, 1 µl reverse primer, 2 µl nuclease free water (NFH_2O) resulting in a 9 µl solution that was thoroughly mixed

and then added to each well of a MicroAmp™ Fast Optical 96-Well Reaction Plate (0.1 ml). Next, was the addition of 1 µl of thawed and mixed cDNA sample into each reaction well resulting in a final 10 µl reaction volume per well. The samples were run in duplicate, and the plate was sealed, mixed, centrifuged, and incubated in the QuantStudio™ 7 Flex under the following conditions: enzyme activation at 95 °C for 2 min (1 cycle), denaturation at 95 °C for 15 s (40 cycles), annealing at 55 °C for 40 s (40 cycles), extension at 72 °C for 30 s (40 cycles). *SIRT1* expression data was normalised using the housekeeping gene, β-actin which was also run in duplicate for each sample under the same incubation conditions. The duplicate CT values obtained for each sample, were averaged for both the target and the housekeeping genes. The data was then categorized into control and test groups according to their BMI and WC status, with the normal weight group considered as the control group. *SIRT1* expression levels for each sample were calculated using the ΔΔCT method where:

$$\Delta\mathrm{CT}_{sample} = Average\ CT_{SIRT1} - Average\ CT_{\beta-\mathrm{actin}}$$

With $2^{-\Delta CT}$ being the measure of the average amount of *SIRT1* in each sample compared to the average amount of the housekeeping gene, β-actin, for that sample.

And,

$$\Delta\Delta\mathrm{CT} = \Delta\mathrm{CT}_{sample} - \Delta\mathrm{CT}_{mean\ normal\ group}$$

With $2^{-\Delta\Delta CT}$ representing the change in gene expression between each sample in the overweight, obese, and central adiposity groups when compared to the average expression in the normal group.

2.8.4 ELISA

SIRT 1 protein was measured from blood samples that were collected in serum separation tubes and centrifuged at 3000 revolutions per minute (rpm) for 10 m. The separated serum was then aliquoted into cryovials which were stored at -80 °C until needed for further testing. Before proceeding with ELISA testing, the serum samples were removed from the freezer and thawed at room temperature, then mixed thoroughly by aspirating and dispensing the serum with a pipette to homogenise.

The Invitrogen Human SIRT1 ELISA Kit (Thermo Fisher Scientific) assay followed a

sandwich ELISA format, involving antigen binding, biotin conjugate addition, and subsequent detection using a streptavidin-HRP conjugate, followed by tetramethylbenzidine (TMB) substrate reaction.

The measurement of SIRT1 protein levels was carried out on serum samples and the assay was performed in a 96-well plate, with all samples and standards run in duplicate. Serum samples were prepared by a 2-fold dilution in assay diluent C. Seven standards were prepared from lyophilized recombinant human SIRT1 using serial dilutions to generate concentrations ranging from 1.229 ng/mL to 300 ng/mL, with assay diluent C serving as the zero or blank standard. In the pre-coated plate, 100 µL of the prepared standards and samples were pipetted into the 96-well plate and incubated for 2,5 h at room temperature (RT) on a lidded automated shaker set at 400 rpm. Following this, the 96-plate wells were washed four times using an automated plate washer to remove unbound proteins. After washing, 100 µL of biotin-conjugated detection antibody specific to SIRT1 was added to each well and incubated for 1 h at RT on a gentle shake. After incubation, the 96-well plate was washed four times to remove excess detection antibody. Thereafter, 100 µL of streptavidin-HRP conjugate was added to each well, followed by a 45 min incubation period with shaking.

After a third round of washing the 96-well plate was done, 100 µL of TMB substrate solution was added to the wells, and the reaction was allowed to incubate for 30 min in the lidded shaker. The colorimetric reaction was stopped by adding 50 µL of stop solution. Absorbance was read at 450 nm using the Thermo Fisher Multiskan Go reader (Thermo Fisher Scientific). The blank-corrected absorbances were used for analysis.

To interpret the SIRT1 levels, a standard curve was generated from the absorbance values of the known standards using a four-parameter logistic (4PL) regression model, which is described by the equation below, as suggested by the manufacturer:

$$y = \frac{(A-D)}{1+\left(\frac{x}{C}\right)^{B}} + D,$$

where,

A: represents the minimum asymptotes

B: the slope

C: the inflection point
D: the maximum asymptotes

This curve was used to interpolate the SIRT1 concentrations in the unknown serum samples. GraphPad Prism software version 10.3.0 (507) was employed to fit the 4PL curve and calculate the unknown sample concentrations based on their absorbance values.

2.9 Ethical consideration

The VMH study obtained ethical approval from the Research Ethics Committees of the Cape Peninsula University of Technology (NHREC: REC – 230, 408 – 014) and Stellenbosch University (N14/01/003). All procedures were explained, in the participants preferred language, prior to informed consent being given. Data was stored in a password protected file on password protected computer. Given this study, ethical clearance was sought and approved by the CPUT Research Ethics Committee (Approval reference number: CPUT/HWS-REC 2024/H1) (Appendix 1). Both studies followed the World Medical Association code of ethics (Declaration of Helsinki).

2.10 Statistical analysis

The data was analysed using Statistical Package for the Social Sciences (SPSS) software, version 29.0. Normal Q-Q Plots were used to determine whether the data is normal with a $p > 0.050$ being considered normally distributed variables. Skewed variables were presented as the median with 25^{th} – 75^{th} percentiles. The categorical variable results are reported as counts and percentages. The Mann-Whitney (two categories) or Kruskal-Wallis (more than two categories) tests were used for comparison of the continuous variables, while the chi-square test of association was used for categorical variables. Spearman's correlations were carried out to assess the relationship between variables. Furthermore, binary logistic regression models were used to investigate the differences in miRNA 30a-5p and *SIRT1* expression with BMI and WC statuses. In order to assess whether miRNA 30a-5p and *SIRT1* has the ability to predict presence of overweight, obesity and central adiposity, the receiver operator curve (ROC) was generated to determine the area under the curve (AUC). A *p*-value <0.050 was considered to be statistically significant.

Chapter 3: Results

For this study we assessed two variables of adiposity, namely BMI and WC.

3.1 BMI analysis

3.1.1 Characteristics

In this study, 300 participants were grouped into categories based on their BMI: normal weight (n=100), overweight (n=100), and obese (n=100). Table 3.1 shows that participants in the obese category had significantly higher body weight, BMI, and waist and hip circumferences compared to both the normal and overweight groups. Both waist and hip measurements were elevated in the obese group, with median WC reaching 104 cm compared to 92.5 cm in the overweight group and 74.7 cm in the normal group. The median hip circumference in the obese group was 110.75 cm, compared to 100.6 cm in the overweight group and 89.7 cm in the normal group. Overall, age exhibited borderline statistical significance with obese individuals being older than those who were normal weight and overweight. There was no significant difference between males and females with respect to BMI (p = 1.00 respectively).

When assessing the various BMI categories and glucose metabolism, i.e., fasting blood glucose, 2 h blood glucose, HbA1c, insulin fasting, 2 h insulin, it was found that obese individuals had higher levels in comparison to overweight and normal individuals. Liver enzyme tests revealed that alanine aminotransferase (ALT) levels were significantly elevated in the obese group, with a median of 23 IU/L, compared to 14.5 IU/L in the normal group. In contrast, aspartate aminotransferase (AST) levels did not show much variation across the different BMI categories. The enzyme gamma-glutamyl transferase (GGT), however, showed significant differences when the normal group was compared to the overweight and obese groups (p = 0.016 and p < 0.01, respectively). Interestingly, there was no statistical significance when the obese and overweight group levels were compared.

Regarding the lipid panel, there was a statistically significant overall difference ($p \leq$ 0.004 for all parameters). However, when assessing between groups i.e., overweight, and obese, there were no significant differences in the various lipid test levels.

No statistically significant results were observed regarding *SIRT1* gene expression;

however, *SIRT1* displayed an increasing pattern across BMI groups with the normal group displaying the lowest levels and the obese group displaying the highest. SIRT1 protein levels showed a trend toward lower values in obese individuals compared to the normal weight group (p = 0.080). In contrast, miR-30a-5p expression was notably higher in the overweight group relative to the normal group, with minimal variation between the normal and obese groups.

3.1.2 Correlation

3.1.2.1 SIRT1 Gene Expression (SIRT1 $2^{-\Delta CT}$)

Table 3.2 shows no significant association between *SIRT1* gene expression and metabolic markers across BMI groups. For instance, the correlation of *SIRT1* with WC (r = 0.027, p = 0.644) and BMI (r = 0.006, p = 0.923) is weak and non-significant. Systolic blood pressure and *SIRT1* have an extremely weak positive correlation (r = 0.019, p = 0.742) across BMI groups, with no notable difference when analysed by weight status. An interesting finding was the positive correlation (r = 0.117) between *SIRT1* expression and CRP which indicated statistical significance (p = 0.046).

