NOVEL METABOLIC RISK MARKERS IN OBESITY AND TYPE TWO DIABETES MELLITUS

By

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SUMMARY

Introduction

The global prevalence of overweight and obese individuals has increased dramatically over the past several decades. Since obesity is one of the strongest risk factors for developing type 2 diabetes (T2DM), it is not surprising that the prevalence of T2DM has also risen sharply with this obesity epidemic. Nevertheless, increase in total body fat mass alone does not fully explain the relationship between obesity and T2DM, and remains an area of interest and debate. The causal link is thought to be a combination of insulin resistance, ectopic fat accumulation, and systemic inflammation.

MicroRNAs (miRNAs) are small, noncoding RNA molecules that regulate gene expression by targeting specific messenger RNA. Dysregulation of the expression of these miRNAs have been associated with several metabolic diseases including T2DM. Since the discovery of their presence in extracellular body fluids, a considerable body of evidence has been generated showing the potential use of these circulating miRNAs as diagnostic biomarkers for several diseases including T2DM.

This thesis investigates the role of body fat distribution and White blood cells (WBC) in the pathogenesis of T2DM and further investigates putative circulating miRNAs as novel biomarkers for metabolic dysfunction leading to T2DM.

The associations of circulating miRNA expression with markers of metabolic health: CAMERA trial

A cross-sectional analysis was conducted to investigate the associations of circulating miRNAs mir-221, mir-222, mir-192, mir-193b, mir-144, and mir-155, with anthropometric and metabolic biomarkers using baseline plasma samples from the CAMERA trial. To do this required the development of techniques not used previously to measure miRNA biomarkers at scale. These circulating miRNAs were chosen based on previous evidence showing that their dysregulation was associated with change in glycaemic status or body mass index (BMI). I observed

broad associations of the targeted circulating miRNAs with biomarkers of metabolic risk in a population without diabetes and with coronary heart disease and large waist circumference. I showed promising utility of these targeted circulating miRNAs as reproducible biomarkers for metabolic dysfunction, worthy of further study.

The effect of metformin on the expression of circulating miRNA: The Carotid Atherosclerosis: MEtformin for insulin ResistAnce (CAMERA) trial

Metformin is the first line therapy for type 2 diabetes, inducing modest weight loss and improved insulin sensitivity. In this study, the effect of metformin on the expression of these circulating miRNAs was explored using both the baseline samples and the 18-month plasma samples from the CAMERA randomised control trial (RCT). Randomisation to metformin failed to show any effect on the expression of circulating mir-222, mir-221, mir-192, mir-193b, mir-144, and mir-155 in a population without diabetes and with coronary heart disease, and large waist but without diabetes, although the study was perhaps underpowered, and the effect of metformin in the study in general was very modest.

The association of body fat distribution with inflammation and T2DM:UK Biobank study

In this study, body fat distribution was examined using all adiposity measurement available in the UK Biobank study: anthropometric measurements, bio-impedance measurements, Dual-energy X-ray absorptiometry (DEXA) scans, and Magnetic resonance imaging (MRI) scans. A cross-sectional analysis was done to investigate the different pattern of body fat distribution between sexes and their association with T2DM and White blood cells (WBC) concentration as a marker of inflammation. Generally, central obesity was independently associated with T2DM in both genders and was a strong predictor for the development of T2DM than total body fat. Out of all the central obesity measures, high visceral adipose tissue (VAT) deposition was the strongest independent risk factor for T2DM and was also associated with total and differential WBC concentration. It was also showed that inflammation associated with T2DM is more strongly associated with VAT

deposition. In this study it was demonstrated that body fat distribution is likely to be more important for T2DM risk and systemic inflammation than total body fat.