Table 3.1: Body Mass Index Characteristics

	Overall (n=300) median (25th; 75th percentiles)	**Normal (N=100) median (25th; 75th percentiles)**	**Overweight (N=100) median (25th; 75th percentiles)**	**Obese (N=100) median (25th; 75th percentiles)**	***p*-value**			
					Overall	**N vs Ov**	**N vs Ob**	**Ov vs Ob**
SIRT1 $2^{-\Delta CT}$	0.4178 (0.1286; 1.6725)	0.3456 (0.1213; 1.3206)	0.4624 (0.1192; 2.0351)	0.4654 (0.1531; 1.6262)	0.756	0.706	0.452	0.719
miR-30a-5p BMI $2^{-\Delta CT}$	0.0016 (0.0005; 0.0058)	0.001 (0.0002; 0.0038)	0.0022 (0.0007; 0.0067)	0.0017 (0.0007; 0.0058)	0.032	0.015	0.051	0.558
SIRT1 concentration ng/mL	4.056 (0.7275; 25.711)	4.7745 (1.2813; 30.0505)	5.027 (0.736; 32.67)	2.2415 (0.523; 8.643)	0.157	0.982	0.080	0.113
Age (years)	50 (34; 59)	49.5 (33; 58)	49 (33; 58)	53 (38.5; 63)	0.053	0.980	0.036	0.036
Weight (kg)	71 (59.275; 80.95)	55.2 (50.425; 60.95)	72.5 (66.25; 77.875)	86.3 (76.8; 98.4)	<0.001	<0.001	<0.001	<0.001
Height (cm)	163 (158; 169)	163.25 (158.625; 168.5)	163 (159.125; 169.375)	162.5 (156.625; 169)	0.450	0.485	0.579	0.206
BMI	26.6473 (22.3761; 30.7965)	20.2402 (19.3187; 22.392)	26.6473 (25.838; 27.6729)	32.1063 (30.7894; 35.3577)	<0.001	<0.001	<0.001	<0.001
Waist circumference (cm)	91.0833 (77.6917; 101)	74.6667 (69.9167; 79.6)	92.5 (84.125; 98.0333)	104 (94.75; 111.1917)	<0.001	<0.001	<0.001	<0.001
Hip (cm)	100.0333 (90.5; 108.5)	89.6667 (83.5625; 93.65)	100.625 (96; 105.5)	110.75 (104.8333; 116.825)	<0.001	<0.001	<0.001	<0.001
SBP (mmHg)	127 (110; 144)	121.5 (107; 140.5)	123.5 (109; 145)	133 (116; 146.75)	0.009	0.267	0.002	0.055
DBP (mmHg)	82 (73; 91)	78.5 (67.25; 86.75)	81 (72; 90.25)	85 (80; 94)	<0.001	0.074	<0.001	0.003
Glucose 2 h (mmol/L)	5.4 (4.5; 6.7)	4.9 (3.975; 5.825)	5.2 (4.5; 6.35)	6.35 (5.3; 7.8)	<0.001	0.070	<0.001	<0.001
Glucose Fasting Blood (mmol/L)	4.9 (4.5; 5.55)	4.65 (4.3; 5.075)	4.8 (4.5; 5.4)	5.35 (4.8; 6.3)	<0.001	0.018	<0.001	<0.001
HbA1c (%)	5.7 (5.4; 6.1)	5.5 (5.2; 5.8)	5.65 (5.4; 6)	6 (5.525; 6.5)	<0.001	0.010	<0.001	0.015
Insulin 120 (mIU/L)	32.15 (14.875; 69.05)	18.1 (8.9; 31.75)	32 (15; 63.6)	72.4 (36.3; 104.7)	<0.001	0.001	<0.001	<0.001
Insulin Fasting (mIU/L)	6.5 (4.2; 11)	3.9 (2.9; 5.525)	6.55 (4.4; 9.725)	11 (7.5; 14.9)	<0.001	<0.001	<0.001	<0.001
Triglycerides (mmol/L)	1.195 (0.9; 1.7375)	1.05 (0.7725; 1.3675)	1.185 (0.9525; 1.77)	1.425 (1.0025; 1.97)	<0.001	0.006	<0.001	0.079
LDL-c (mmol/L)	3.1 (2.4; 3.8)	2.7 (2; 3.4)	3.4 (2.5; 4.1)	3.3 (2.7; 3.8)	<0.001	<0.001	<0.001	0.429
HDL-c (mmol/L)	1.2 (1.1; 1.5)	1.37 (1.1; 1.6)	1.3 (1.1; 1.5)	1.2 (1.02; 1.4)	0.004	0.126	0.001	0.051
Total cholesterol (mmol/L)	5 (4.2; 5.8)	4.75 (3.9; 5.4)	5.3 (4.325; 6.2)	5.1 (4.5; 5.8)	0.001	0.001	0.004	0.423
C-reactive protein (mg/L)	3.78 (1.45; 7.74)	2.735 (1.015; 8.0925)	2.69 (1.0225; 5.9775)	5.49 (2.76; 9.74)	<0.001	0.726	0.001	<0.001
Cotinine (ng/mL)	83.5 (10; 258)	215 (79.2; 337.5)	10 (10; 239.75)	10 (10; 149)	<0.001	<0.001	<0.001	0.059
ALT (IU/L)	19 (14; 28.75)	14.5 (11; 22)	18 (14; 30.5)	23 (17; 35)	<0.001	0.002	<0.001	0.009
AST (IU/L)	23 (20; 29)	23 (20; 29)	23 (19; 28)	25 (20; 30.75)	0.202	0.351	0.394	0.074
Creatinine (µmol/L)	64 (54; 76)	61 (51; 70)	66.5 (55; 76.75)	65 (54.25; 82.75)	0.040	0.021	0.038	0.925

Gamma GT (IU/L)	30 (21; 45)	25 (17.25; 37.75)	28.5 (22; 45)	36 (25; 51)	<0.001	0.016	<0.001	0.078
JIS Criteria					<0.001	<0.001	<0.001	0.002
No, %(n)	57.9 (173)	83 (83)	56 (56)	34.3 (34)				
Yes, %(n)	42.1 (126)	17 (17)	44 (44)	65.7 (65)				
Sex					1.000	1.000	1.000	1.000
Female, %(n)	50 (150)	50 (50)	50 (50)	50 (50)				
Male, %(n)	50 (150)	50 (50)	50 (50)	50 (50)				
Alcohol use					0.051	0.569	0.019	0.076
No, %(n)	52.3 (156)	45.5 (45)	49.5 (49)	62 (62)				
Yes, %(n)	47.7 (142)	54.5 (54)	50.5 (50)	38 (38)				
Diabetes status					<0.001	0.031	<0.001	0.086
Normal, %(n)	71 (213)	84 (84)	72 (72)	57 (57)				
Prediabetes, %(n)	11.7 (35)	10 (10)	10 (10)	15 (15)				
Diabetes, %(n)	17.3 (52)	6 (6)	18 (18)	28 (28)				

N: Normal; Ov: Overweight; Ob: Obese; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; HbA1c: Haemoglobin A1c; LDL-c: Low density lipoprotein-cholesterol ; HDL-c: High density lipoprotein -cholesterol; ALT: alanine aminotransferase ; AST: aspartate aminotransferase ; Gamma GT: gamma-glutamyl transferase

3.1.2.2 miR-30a-5p Gene Expression (miR-30a-5p $2^{-\Delta CT}$)

The data (Table 3.2 and Table 3.3) indicate a positive correlation between miR-30a-5p and *SIRT1* expression across participants (r = 0.145, *p* = 0.023). Although the correlation between the miRNA and *SIRT1* is weak, it is statistically significant. However, in specific BMI subgroups (e.g., obese individuals), this correlation was noted to lose significance (*p* = 0.631). miR-30a-5p also shows significant associations with age (r = -0.145, *p* = 0.022) and weight (r = 0.143, *p* = 0.024). Significant positive correlations, though weak, are evidenced between miR-30a-5p, fasting glucose, and fasting insulin levels across participants (r = 0.135 and r = 0.137 respectively and *p* = 0.034 for both glucose metabolism markers). The liver function markers ALT and AST showed weak positive correlations with miR-30a-5p across the sample (ALT: r = 0.154, *p* = 0.015 and AST: r = 0.191, *p* = 0.002). The normal BMI groups displayed positive correlation with statistical significance, (r = 0.21, *p* = 0.047 and r = 0.239, *p* = 0.023 respectively). Interestingly the obese group also showed a significant positive correlation between miR-30a-5p and AST with r = 0.346 and *p* = 0.002, this was not seen with ALT.