Reduction of adipose levels of inflammation when treating obesity (REALITY): Feasibility study

From the previous study, it was concluded that obesity-related adverse health consequences appear to be related to fat distribution rather than total amount of fat gained. For a better understanding of the role of adipose tissue expandability and inflammation in the pathogenesis of T2DM, a study was proposed where bariatric surgery can be used as a model of metabolic change (before and after weight loss) to study adipocyte size, inflammation, and miRNA expression, the REALITY study. A feasibility study was performed to test the possibility of conducting such study in Glasgow. First, samples collection, transport, processing, and storage were worked out. This was done by reviewing the literature, consulting experts in the field, and then developing a Standard Operating Procedure (SOP). After that, specimen collection was discussed and coordinated with clinical staff. Overall, the feasibility of the REALITY study was tested, and the results showed that the designed protocol was accomplishable and ready to be tested in a full-scale study.

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Thank you all, I couldn't have done it without you

Author's Declaration

I declare that this thesis has been written by myself and is a record of research performed by myself apart from participants recruitment, sample collection and biochemical laboratory measurement of the CAMERA study. The work represented in my thesis has not been previously submitted for any degree to the University of Glasgow or any other institutions.

Tahani Y. M. Alramah

April, 2020

Definitions/Abbreviations

ΔΔCt	delta Ct
ADA	American Diabetes Association
Adipo	Adiponectin
ADIPOR1	Adiponectin receptor 1
AHEAD	Action for Health in Diabetes
AHEI-2010	The Alternate Healthy Eating Index 2010
ALT	Alanine aminotransferase
AMED	Alternate Mediterranean Diet
ASAT	Abdominal subcutaneous adipose tissue
AT	Adipose Tissue
ATM	Adipose Tissue Macrophages
BIA	Bioelectrical Impedance Analysis
BMI	Body Mass Index
BPD	Biliopancreatic diversion
BPD+DS	Biliopancreatic diversion with or without duodenal switch
BS	Bariatric surgery
C. elegans	Caenorhabditis elegans
CAMERA	**C**arotid **A**therosclerosis: **ME**tformin for insulin **R**esist**A**nce
CBC	Complete Blood Count
cDNA	Complementary DNA
CHD	Coronary Heart Disease
CI	Confidence Interval
CIMT	Carotid intima-media thickness
CLS	Crown-Like Structures
CRP	C-Reactive Protein
CT	Computed X-ray Tomography
Ct	Threshold Cycle
CV	Coefficient of Variation
CVD	Cardiovascular Disease
DASH	Dietary Approach to Stop Hypertension
dCt	delta Ct
DEXA	Dual-Energy X-ray Absorptiometry
DHS	Dallas Heart Study
DiRECT	Diabetes Remission Clinical Trial
EASD	European Association for the Study of Diabetes
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
ERFC	The Emerging Risk Factor Collaboration
ER-α	Oestrogen receptor α
FBG	Fasting Blood Glucose
FFM	Fat-Free Mass
FHS	Framingham Heart Study
FLI	Fatty Liver Index
FM	Fat Mass
GDP	Global Gross Domestic Product
GGT	γ-glutamyl transferase
GLUT4	Glucose transporter 4
H&E	Hematoxylin and Eosin
HbA1c	Hemoglobin A1c
HC	Hip circumference