Table 3.2: Spearman correlation between SIRT1 and BMI

	Overall		Normal		Overweight		Obese	
	r-value	***p*-value**	**r-value**	***p*-value**	**r-value**	***p*-value**	**r-value**	***p*-value**
SIRT1 $2^{-\Delta CT}$	1.000		1.000		1.000		1.000	
miR-30a-5p BMI $2^{-\Delta CT}$	0.145	0.023	0.229	0.032	0.114	0.317	0.055	0.631
SIRT1 concentration ng/mL	-0.053	0.493	0.069	0.618	-0.047	0.721	-0.113	0.400
Age (years)	-0.053	0.368	-0.009	0.931	-0.043	0.677	-0.125	0.218
Weight (kg)	0.006	0.923	0.013	0.898	-0.040	0.693	-0.108	0.288
Height (cm)	0.028	0.633	-0.005	0.959	-0.022	0.830	0.104	0.307
Waist circumference (cm)	0.027	0.644	0.014	0.893	0.010	0.926	-0.059	0.560
Hip (cm)	0.061	0.300	-0.006	0.954	0.178	0.080	-0.088	0.385
Systolic blood pressure(mmHg)	0.019	0.742	0.079	0.442	-0.043	0.677	0.013	0.902
Diastolic blood pressure (mmHg)	0.003	0.956	0.061	0.548	-0.099	0.331	0.014	0.892
Glucose 2 HRs (mmol/L)	0.035	0.579	-0.062	0.558	0.062	0.586	0.108	0.345
Glucose Fasting Blood (mmol/L)	-0.002	0.970	-0.058	0.572	-0.018	0.860	0.051	0.622
HbA1c (%)	0.002	0.977	-0.056	0.585	-0.019	0.849	0.067	0.512
Insulin 120 (mIU/L)	0.033	0.609	-0.073	0.493	0.053	0.642	0.039	0.742
Insulin Fasting (mIU/L)	0.059	0.322	0.039	0.713	0.163	0.113	-0.095	0.363
Triglycerides (mmol/L)	-0.065	0.265	-0.122	0.232	-0.127	0.214	0.031	0.764
LDL-c (mmol/L)-M	-0.035	0.555	0.011	0.918	-0.112	0.273	-0.022	0.828
HDL-c (mmol/L)	-0.105	0.074	0.010	0.918	-0.171	0.094	-0.140	0.169
Total cholesterol (mmol/L)	-0.044	0.454	0.025	0.809	-0.157	0.122	0.004	0.971
C-reactive protein (mg/L)	0.117	0.046	0.031	0.763	0.319	0.001	-0.059	0.564
Cotinine (ng/mL)	0.098	0.096	0.142	0.168	-0.009	0.928	0.289	0.005
ALT (IU/L)	-0.086	0.142	-0.111	0.277	-0.173	0.088	-0.023	0.824
AST (IU/L)	-0.134	0.021	-0.054	0.600	-0.257	0.011	-0.091	0.371
Creatinine (µmol/L)	0.019	0.748	-0.014	0.893	-0.023	0.824	0.076	0.453
Gamma GT (IU/L)	-0.073	0.210	-0.079	0.437	-0.096	0.346	-0.087	0.389

HbA1c: *Haemoglobin A1c*; LDL-c: Low density lipoprotein -cholesterol; HDL-c: High density lipoprotein -cholesterol ; ALT: Alanine aminotransferase ; AST: Aspartate aminotransferase ; Gamma GT: gamma-glutamyl transferase

Table 3.3: Spearman correlation between miR-30a-5p and BMI

	Overall		Normal		Overweight		Obese	
	r-value	***p*-value**	**r-value**	***p*-value**	**r-value**	***p*-value**	**r-value**	***p*-value**
SIRT1 $2^{-\Delta CT}$	0.145	0.023	0.229	0.032	0.114	0.317	0.055	0.631
miR-30a-5p BMI $2^{-\Delta CT}$	1.000		1.000		1.000		1.000	
SIRT1 concentration ng/mL	0.121	0.148	0.078	0.600	0.173	0.234	0.101	0.496
Age (years)	-0.145	0.022	-0.059	0.578	-0.190	0.091	-0.232	0.040
Weight (kg)	0.143	0.024	0.077	0.473	-0.034	0.766	0.151	0.184
Height (cm)	0.077	0.227	0.148	0.164	0.027	0.814	0.057	0.619
Waist circumference (cm)	0.059	0.355	-0.078	0.463	-0.198	0.079	0.075	0.510
Hip (cm)	-0.002	0.976	-0.244	0.020	-0.238	0.034	0.051	0.652
Systolic blood pressure(mmHg)	0.046	0.470	0.104	0.331	-0.111	0.325	0.065	0.571
Diastolic blood pressure (mmHg)	0.017	0.790	0.001	0.994	-0.130	0.249	0.032	0.780
Glucose 2 HRs (mmol/L)	-0.026	0.687	-0.172	0.106	0.055	0.634	-0.105	0.360
Glucose Fasting Blood (mmol/L)	0.135	0.034	0.044	0.684	0.020	0.860	0.253	0.025
HbA1c (%)	-0.048	0.447	-0.180	0.090	-0.045	0.693	0.025	0.827
Insulin 120 (mIU/L)	-0.062	0.335	-0.148	0.168	-0.023	0.844	-0.286	0.014
Insulin Fasting (mIU/L)	0.137	0.034	0.126	0.249	0.032	0.777	0.118	0.318
Triglycerides (mmol/L)	0.018	0.781	0.100	0.350	-0.142	0.209	0.016	0.889
LDL-c (mmol/L)-M	0.017	0.787	-0.115	0.280	0.049	0.665	-0.023	0.844
HDL-c (mmol/L)	0.111	0.082	0.166	0.117	0.075	0.511	0.161	0.159
Total cholesterol (mmol/L)	0.075	0.237	0.009	0.930	0.041	0.721	0.051	0.657
C-reactive protein (mg/L)	0.031	0.632	-0.110	0.303	0.064	0.573	0.177	0.122
Cotinine (ng/mL)	-0.046	0.477	0.090	0.407	-0.073	0.525	-0.032	0.785
ALT (IU/L)	0.154	0.015	0.21	0.047	-0.016	0.886	0.168	0.139
AST (IU/L)	0.191	0.002	0.239	0.023	0.003	0.981	0.346	0.002
Creatinine (µmol/L)	0.017	0.786	0.124	0.248	-0.119	0.292	-0.036	0.754
Gamma GT (IU/L)	0.064	0.315	0.179	0.092	-0.127	0.262	0.032	0.781

HbA1c: Haemoglobin A1c; LDL-c: Low density lipoprotein -cholesterol; HDL-c: High density lipoprotein -cholesterol; ALT: alanine aminotransferase; AST: Aspartate aminotransferase ; Gamma GT: gamma-glutamyl transferase

3.1.3 Logistic regression

3.1.3.1 SIRT1

The logistic regression results in Table 3.4 indicates no statistically significant associations were observed between *SIRT1* expression and transitions across any of the BMI category pairings (Normal to Overweight, Normal to Obese, Overweight to Obese). All *p*-values remain above the standard threshold for significance ($p > 0.05$), indicating no significant relationship between *SIRT1* expression and BMI transitions in this analysis. Variables for the models 2 to 4 was based on correlation analysis. The results found that these variables, when assessed collectively, yield non-significant results (all $p \geq 0.172$). This suggests that when the variables are combined, they do not add predicative value unlike when being assessed alone.

3.1.3.2 miR-30a-5p

In Table 3.5, miR-30a-5p shows significant results in Model 1 and Model 2. Both models show statistical significance for the normal to overweight transition (model 1 $p = 0.046$ and model 2 $p = 0.047$, respectively). Model 5 reveals statistical significance for the normal to overweight transition ($p = 0.014$), indicating a stronger association for miR-30a-5p when adjusting for hip, ALT, AST and SIRT1. No statistically significant results were found for transitions from Normal to Obese or Overweight to Obese in any of the models.

Table 3.4: Logistic regression of SIRT1 and BMI

	Normal ->Overweight		Normal ->Obese		Overweight ->Obese	
	OR (95% CI)	***p*-value**	**OR (95% CI)**	***p*-value**	**OR (95% CI)**	***p*-value**
Model 1	0.99 (0.98; 1.01)	0.359	0.99 (0.97; 1.01)	0.172	1 (0.98; 1.01)	0.648
Model 2	0.99 (0.98; 1.01)	0.361	0.99 (0.97; 1.01)	0.212	1 (0.98; 1.02)	0.821
Model 3	0.99 (0.98; 1.01)	0.321	0.99 (0.97; 1.01)	0.196	1 (0.98; 1.02)	0.840
Model 4	0.99 (0.98; 1.01)	0.441	0.99 (0.97; 1.01)	0.468	1 (0.98; 1.02)	0.635

OR: Odds Ratio; Model 1: Crude; Model 2: Model 1 + age; Model 3: Model 1 + c-reactive protein + AST; Model 4: Model 3 + cotinine

Table 3.5: Logistic Regression of miR-30a-5p and BMI

	Normal ->Overweight		**Normal ->Obese**		**Overweight ->Obese**	
	OR (95% CI)	***p*-value**	**OR (95% CI)**	***p*-value**	**OR (95% CI)**	***p*-value**
Model 1*	1.74 (1.01; 3.00)	0.046	1.60 (0.95; 2.70)	0.079	0.90 (0.72; 1.12)	0.351
Model 2*	1.74 (1.01; 3.01)	0.047	1.59 (0.94; 2.69)	0.082	0.92 (0.73; 1.14)	0.432
Model 3*	1.69 (0.66; 4.32)	0.273	0.53 (0.04; 7.74)	0.643	0.64 (0.39; 1.06)	0.082
Model 4*	1.72 (0.59; 5.05)	0.323	6.27 (0.05; 757)	0.453	0.76 (0.45; 1.29)	0.315
Model 5*	2.97 (1.24; 7.1)	0.014	2.45 (0.78; 7.75)	0.126	0.94 (0.67; 1.31)	0.709

OR: Odds Ratio; Model 1: crude, Model 2: Model 1+age, Model 3: Model1 + weight + fasting blood glucose + insulin fasting + ALT + AST, Model 4: Model 3 + hip + insulin 120, Model 5: Model1 + hip + ALT + AST +SIRT1, *calculated for 0.01-unit increase

3.1.4 Receiver operating curves

For the ROC curves, BMI was used as the gold standard to compare with miR-30a-5p and *SIRT1. Based on this,* miR-30a-5p displayed a modest but statistically significant ability to distinguish between these groups, with an AUC of 0.603 and $p = 0.019$, see Figure 3.1. Conversely, inverse *SIRT1* did not demonstrate a significant discriminative power, with an AUC of 0.501 and $p = 0.990$.In Figure 3.2., miR-30a-5p exhibited a limited ability to differentiate these groups, with an AUC of 0.583 and a $p = 0.060$, falling short of statistical significance. Similarly, SIRT1 did not demonstrate significant discriminative capacity, showing an AUC of 0.510 and a $p = 0.830$. When assessing overweight and obese categories, Figure 3.3, neither inverse miR-30a-5p (AUC = 0.528, $p = 0.538$) nor *SIRT1* (AUC = 0.519, $p = 0.676$) demonstrated statistically significant discriminatory power for this comparison. Based on all three figures, miR-30a-5p and *SIRT1* did not outperform BMI as potential alternative diagnostic markers.

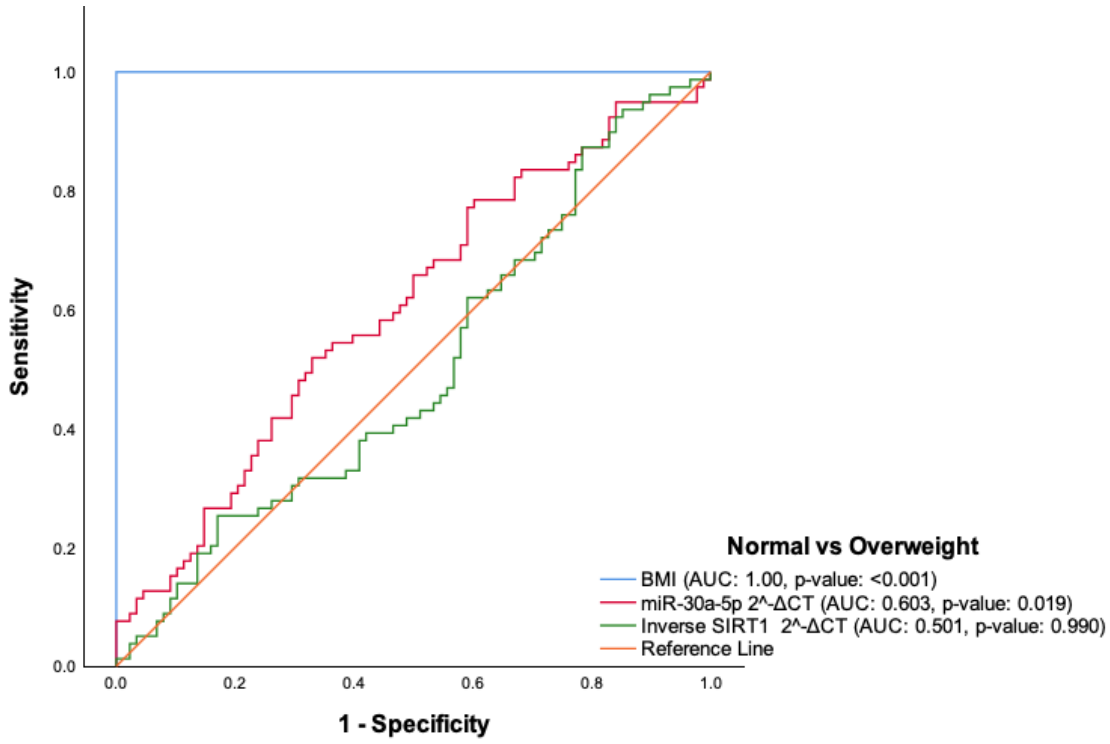

Figure 3.1: *Receiver operating curve of normal vs overweight*

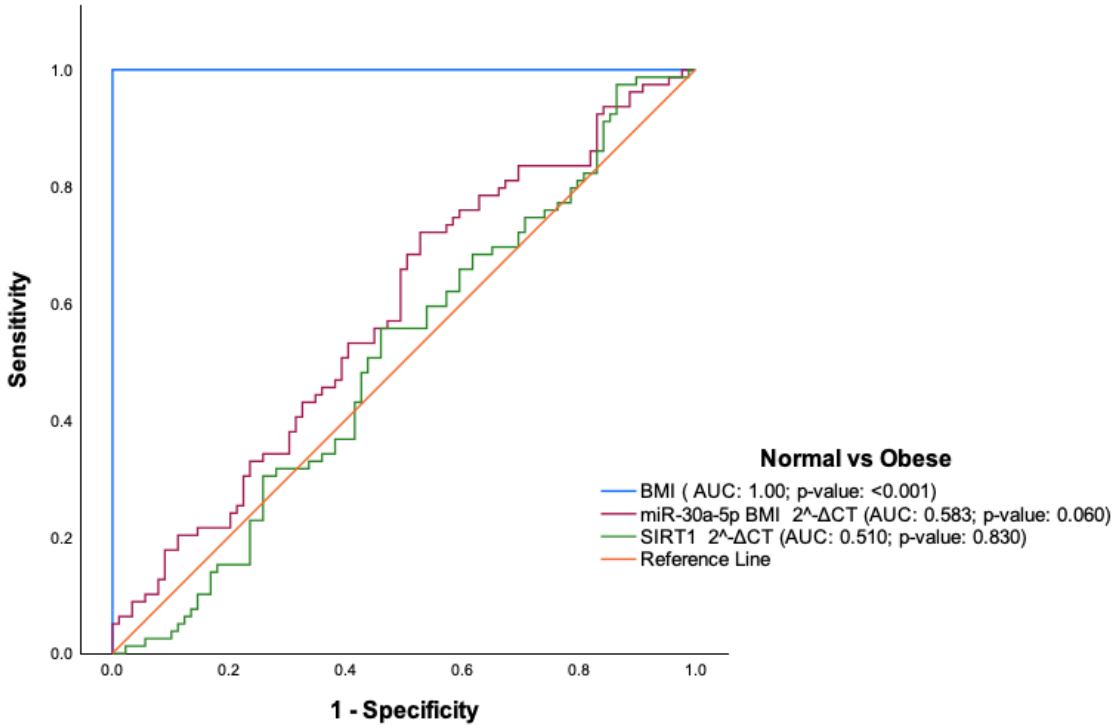

Figure 3.2: *Receiver operating curve of normal vs obese*

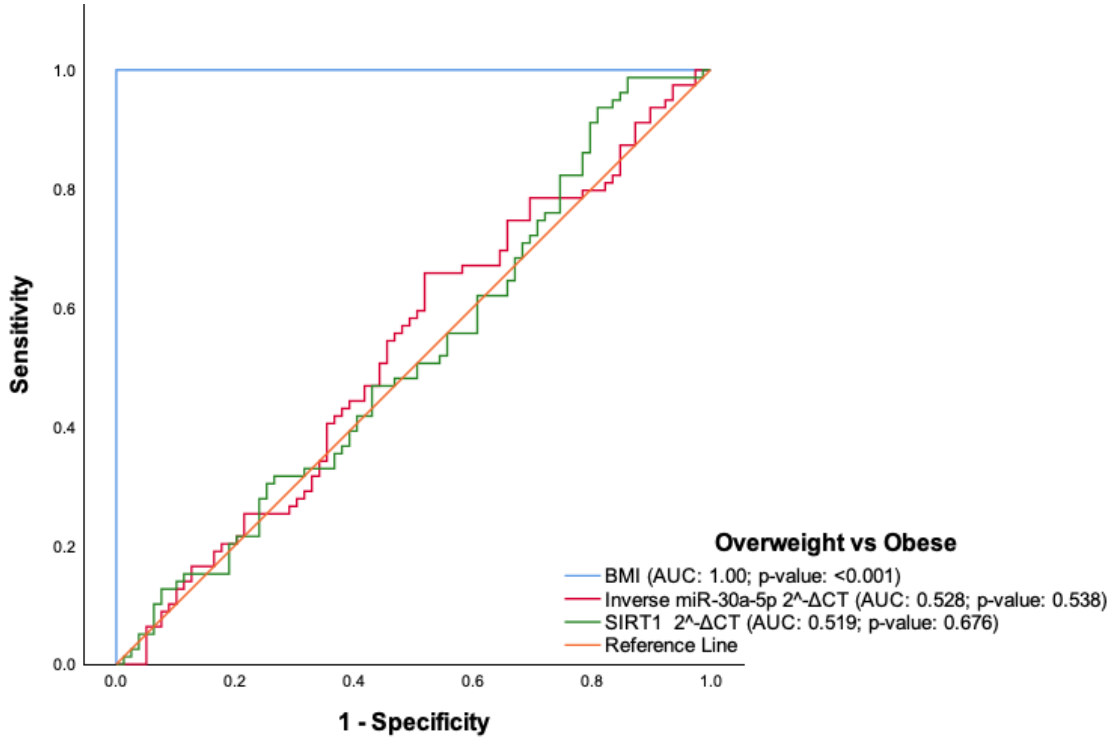

Figure 3.3: *Receiver operating curve of overweight vs obese*

3.2 Waist Circumference

3.2.1 Characteristics

When the participants were grouped by WC, 144 had normal measurements, while 156 had central adiposity or high WC. The data, summarized in Table 3.6, revealed that those with high WC had considerably greater weight, BMI, waist and hip circumferences compared to those with normal range WC. The median BMI was 30.5 kg/m² in the high WC group and 22.3 kg/m² in the normal WC group. Likewise, the median WC in the central adiposity group was much higher, at 100.8 cm, compared to 77.2 cm in the normal group. Age was shown to differ significantly between the two groups (p = 0.007) with central adiposity observed in older participants. There was no variation in WC between males and females.