HDL	High Density Lipoprotein
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
HT	Hypertension
ICAMS	Institute of Cardiovascular and Medical Sciences
IDF	International Diabetes Federation
IDV	Independent variable
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IL-1β	Interleukin-1β
IL-6	Interleukin-6
ILI	Intensive Lifestyle Intervention
IMA	Internal Mammary Artery
IMAs	Internal mammary artery
INS	Insulin
IR	Insulin Resistance
IRS1	Insulin receptor substrate 1
IS	Insulin Sensitive
LAGB	Laparoscopic adjustable gastric banding
MCP-1	Monocyte Chemotactic Protein-1
MetS	Metabolic Syndrome
miRNAs	MicroRNAs
MRI	Magnetic Resonance Imaging
mRNAs	messenger RNAs
MT	Medical Therapy
NAFLD	Non-Alcoholic Fatty Liver Disease
ND	No diabetes
NEFA	Non-Esterified Fatty Acid
NHS	National Health Service
NIH	National Institutes of Health
OR	Odds Ratio
PBMC	Peripheral Blood Mononuclear Cell
PCOS	Polycystic Ovary Syndrome
PCR	Polymerase Chain Reaction
PODOSA	Prevention of Diabetes and Obesity in South Asians
pre-miRNA	precursor miRNA
pri-miRNA	Primary miRNA
QC	Quality Control
QEUH	Queen Elizabeth University Hospital
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
r	Pearson correlation
RA	Rheumatoid arthritis
RCT	Randomised Control Trial
REALITY	Reduction of adipose levels of inflammation when treating obesity
RISC	RNA-Induced Silencing Complex
RL	RNA*Later*
RNA	Ribonucleic acid
RQ	Relative Quantification
RR	relative risk
RT	Reverse Transcription
RYGB	Roux-en-Y gastric bypass
SAT	Subcutaneous Adipose Tissue
SBC	Standardized beta coefficient

SCOTS	**S**urgical **O**besity **T**reatment Study
SD	Standard Deviation
SF	Snap Freezing
SG	Sleeve gastrectomy
SOP	Standard Operating Procedure
SPSS	Statistical Package for Social Sciences
T1DM	Type one diabetes mellitus
T2DM	Type two Diabetes Mellitus
TAGs	Triacyl glycerides
TBW	Total Body Water
TC	Total cholesterol
TNF-α	Tumour Necrosis Factor-α
UK	United Kingdom
UKPDS	UK Prospective Diabetes Study
USA	United states of America
USBC	Unstandardized beta coefficient
UTR	Untranslated Region
VAT	Visceral Adipose Tissue
VIVIT	Vorarlberg Institute for Vascular Investigation and Treatment
WBC	White Blood Cells
WC	Waist Circumference
WHO	World Health Organization
WHR	Waist to Height Ratio
XPO5	Exportin 5

Oral Presentations, Posters, And Awards

Abstracts for Poster Presentation

Tahani Alramah, Karine Pinel, David Preiss, Naveed Sattar, Jennifer Logue, Paul Welsh. 53rd EASD Annual Meeting, Lisbon, Portugal, 11 - 15 September 2017. Putative miRNA biomarkers of insulin resistance and the effect of metformin: data from the CAMERA trial (Appendix 9).

Tahani Alramah, David Preiss, Naveed Sattar, Jennifer Logue, Paul Welsh. Diabetes UK professional conference, 8 – 6 March 2019. Associations of targeted circulating microRNAs with insulin resistance in the CAMERA (Carotid Atherosclerosis: MEtformin for insulin ResistAnce) trial (Appendix 10).

Tahani Alramah, David Preiss, Naveed Sattar, Jennifer Logue, Paul Welsh. Association of Physicians Annual Meeting 2019. Putative miRNA biomarkers of insulin resistance and the effect of metformin: data from the CAMERA trial.

Abstracts for Oral Presentation

Tahani Alramah, David Preiss, Naveed Sattar, Jennifer Logue, Paul Welsh. Diabetes UK professional conference, 8 – 6 March 2019. Associations of targeted circulating microRNAs with insulin resistance in the CAMERA (Carotid Atherosclerosis: MEtformin for insulin ResistAnce) trial.

Awards

MVLS Conference support funding award 2018/2019 (Appendix 11).

Publication

The main findings of this study are being prepared for a paper to be submitted to international journal.

1 GENERAL INTRODUCTION

1.1 Obesity

Obesity is one of the most significant public health problems affecting populations worldwide; the prevalence of obesity continues to increase. The concern is not about the extra fat tissue gained but instead about the significant health consequences that accompany obesity. During the past four decades, experts, scientists, and health professionals have attempted to reverse the obesity epidemic, but unfortunately it has been increasing gradually worldwide. What makes it challenging to tackle is that causes of obesity are multifactorial and no single approach will reverse the trend.

Since obesity is considered a global public health concern, an effort is needed to understand both the reason behind the rapid increase in numbers affected and its relationship with chronic diseases, to find appropriate pathophysiological pathways that might be susceptible to intervention.

1.1.1 Definition

Obesity and being overweight are defined as conditions of excessive fat accumulation in the body leading to adverse health problems, with overweight being a pre-obesity state where intervention is recommended. Although obesity has been long considered as a complicating factor in a variety of diseases, it is now recognized as a disease itself. This was agreed on after the World Health Organization (WHO) published a ground-breaking document in 2000 clearly stating that obesity was a disease specifically a “complex, incompletely understood, serious and ‘chronic’ disease” and considered it as part of the non-communicable diseases that required prevention and management strategies at both individual and society level (World Health Organization, 2000).

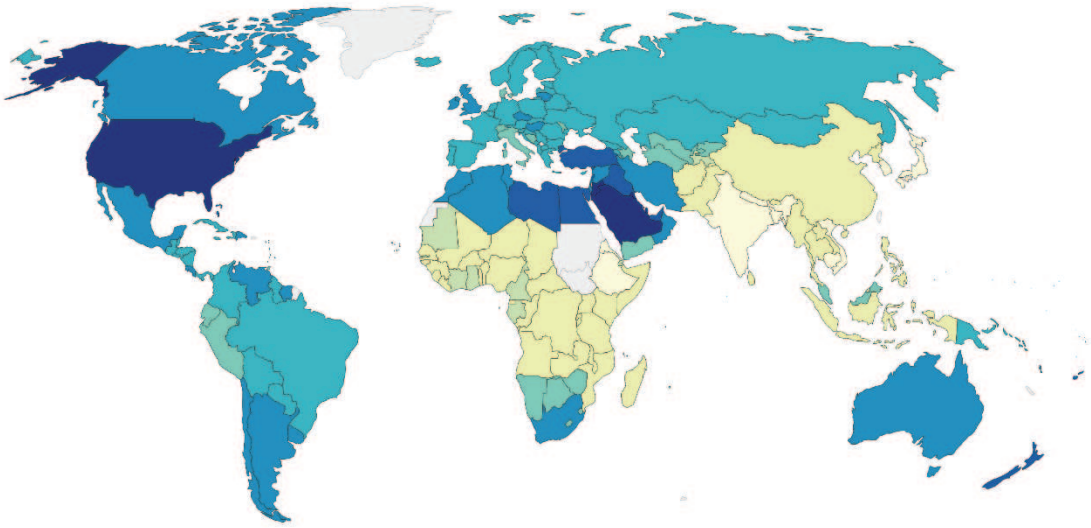
1.1.2 Prevalence

Several epidemiological studies have reported that the prevalence of obesity is rising globally and rapidly worldwide across all age groups: adults, adolescents, and children. The WHO recent estimation for adults with overweight and obesity is that in 2016, more than 1.9 billion adults had a BMI in the overweight range and of these over 650 million adults had obesity (Figure 1-1a);and over 340 million children and adolescents aged 5-19 years had overweight or obesity (World Health

Organization, 2018). Overall, in 2016, about 39% of the adult population had a BMI in the overweight range, and 13% had obesity, and just over 18% of children and adolescents aged 5-19 years had overweight or obesity. A systematic analysis published in the Lancet 2014 on the global prevalence of obesity and overweight between the years 1980 and 2013, estimated that the number of individuals with overweight and obesity has increased from 921 million in 1980 to 2.1 billion in 2013 worldwide (Ng *et al.*, 2014). In Scotland, the 2017 Scottish Health Survey showed that 65% of adults age 16 and above were overweight, and of this, 29 % were obese (Scottish Government, 2017). Moreover, the Scottish Government suggests that the prevalence of adult obesity could reach over 40% by the year 2030 (Scottish Government, 2010). It was a problem in the high income countries but now it expanded to middle and low income countries too (World Health Organization, 2018).