Participants with high WC had significantly higher results in all glucose metabolism tests, including fasting glucose, 2 h post-prandial glucose, HbA1c, and insulin levels, with all p < 0.001. The normal WC group showed lower levels of triglycerides, LDL, and total cholesterol, while the high WC exhibited lower levels of HDL.

The liver enzymes, ALT and GGT, were significantly elevated in the high WC group, both with p < 0,001. In contrast, AST did not show a remarkable difference between the two groups. Although statistical significance was not reached, (p = 0.086), SIRT1 protein concentration tended to be higher in the normal WC group compared to the high WC group, while *SIRT1* gene expression remained comparable between the two groups ($p \leq 0.854$). In contrast, miR-30a-5p showed a pattern of increased levels in the central adiposity group rather than the normal group ($p \leq 0.082$).

Table 3.6 Waist Circumference Characteristics

	Overall (N=300)	**Normal (N=144)**	**High WC (N=156)**	***p*-value**
	Median (25^{th}; 75^{th} percentile)	**Median (25^{th}; 75^{th} percentile)**	**Median (25^{th}; 75^{th} percentile)**	
SIRT1 WC $2^{-\Delta CT}$	0.42 (0.13; 1.64)	0.41 (0.14; 1.62)	0.45 (0.12; 1.68)	0.854
miR-30a-5p WC $2^{-\Delta CT}$	0.0016 (0.0005; 0.0053)	0.0013 (0.0003; 0.0045)	0.0019 (0.0007; 0.0061)	0.082
SIRT1 concentration ng/mL	4.1 (0.7; 25.7)	4.8 (1.2; 28.5)	2.6 (0.4; 21.5)	0.086
Age (years)	50 (34; 59)	49 (32; 58)	51.5 (40.5; 61.8)	0.007
Weight (kg)	71 (59.3; 81)	58.6 (51.9; 67.2)	80.2 (74.9; 92.7)	<0.001
Height (cm)	163 (158; 169)	161.5 (156.5; 166.5)	165 (159; 171.4)	0.001
BMI (m^2/kg)	26.7 (22.4; 30.8)	22.3 (19.8; 25.4)	30.5 (27.3; 33.4)	<0.001
Waist (cm)	91.1 (77.7; 101)	77.2 (71.5; 84)	100.8 (94.5; 108.6)	<0.001
Hip (cm)	100.1 (90.5; 108.5)	91.1 (85.5; 96.7)	106.5 (102.5; 113.6)	<0.001
SBP (mmHg)	127 (110; 144)	121.5 (107; 142.8)	129 (115; 145.8)	0.008
DBP (mmHg)	82 (73; 91)	80.5 (68.3; 87)	83.5 (77; 93)	0.001
Glucose 2 HRs (mmol/L)	5.4 (4.5; 6.7)	4.9 (4; 5.9)	6 (4.9; 7.7)	<0.001
Glucose Fasting Blood (mmol/L)	4.9 (4.5; 5.6)	4.7 (4.3; 5.1)	5.1 (4.7; 6)	<0.001
HbA1c (%)	5.7 (5.4; 6.1)	5.6 (5.3; 5.9)	5.9 (5.5; 6.4)	<0.001
Insulin 120 (mIU/L)	32.2 (14.9; 69.1)	19.3 (10.1; 39.3)	51.1 (30.4; 86.2)	<0.001
Insulin Fasting (mIU/L)	6.5 (4.2; 11)	4.3 (3; 6.9)	9.6 (6.2; 14.1)	<0.001
Triglycerides (mmol/L)	1.2 (0.9; 1.7)	1 (0.8; 1.4)	1.5 (1; 1.9)	<0.001
LDL-c (mmol/L)	3.1 (2.4; 3.8)	2.7 (2.1; 3.6)	3.4 (2.8; 3.9)	<0.001
HDL-c(mmol/L)	1.2 (1.1; 1.5)	1.4 (1.1; 1.6)	1.2 (1.1; 1.4)	<0.001
Total cholesterol (mmol/L)	5 (4.2; 5.8)	4.8 (4; 5.6)	5.2 (4.5; 6.1)	0.001
C-reactive protein (mg/L)	3.8 (1.5; 7.7)	2.4 (0.9; 6.3)	4.7 (2.1; 8.8)	<0.001
Cotinine (ng/mL)	83.5 (10; 258)	187 (10; 295.8)	10 (10; 185)	<0.001
ALT (IU/L)	19 (14; 28.8)	16 (12; 24)	23 (16; 34)	<0.001
AST (IU/L)	23 (20; 29)	23 (20; 29)	25 (20; 29.8)	0.476
Creatinine (µmol/L)	64 (54; 76)	62 (53; 71)	67.5 (54.3; 80.8)	0.020
Gamma GT-S* (IU/L)	30 (21; 45)	26 (19; 37.8)	35 (24; 49)	<0.001
Sex				0.065
Female, %(n)	50 (150)	55.6 (80)	44.9 (70)	
Male, %(n)	50 (150)	44.4 (64)	55.1 (86)	
JIS Criteria				<0.001
No, %(n)	57.9 (173)	81.3 (117)	36.1 (56)	
Yes, %(n)	42.1 (126)	18.8 (27)	63.9 (99)	
Alcohol use				0.087
No, %(n)	52.3 (156)	47.2 (68)	57.1 (88)	
Yes, %(n)	47.7 (142)	52.8 (76)	42.9 (66)	

BMI: body mass index; SBP: Systolic blood pressure; DBP: diastolic blood pressure; HbA1c: Haemoglobin A1c ; LDL-c: Low density lipoprotein -cholesterol; HDL-c: High density lipoprotein -cholesterol ; ALT: alanine aminotransferase ; AST: aspartate aminotransferase ; Gamma GT: gamma-glutamyl transferase

3.2.2 Correlations

3.2.2.1 SIRT1 Gene Expression

Table 3.7 shows weak and non-significant correlations for *SIRT1* with key adipose markers such as WC (r = 0.018, *p* = 0.753) and hip circumference (r = 0.052, *p* = 0.375). These correlations suggest that *SIRT1* gene expression may not change markedly with central obesity. The correlation between *SIRT1* and CRP (r = 0.109, *p* = 0.062) approaches significance, suggesting that *SIRT1* may have a limited association with inflammation markers, especially in the high WC group. This may reflect a subtle connection between *SIRT1* expression and inflammatory responses in high WC. Another finding illustrated in the table suggested a weakly negative relationship between *SIRT1* and AST (r = -0.127 and *p* = 0.029).

3.2.2.2 miR-30a-5p Gene Expression

Table 3.8 demonstrates a positive correlation between miR-30a-5p and *SIRT1* (r = 0.153, *p* = 0.010). Although the correlation is weak (r <1), the statistically significant value (*p* = 0.010) suggests that this association is unlikely to be due to chance. However, despite the statistical significance, the weak correlation indicates that this relationship may have limited practical impact. Interestingly, miR-30a-5p has a significant positive association with fasting blood glucose in the high WC group (r = 0.263, *p* = 0.001). This finding suggests that higher levels of miR-30a-5p may correlate with increased blood glucose levels, especially in individuals with central obesity. This pattern may point to a role for miR-30a-5p in glucose metabolism within this population. When the sample population findings are viewed in their entirety for miR-30a-5p correlation with ALT and AST in the WC groups, weak positive but significant correlations were displayed, (r = 0.142, *p* = 0.016 and r = 0.143, *p* = 0.015, respectively). This positive correlation trend with ALT was seen in the normal group (r = 0.190 and *p*= 0.026).