All these data showed that the worldwide prevalence of obesity has almost tripled since 1975 (Figure 1-1b), and this trend is projected to continue over the coming years. Despite the best intentions to overcome this problem, no country has been successful in reversing their rates of obesity.

a. Obese adults, 2016



b. Obese adults, 1975

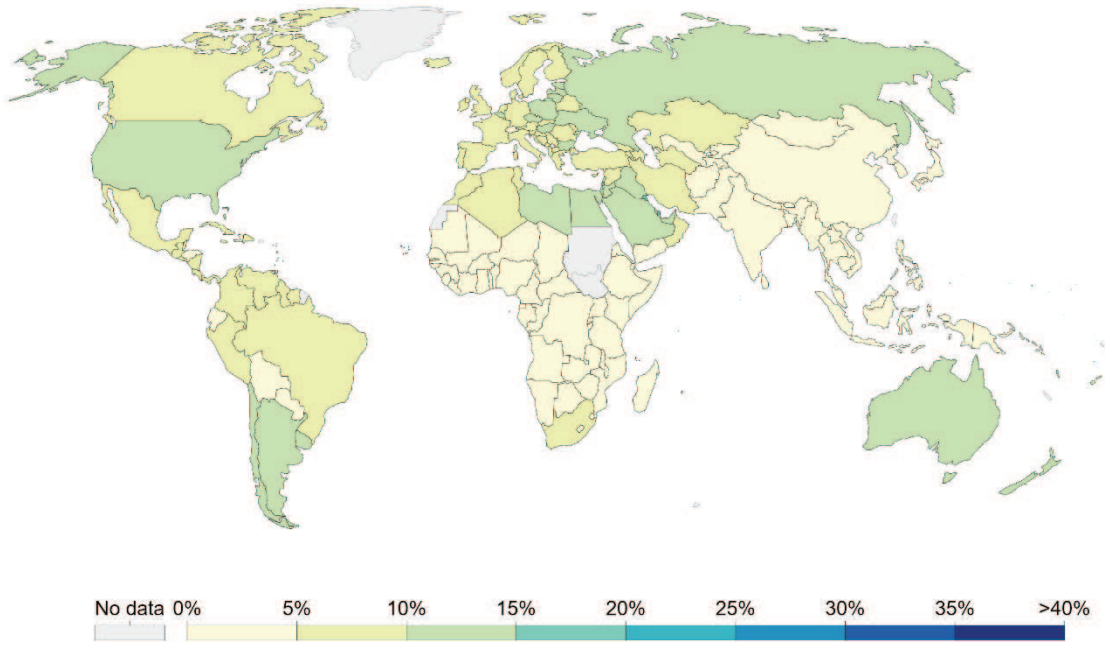


Figure 1-1: The differences in the global distribution of adults with obesity
Global distribution of obese adults (18+ years) between the years 1975 and 2016.The definition of obesity based on body mass index (BMI ≥ 30). a. Map for the year 1975, b. map for the year 2016 reproduced from (Ritchie & Roser, 2019) with permission.

1.1.3 Burden

Obesity is the underlying cause of metabolic disturbances that lead to type two diabetes mellitus (T2DM), as well as being a risk factor for hypertension (HT), dyslipidaemias, non-alcoholic fatty liver disease (NAFLD), cardiovascular diseases (CVD), and cancer. These chronic conditions placed a massive burden on the health care system because of the increased health care costs and prolonged medical care. The WHO reported that 44% of the diabetes burden, 23% of the ischemic heart disease burden, and approximately 7 – 41% of certain cancer burdens are related to overweight and obesity (World Health Organization, 2014).

The Emerging Risk Factor Collaboration (ERFC) individual participants meta-analysis showed, in 221,934 participants with no baseline CVD and ten years of follow-up, that every increase of 1 standard deviation (SD) in body mass index (BMI) (4.3 kg/m^2 increase) was associated with a 23% increased risk of CVD (95% confidence interval (CI): 1.17-1.29) after adjusting for age, sex, and smoking status (Wormser *et al.*, 2011). Although this study concluded that adiposity indices [BMI, waist circumference (WC), and waist to height ratio (WHR)] are not essential predictors for use in CVD risk scores, as intermediate factors, they are strongly associated with these risk factors for other chronic conditions such as blood pressure, lipid, and diabetes.

Obesity affects more than just health but also has a severe impact on economic development. In 2014, it was estimated that the global expenditure on obesity health care was $2.0 trillion, or 2.8% of the global gross domestic product (GDP) (Dobbs *et al.*, 2014). In the United Kingdom (UK), the overall economic cost of obesity was estimated at £27 billion (Fenton, 2017). Moreover, there is an indirect hidden burden caused by obesity which is loss of work productivity because of sick leave, lower performance, permanent disability, and premature death (Goettler, Grosse & Sonntag, 2017).

Finkelstein *et al.* showed that even a small reduction in obesity prevalence could save a lot in medical expenditure. They estimated that even a one percent reduction from the predicted obesity rate trend to 2030 would reduce medical spending by $84.9 ± $9.3 billion over two decades (Finkelstein *et al.*, 2012).

1.1.4 Causes of Obesity

The aetiology of obesity is complex and involves many factors that interact with each other such as dietary patterns, sedentary lifestyle, socioeconomic status, genes, and psychological profile. All these factors have participated in the dramatic global increase in obesity. Apart from a relatively small number of people with specific metabolic disorders, the fundamental cause of obesity is the "modern lifestyle" that encourages increased caloric consumption and discourages physical activity (Figure 1-2). The term "toxic environment" was suggested by Brownell and colleagues to describe a series of social and economic changes that have occurred and have led to the rising prevalence of obesity (Battle & Brownell, 1996). The toxic environment promotes obesity by encouraging the consumption of excess calories through increasing meal portion size, the availability of pre-packaged energy-dense food, and sugar-sweetened soft drinks. Also, this toxic environment encourages a sedentary lifestyle.

Genes play a part in obesity as shown by a sub-population with a pre-existing genetic predisposition to excess fat accumulation (Walley, Blakemore & Froguel, 2006). Researchers have identified genes with significant roles in obesity (Choquet & Meyre, 2011). These genes are mostly found within families or groups from the same ethnic background. The differences in how people respond to the same environment suggest that genes do play a role in the development of obesity.

Wang and colleagues did a prospective cohort study to examine the interaction between adherence to a healthy dietary pattern and genetic predisposition to obesity (Wang *et al.*, 2018). The authors assessed two large cohorts of United states of America (USA) health professionals (The Nurses' Health Study and The Health Professionals Follow-up Study) between the years 1986 and 2006 including 8828 females and 5218 males of European ancestry and were free from diabetes, cancer, or cardiovascular at baseline. Healthy dietary patterns were assessed using three dietary indices: The Alternate Healthy Eating Index 2010 (AHEI-2010), Dietary Approach to Stop Hypertension (DASH), and the Alternate Mediterranean Diet (AMED). The authors concluded that adherence to a healthy dietary pattern could attenuate the genetic association with weight gain, and it was stronger in participants with a higher genetic predisposition to obesity.