Table 3.7: Spearman correlation between SIRT1 and WC

	Overall		Normal		High WC	
	r-value	***p*-value**	**r-value**	***p*-value**	**r-value**	***p*-value**
SIRT1 WC 2^-ΔCT	1.000		1.000		1.000	
miR-30a-5p WC 2^-ΔCT	0.153	0.010	0.196	0.023	0.113	0.168
SIRT1 concentration ng/mL	-0.071	0.358	-0.048	0.672	-0.108	0.313
Age (years)	-0.055	0.343	0.004	0.960	-0.110	0.173
Weight (kg)	0.010	0.867	0.044	0.604	0.027	0.736
Height (cm)	0.035	0.545	0.000	0.998	0.072	0.372
BMI	-0.003	0.954	0.055	0.520	-0.068	0.401
Waist (cm)	0.018	0.753	0.110	0.197	0.013	0.877
Hip (cm)	0.052	0.375	0.107	0.209	0.063	0.440
SBP (mmHg)	0.011	0.855	0.046	0.587	-0.016	0.843
DBP (mmHg)	0.005	0.925	-0.014	0.871	0.024	0.765
Glucose 2 HRs (mmol/L)	0.025	0.694	0.062	0.485	0.037	0.689
Glucose Fasting Blood (mmol/L)	-0.009	0.876	-0.018	0.834	0.045	0.584
HbA1c (%)	-0.005	0.930	-0.055	0.515	0.075	0.354
Insulin 120 (mIU/L)	0.023	0.723	0.048	0.595	0.037	0.690
Insulin Fasting (mIU/L)	0.054	0.371	0.118	0.177	0.003	0.976
Triglycerides (mmol/L)	-0.063	0.285	-0.123	0.147	0.001	0.994
LDL-c (mmol/L)	-0.038	0.516	-0.032	0.708	-0.060	0.463
Chol LDL-S (mmol/L)	-0.040	0.500	-0.021	0.809	-0.068	0.411
HDL-c(mmol/L)	-0.104	0.077	-0.131	0.123	-0.087	0.287
Total cholesterol (mmol/L)	-0.048	0.417	-0.056	0.514	-0.049	0.544
C-reactive protein (mg/L)	0.109	0.062	0.096	0.258	0.166	0.040
Cotinine (ng/mL)	0.105	0.076	0.093	0.276	0.140	0.091
ALT (IU/L)	-0.084	0.150	-0.111	0.190	-0.051	0.531
AST (IU/L)	-0.127	0.029	-0.122	0.151	-0.122	0.131
Creatinine (µmol/L)	0.025	0.668	0.040	0.643	-0.007	0.935
Gamma GT-S* (IU/L)	-0.064	0.271	-0.114	0.179	-0.015	0.851

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; HbA1c: Haemoglobin A1c; LDL-c: Low density lipoprotein -cholesterol; HDL-c: High density lipoprotein-cholesterol; ALT: alanine aminotransferase; AST: aspartate aminotransferase; Gamma GT: gamma-glutamyl transferase

Table 3.8: *Spearman correlation between miR-30a-5p and WC*

	Overall		Normal		High WC	
	r-value	***p*-value**	**r-value**	***p*-value**	**r-value**	***p*-value**
SIRT1 WC $2^{\wedge-\Delta CT}$	0.153	0.010	0.196	0.023	0.113	0.168
miR-30a-5p WC $2^{\wedge-\Delta CT}$	1.000		1.000		1.000	
SIRT1 concentration ng/mL	0.075	0.338	0.071	0.537	0.089	0.407
Age (years)	-0.108	0.068	-0.127	0.139	-0.137	0.093
Weight (kg)	0.136	0.021	0.104	0.226	0.064	0.437
Height (cm)	0.111	0.059	0.181	0.034	0.025	0.764
BMI	0.088	0.134	0.038	0.657	0.024	0.765
Waist (cm)	0.082	0.164	-0.004	0.965	-0.021	0.802
Hip (cm)	0.011	0.849	-0.147	0.086	0.014	0.868
SBP (mmHg)	0.039	0.508	-0.066	0.442	0.106	0.193
DBP (mmHg)	-0.023	0.703	-0.082	0.340	-0.026	0.752
Glucose 2 HRs (mmol/L)	-0.023	0.723	-0.090	0.314	-0.025	0.791
Glucose Fasting Blood (mmol/L)	0.152	0.010	-0.031	0.721	0.263	0.001
HbA1c (%)	-0.026	0.661	-0.152	0.076	0.033	0.683
Insulin 120 (mIU/L)	-0.071	0.271	-0.078	0.389	-0.163	0.079
Insulin Fasting (mIU/L)	0.118	0.049	0.156	0.075	0.004	0.963
Triglycerides (mmol/L)	0.074	0.212	0.073	0.395	0.029	0.722
LDL-c (mmol/L)	0.048	0.418	-0.022	0.802	0.069	0.403
Chol LDL-S (mmol/L)	0.053	0.376	-0.020	0.820	0.096	0.253
HDL-c(mmol/L)	0.080	0.175	0.046	0.598	0.192	0.018
Total cholesterol (mmol/L)	0.103	0.081	0.033	0.704	0.139	0.088
C-reactive protein (mg/L)	0.040	0.500	0.030	0.727	-0.007	0.934
Cotinine (ng/mL)	0.013	0.835	0.001	0.994	0.047	0.575
ALT (IU/L)	0.142	0.016	0.19	0.026	0.064	0.430
AST (IU/L)	0.143	0.015	0.159	0.064	0.112	0.168
Creatinine (µmol/L)	0.048	0.417	0.097	0.259	-0.001	0.987
Gamma GT-S* (IU/L)	0.044	0.454	0.123	0.151	-0.095	0.246

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; HbA1c: haemoglobin A1c; LDL-c: low density lipoprotein; HDL-c: high density lipoprotein; ALT: alanine aminotransferase; AST: aspartate aminotransferase; Gamma GT: gamma-glutamyl transferase

3.2.3 Logistic Regression

3.2.3.1 SIRT1

As seen in Table 3.9, *SIRT1* expression displays non-significant associations with high WC classification across all models. For instance, Model 1 (crude) shows an OR of 0.986 ($p = 0.095$), while Model 4 shows an OR of 0.987 ($p = 0.111$) even after adjusting for CRP and AST. This lack of significance reinforces the earlier observation that *SIRT1* does not serve as a major predictor for WC-based obesity.

3.2.3.2 miR-30a-5p

Table 3.10 reveals similar findings, with miR-30a-5p also failing to significantly predict WC classification across models (e.g., Model 5, OR = 0.81, $p = 0.360$). These findings show that miR-30a-5p does not consistently predict WC-based obesity.

Table 3.9: *Logistic regression of SIRT1 and WC*

	Odds ratio (OR)	95% confidence interval		*p*-value
		Lower	Upper	
Model 1	0.986	0.971	1.002	0.095
Model 2	0.988	0.972	1.004	0.141
Model 3	0.987	0.971	1.003	0.107
Model 4	0.987	0.971	1.003	0.111

Model 1: Crude, Model 2: Model 1 + age, Model 3: Model 1+AST, Model 4: Model 3 + CRP

Table 3.10: *Logistic regression of miR-30a-5p and WC*

	Odds ratio (OR)	95% confidence interval		*p*-value
		Lower	Upper	
Model 1	1.04	0.83	1.3	0.749
Model 2	1.06	0.85	1.33	0.603
Model 3	0.79	0.49	1.27	0.329
Model 4	1.03	0.81	1.3	0.836
Model 5	0.81	0.51	1.28	0.360

Model 1: Crude, Model 2: Model 1 + age, Model 3: Model 1+ weight + fasting blood glucose + insulin fasting + ALT + AST, Model 4: Model 1 + height + HDL-c, Model 5: Model 3 + Model 4, calculated for 0.01-unit increase.

3.2.4 Receiver operating curves

We performed a receiver operating curve using WC as the gold standard in comparison with miR-30a-5p and *SIRT1*, see Figure 3.4. from the figure, it was revealed that miR-30a-5p (AUC = 0.550, *p* = 0.143) and inverse *SIRT1* (AUC = 0.505, *p* = 0.889) did not reach statistical significance for use as a potential alternative diagnostic marker for WC categories.

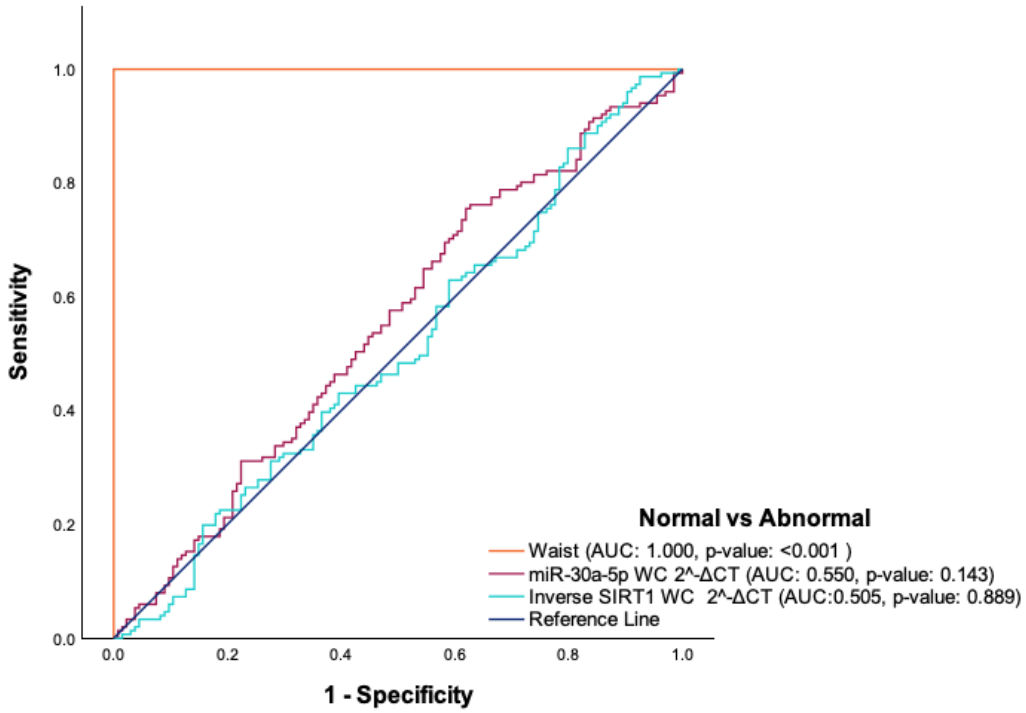

Figure 3.4: *Receiver operating curve of normal Waist Circumference vs High Waist Circumference*

Chapter 4: Discussion and Conclusion

4.1 Overview

The rising prevalence of obesity and its associated health risks, including MetS, CVD, and T2DM, continues to pose a significant global health challenge, with unique impacts on specific populations. Understanding the molecular mechanisms underlying adiposity has become a priority for identifying effective intervention points for obesity-related diseases. In this study, we focused on the potential roles of miR-30a-5p and SIRT1—a key metabolic regulator—in adiposity, specifically within a mixed-ancestry population in Cape Town, which has distinct genetic, socio-economic, and lifestyle factors influencing obesity prevalence and metabolic health (Barata Cavalcanti et al., 2022; Sartorius et al., 2015).

This investigation was motivated by the dual recognition that SIRT1 plays a critical role in regulating energy balance, lipid metabolism, and adipocyte differentiation, and that miR-30a-5p may regulate *SIRT1* expression, potentially contributing to adiposity. The study's findings potentially begin to bridge the gap in understanding the relationship in humans, between miR-30a-5p and SIRT1 in the context of adiposity.

Our findings reveal trends that support the hypothesis of miR-30a-5p regulation of SIRT1 even though the significance of the trends varies across BMI and WC groups. Further investigation of complex molecular mechanisms will narrow the knowledge gap in the metabolic pathways involved in obesity development and this study particularly contributes by focusing on one epigenetic relationship that may influence adiposity outcomes in diverse populations.

4.2 Relationship between miR-30a-5p and Adiposity

miR-30a-5p expression demonstrated significant differences across BMI categories, with higher levels observed in the overweight group compared to the normal weight group. This finding agrees with other studies that noted that miRNAs, including miR-30a-5p, modulate adipocyte maturation and function (Cui et al., 2018; Saha et al., 2020; Koh et al., 2018). miR-30a-5p is documented specifically to promote preadipocyte differentiation to mature adipocytes thus promoting increased fat storage. Increase in the expression of this miRNA likely contributes to overweight and

obesity development because an increase in mature adipocytes capacitates increased fat storage (Baraniskin et al., 2012; Zhang et al., 2016). In our population the increased expression of miR-30a-5p in the overweight group may be influenced by a combination of poor dietary habits, limited access to healthy foods and socioeconomic challenges. In the ever-rising food cost economic climate of South Africa, high-energy and low nutrition foods tend to be more affordable and easily accessible to the less economically affluent populations. The increased caloric intake leads to excess energy storage and thus increased lipid formation (Steyn et al., 2004; Brennan et al., 2009; Matsha et al.,2012; Markwick et al., 2013; Alaba & Chola, 2014; Reardon et al., 2021; Okoye et al.,2024). The resulting lipid accumulation likely encourages miR-30a-5p dysregulation as the body attempts to adapt by increasing adipocyte production, especially during the initial stages of weight gain.

Additionally, we found BMI to be closely associated with elevated metabolic markers. Participants in the obese category had significantly higher levels of fasting glucose, HbA1c, and insulin when compared to participants in the normal-weight group. The correlational relationship between glucose and insulin suggests the development of insulin resistance. The participants in the obese category also showed increased ALT and GGT levels, which suggests hepatic stress potentially due to fat accumulation and associated inflammation (Boachie et al., 2022: Gray & Vidal-Puig,2007). These metabolic abnormalities associated with increased BMI, combined with elevated miR-30a-5p expression highlight this miRNA's role in adiposity.

The elevation of miR-30a-5p in overweight individuals may represent a molecular signal of metabolic adaptation, where miR-30a-5p enhances lipid storage to protect against ectopic fat deposition. However, as adiposity advances, this mechanism may become maladaptive, contributing to systemic metabolic dysfunction. This aligns firstly with the theory of an inverse relationship between miRNA upregulated in adipogenesis and those upregulated in obesity and, secondly, that miRNA that modulate fat cell maturation may have a role in obesity development (O'Brien et al., 2018; Weber et al., 2010; Ortega et al., 2010).

When examining WC, a similar pattern was observed: individuals with high WC had elevated miR-30a-5p levels compared to those with normal WC, although this difference did not reach statistical significance. This pattern suggests that miR-30a-5p

may be associated not only with overall body fat but also with fat distribution, particularly abdominal fat, which is strongly linked to metabolic risk factors (Hilton et al., 2013; Miranda et al., 2018). Interestingly, age emerged as a significant determinant of high WC, with older participants more likely to have central adiposity. This age-related increase in WC aligns with evidence that adiposity tends to shift toward central fat accumulation as individuals age, due to factors such as reduced basal metabolic rate, hormonal changes (e.g., decreased growth hormone or oestrogen/testosterone), and lifestyle changes (Kuk et al., 2009; Mentzel et al., 2015; Ponti et al., 2020; Hunter et al., 2010). Since aging is known to affect miRNA expression and regulation, and miR-30a-5p has been reported to be upregulated in different aged non-adipose tissues, it can be hypothesized that similar patterns could occur in adipose tissue (Noren Hooten et al., 2013; Muther et al., 2017; Naguib et al., 2023; Pedraza-Vázquez et al., 2023; Tak et al., 2024). Over time, the effects of a high-energy, low nutrient diet combined with a sedentary lifestyle may be worsened resulting in a positive feedback loop between miR-30a-5p and high WC. In the high WC group this is demonstrated by the elevated CRP levels seen in the participants. CRP is a biomarker of systemic inflammation and the elevation of CRP levels in this context highlights the inflammatory nature of adiposity. The inflammation is coupled with the elevated liver enzymes, ALT and GGT, which draws attention to hepatic stress due to the abdominal or visceral fat which encroaches on normal liver function. The theme of upregulated miR-30a-5p to promote the accommodation of the increased caloric intake resulting in increased fat content within adipocytes and increased mature adipocytes to house the fat resulting in metabolic dysfunction is seen in high WC as was demonstrated in the overweight and obese BMI groups.

These findings align with literature suggesting that central adiposity contributes to both metabolic and inflammatory dysfunctions. Overall, the findings suggest that miR-30a-5p may play a dual role – it may serve as an early marker of overweight and signal the development of central adiposity particularly in older individuals.

4.3 SIRT1 Expression and Concentration Trends

SIRT1 gene expression across BMI categories showed a trend of increasing levels from normal to obese individuals, although this difference was not statistically

significant. Interestingly, SIRT1 protein levels tended to decrease in obese individuals compared to the normal-weight group, consistent with the hypothesis that SIRT1 activity may inversely correlate with adiposity due to its role in regulating lipid metabolism and energy homeostasis during prolonged caloric deficit and suppression in excess energy states (Song et al., 2013; Chang & Guarente, 2014). The overweight and obese participants in this population showed significantly elevated serum triglyceride and LDL-c levels when compared to the normal-weight group, as well as significantly decreased HDL-c levels. This lipid profile aligns with patterns of reduced SIRT1 activity observed in other adiposity studies. SIRT1 is known to regulate lipid metabolism by modulating certain pathways, such as PPARγ and sterol regulatory element-binding protein 1c (SREBP-1c), which influence triglyceride synthesis, fat storage, and cholesterol regulation (Picard et al., 2004; Han et al., 2010; Mayoral et al., 2015; Jang et al., 2017; Shen et al., 2024).

Similar to the trends seen in the BMI group analysis, the high WC cohort displayed lower trends in SIRT1 concentration when compared to the normal WC counterparts. Although this finding did not reach statistical significance, it aligns with the theme relayed in various studies, linking lowered SIRT1 levels with central adiposity and the metabolic consequences thereof. Furthermore, documented research shows that low SIRT1 activity is associated with increased visceral fat and is pro-inflammatory. Our study findings agreed with literature as evidenced by the elevated levels of fasting glucose, HbA1c, and CRP in the high WC group. Elevated glucose and HbA1c levels are in direct contrast with the insulin-sensitizing effects of SIRT1 activity. Additionally, the increased CRP levels seen in our high WC cohort further supports the lack of effective SIRT1 action (Elibol & Kilic, 2018; Mariani et al., 2016; Nakagawa & Guarente, 2011; Bosch-Presegué & Vaquero, 2015). Excessive abdominal fat, as seen with high WC individuals, has been purported to cause liver dysfunction. This hypothesis is supported in our study by the increased levels of the liver enzymes ALT and GGT seen in the high WC group (Liu et al., 2011; Saponaro et al., 2022).

In our study, the lower SIRT1 protein concentrations observed in individuals with high WC might similarly reflect suppressed SIRT1 function, leading to increased inflammatory cytokine production and reduced insulin sensitivity, as suggested in previous studies (Schug & Li, 2011; Song et al., 2013). However, unlike previous

studies that reported significant reductions in SIRT1 levels in individuals with central adiposity, the trends observed in this population did not reach statistical significance. This difference may be due to population-specific factors, such as genetic variations. Moreover, the abdominal fat range of our high WC group may constitute the early stages where though SIRT1 trends toward lowered activity it is not yet reached the threshold of significant reduction. The SIRT1 protein findings across this population is suggestive of this protein playing a role in lipid breakdown and involvement in the inhibition of inflammation and insulin resistance. However, due to the findings not reaching significance, further investigations need to be carried out to determine true correlations between SIRT1 and adiposity in this population as understanding SIRT1 associations could assist with the development of targeted interventions for obesity in the mixed-ancestry population.