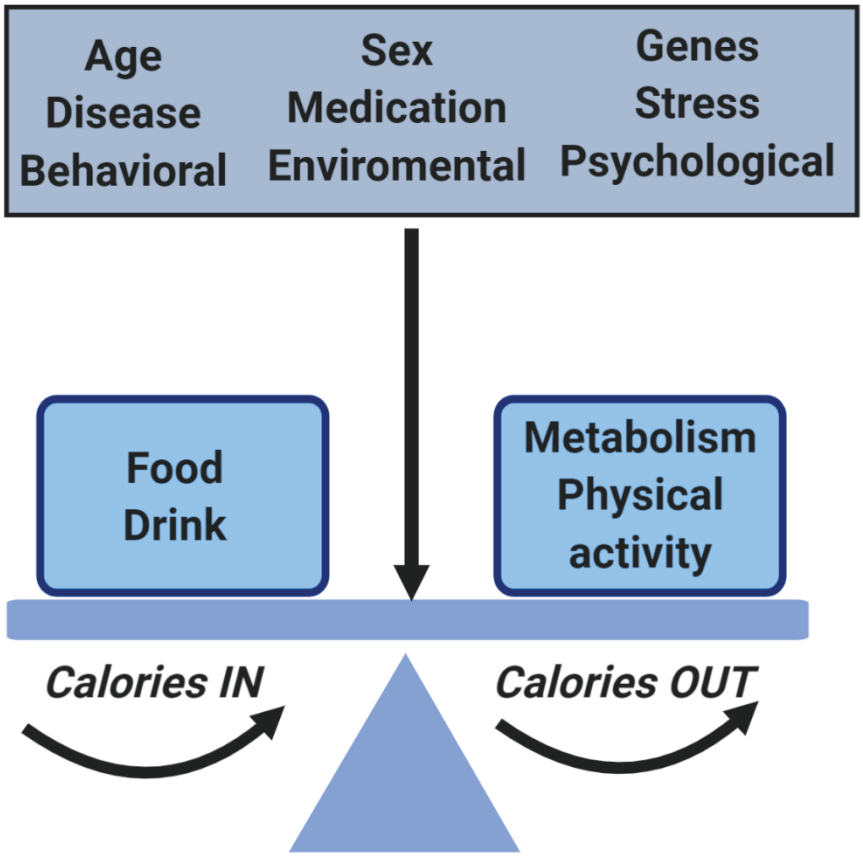


Figure 1-2: Causes of obesity
Overweight, and obesity occurs when energy intake exceeds energy expenditure over an extended period. The rise in overweight and obesity prevalence must be explained by a combination of physiological and behavioural factors triggered by the changes in the food environment and the environmentally driven reductions in physical activity. Depending on the genetic susceptibility, age, and sex of individuals, such changes result in variable weight gain.

1.1.5 Body fat distribution

In humans, adipose tissue is divided into two types according to their anatomical location: subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) (Figure 1-3). These regional differences are of physiological and pathological importance.

1.1.5.1 Subcutaneous Adipose Tissue (SAT)

The subcutaneous adipose tissue (SAT) is present as a continuous layer beneath the skin (Arner, 1997). It accounts for about 80% of body fat in a healthy person. SAT represents the normal body storage for excess energy intake (Drolet *et al.*, 2008; Ibrahim, 2010). Some evidence suggests that excess fat in SAT results in fewer health complications (Fox *et al.*, 2007).

1.1.5.2 Visceral Adipose Tissue (VAT)

Visceral adipose tissue (VAT) is located inside the thorax (mediastinum) and abdominal cavity surrounding the inner organs in distinct areas: omental, mesenteric, perirenal, retroperitoneal, parametrial, periovaric, epididymal, and perivisceral. Visceral adipose tissue accounts for up to 10 – 20% of total fat in healthy men and 5 – 8% in women and this increases with age in both sexes (Karastergiou *et al.*, 2012; Wajchenberg, 2014). Accumulation of fat in this area is considered an independent risk factor for T2DM (Neeland *et al.*, 2012) and leads to metabolic consequences of obesity (Matsuzawa, Funahashi & Nakamura, 2011).

1.1.5.3 The differences between SAT and VAT

Subcutaneous and visceral adipose tissue differ in several aspects, including adipocyte size, adipokines secretion, lipolytic activity, vascularity innervation, and receptors (Wajchenberg, 2000). These differences are more pronounced in obesity. For example, adipokine expression and secretion is different between the two depots. VAT is more infiltrated with macrophages and lymphocytes than SAT in patients with obesity (Lesna *et al.*, 2016). As such, leptin and adiponectin, metabolically beneficial adipokines, are secreted in higher amounts from SAT (Lihn *et al.*, 2004; Sarr *et al.*, 2017), whereas pro-inflammatory adipokines such as monocyte chemotactic protein-1 (MCP-1), interleukin-1β (IL-1β), and

interleukin-6 (IL-6) are more highly secreted from VAT (Bruun *et al.*, 2005; Rakotoarivelo *et al.*, 2018). Additionally, adipokines produced by VAT are secreted directly into the portal vein whereas adipokines produced by SAT are secreted into the circulation. Moreover, VAT is more sensitive to the lipolytic effects of catecholamines, which provide a large amount of free fatty acids (Wajchenberg, 2000).