4.4 Relationship between miR-30a-5p and SIRT1

In this study we found that miR-30a-5p expression was upregulated in overweight and high WC groups, similarly, *SIRT1* expression tended towards increase across the BMI groups. In contrast, SIRT1 levels showed an inversely proportional trend with increasing BMI categories and high WC. Although the changes show a noteworthy trend, they did not reach statistical significance. We hypothesized that miR-30a-5p would have a silencing effect on SIRT1 in overweight, obese and high WC participants. This hypothesis was based on a previous animal study on adipose tissue, which had shown miR-30a-5p to suppress SIRT1, thus promoting adiposity by inducing adipogenesis and fat storage in a murine cell line (Ciu et al., 2018). Another murine study on cardiac tissue found that miR-30a-5p exhibited the same repressive effect on SIRT1 leading to heart failure (Wu et al.,2022). These studies emphasized not only the modulatory effect of miR-30a-5p on SIRT1 but also the tissue specific nature of miR-30a-5p regulation of SIRT1, which suggests that miR-30a-5p regulation of SIRT1 may be activated in response to specific biological or pathological conditions.

Our study sought to determine whether a similar relationship existed between miR-30a-5p and SIRT1 in adiposity in human contexts. We did not find significant suppression of *SIRT1* expression or SIRT1 serum concentration. However, analysis of our high WC participant group shows a trend towards a potential relationship

between elevated miR-30a-5p levels and lowered SIRT1 protein levels in this group. The indirectly proportional relationship between the biomolecules may hint toward the silencing mechanism used by the miRNA on *SIRT1*. miRNAs regulate target genes by either degradation of the gene or inhibition of protein translation. In gene degradation, the miRNA possesses a base sequence that is perfectly complementary to the target gene. This allows for perfect binding between the miRNA and the target gene. The miRNA/mRNA coupling induces the Ago proteins in the RISC complex to cleave and degrade the target mRNA . In contrast, imperfect base pairing between miRNA and target gene results in the inhibition of translation. In our population, the increased *SIRT1* expression suggests that the target has not been degraded. This is further supported by the lowered SIRT1 protein levels which link with inhibition of protein translation (Matsuyama & Suzuki, 2020; O'Brien et al., 2018; Bushati & Cohen, 2007).

Although the findings in this mixed-ancestry population support the hypothesis that there is a negative relationship between miR-30a-5p and SIRT1 in human adiposity, the lack of statistical significance suggests that there may be other factors exerting an effect in the relationship. These factors may primarily include age and diet which are known to influence both miRNA and sirtuin activity. Other major influences may include genetic and environmental factors unique to this population resulting in the observed differences from the pioneering animal studies. Further exploration of the population specific factors that may influence the miR-30a-5p/SIRT1 modulation axis and their mechanisms of action in adiposity is crucial to the development of targeted interventions in this population.

4.5 Study strengths and limitations

The strength of this study lies on its focus on the mixed-ancestry population of Bellville South and Belhar in Cape Town. This is the second largest population group in South Africa and the largest in the Western Cape (Davids,2022). The Bellville South and Belhar suburbs are urban communities that are particularly associated with a high prevalence of cardiometabolic diseases. This makes the population in this community an attractive demographic for studying obesity-related biomarkers as obesity is a commonly associated risk factor for cardiometabolic disease. Exploring biomolecular interrelationships such as those between miR-30a-5p and SIRT1, combined with

investigating the effect specific factors such as genetic, epigenetic and environmental conditions among others have on this community, tailors our study to contribute relevant insights into adiposity and cardiometabolic health mechanisms in an underrepresented demographic (Davids et al., 2018; Weale, 2020). The benefit from the findings of such a tailored study includes the potential to develop targeted public health strategies to improve disease screening, follow-up practices and effectively guide resource allocation and management towards combating obesity-related health risk.

The study also had a few limitations including: (1) Underweight participants exclusion from the study limited the ability to examine miR-30a-5p and SIRT1 interactions in low BMI populations. Future inclusion of this population could enhance understanding of how the biomarkers of interest behave across all BMI groups. (2) The samples used for analysis were collected between 2014 and 2016 and frozen at -80 °C until thawed for this study. Although miRNA and serum proteins have been demonstrated to be stable in frozen conditions, there is no evidence to suggest that prolonged frozen storage conditions could not introduce some variability in the expression levels of the biomolecules (Kupec et al., 2022; Glinge et al., 2017; Valo et al., 2022). In future, the use of freshly collected samples to study miR-30a-5p/SIRT1 interactions could limit the possibility of expression variability due to prolonged storage. (3) the study's cross-sectional nature limits the ability to confidently conclude on the effects of miR-30a-5p on SIRT1. Due to the samples having been collected at a single point in time there is no ability to compare expression level changes in the population over time as BMI and WC fluctuate. Upcoming studies could employ a longitudinal design to the study thereby having more ability to infer causation between miR-30a-5p, SIRT1 and adiposity. Careful consideration of the limitations must be taken when interpreting the study results and addressing them in future studies could greatly improve understanding of epigenetic factors and mechanisms involved in adiposity development.

4.6 Conclusion

This study explored the intricacies of the relationship between miR-30a-5p and SIRT1 in adiposity in a mixed-ancestry population in Cape Town. The population was

selected due to the documented high prevalence of cardiometabolic disease in this community. Adiposity is a major risk factor for many cardiometabolic disease states and is showing steadily growing incidence worldwide. Studies that investigate adiposity mechanisms, especially in underrepresented populations, could prove insightful in understanding obesity development and possibly help identify novel biomarkers for rapid diagnosis. Additionally, these studies could identify novel targets for therapeutic interventions.

Our findings demonstrated the importance of BMI and WC as metabolic health markers, with miR-30a-5p levels showing higher trends in overweight individuals and participants with high WC. SIRT1 concentration was seen to decrease in the obese and high WC groups. Because SIRT1 is associated with the mobilization of energy by breaking down fat stores, it's decrease in overall and central obesity combined with the increase of miR-30a-5p in overweight and high WC corroborates the hypothesis that the miRNA may be involved in downregulating SIRT1 in favour of adipogenesis and fat storage when there is constant excessive energy intake. The increase in miR-30a-5p coupled with lower levels of SIRT1 in high WC may particularly attest to the miRNA's tissue specific regulation of SIRT1.

The findings in this study emphasize the need to consider both BMI and WC when investigating adiposity and its role in cardiometabolic disease, especially within populations with genetics that may be uniquely affected by multiple factors including environment, socioeconomics and lifestyle among others. Overall, the findings highlight how understanding molecular biomarkers like miR-30a-5p and SIRT1 might be crucial to advances in the diagnosis, management and therapy of adiposity and obesity-related complications.

4.7 Recommendations

The findings in this study encourage further exploration of the following:

1) Inclusion of underweight participants in the investigation to assess miR-30a-5p and SIRT1 interactions across all BMI categories. This would supply a clearer view of the role of these biomolecules in adiposity.
2) Structuring a longitudinal study would offer detailed clarity on causal relationships between miR-30a-5p and SIRT1 and measure of adiposity such

as BMI and WC. Secondly this approach would eliminate the variance caused by temporal changes and underscore significant trends associated with obesity development.

3) Use of freshly collected samples in subsequent studies. This will eliminate any potential negative effect of long-term storage on the quality and reliability of results obtained.

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Appendix 1: Ethics Certificate

Cape Peninsula University of Technology

HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HW-REC)
Registration Number NHREC: REC- 230408-014

P.O. Box 1906 • Bellville 7535 South Africa
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Tel: +27 21 959 6917
Email: sethn@cput.ac.za

14 February 2024
HWS-REC Approval Reference No: CPUT/HWS-REC 2024/H1

Faculty of Health and Wellness Sciences

Dear Ms. Babalwa Gaxamba - 216226872

Re: APPLICATION TO THE CPUT HWS-REC FOR ETHICS CLEARANCE

Approval was granted by the Health and Wellness Sciences-REC to **Ms. B Gaxamba** for ethical clearance. This approval is for research activities related to research for **Ms. B Gaxamba** at Cape Peninsula University of Technology.

TITLE: **Assessment of the relationship between adiposity, Sirtuin 1 and miR- 30a-5p in a Mixed-ancestry population in Bellville South, Cape Town**

Supervisor: Dr.S Davids and Dr. S Raghubeer

Comment:
Approval will not extend beyond 15 February 2025. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

The investigator(s) should understand the ethical conditions under which they are authorized to carry out this study and they should be compliant to these conditions. It is required that the investigator(s) complete an **annual progress report** that should be submitted to the CPUT HWS-REC in December of that particular year, for the CPUT HWS-REC to be kept informed of the progress and of any problems you may have encountered.

Kind Regards

Ms. Carolynn Lackay
Chairperson – Research Ethics Committee
Faculty of Health and Wellness Sciences