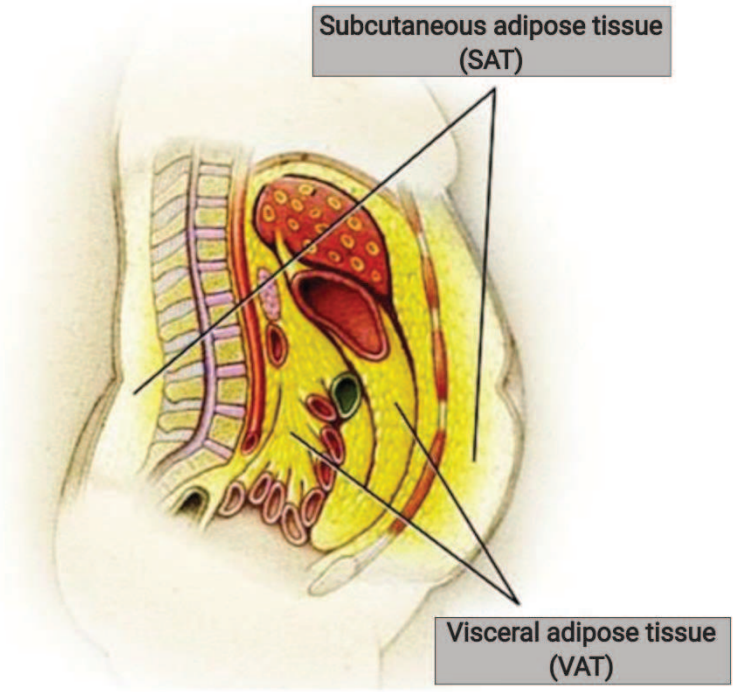


Figure 1-3: Location of visceral and subcutaneous fat deposits; reproduced with permission from (Lipman, 2009)**.**
Visceral adipose tissue (VAT) is located inside the thorax (mediastinum) and abdominal cavity. Subcutaneous adipose tissue (SAT) is located as a continuous layer underneath the skin.

1.1.5.4 Sex differences in body fat distribution

Sex differences in fat distribution and associations with metabolic health are well established, but the biological foundations of these associations remain poorly understood (Karastergiou *et al.*, 2012; Palmer, 2013; Kautzky-Willer, Harreiter & Pacini, 2016). They are determined by a complex interaction of hormones, genes, and environment. Generally, for the same BMI, females typically present with higher body fat compared to males. Additionally, body fat distribution is different between males and females (Chang, Varghese & Singer, 2018). Females tend to store fat on hips, thighs, and buttocks, in the subcutaneous area, giving them a pear shape, also known as gynoid obesity (Figure 1-4a). Males accumulate fat predominately in the abdominal region, mostly in the visceral area, giving them an apple shape, also referred to as android obesity (Figure 1-4b). These differences in body distribution are also observed in lean males and females.

On the contrary, these differences in body fat distribution tend to disappear with age. As they age, both males and females tend to store fat more in the abdominal area with females developing more android obesity, particularly after menopause (Demerath *et al.*, 2007). In addition, hyperandrogenism in females with polycystic ovary syndrome (PCOS) is frequently associated with increased abdominal fat (Carmina *et al.*, 2007). All the above shows the importance of sex hormones in body fat distribution. The factors and mechanisms that direct this sexual dimorphism in body fat distribution are critical to the understanding of metabolic consequences of obesity.

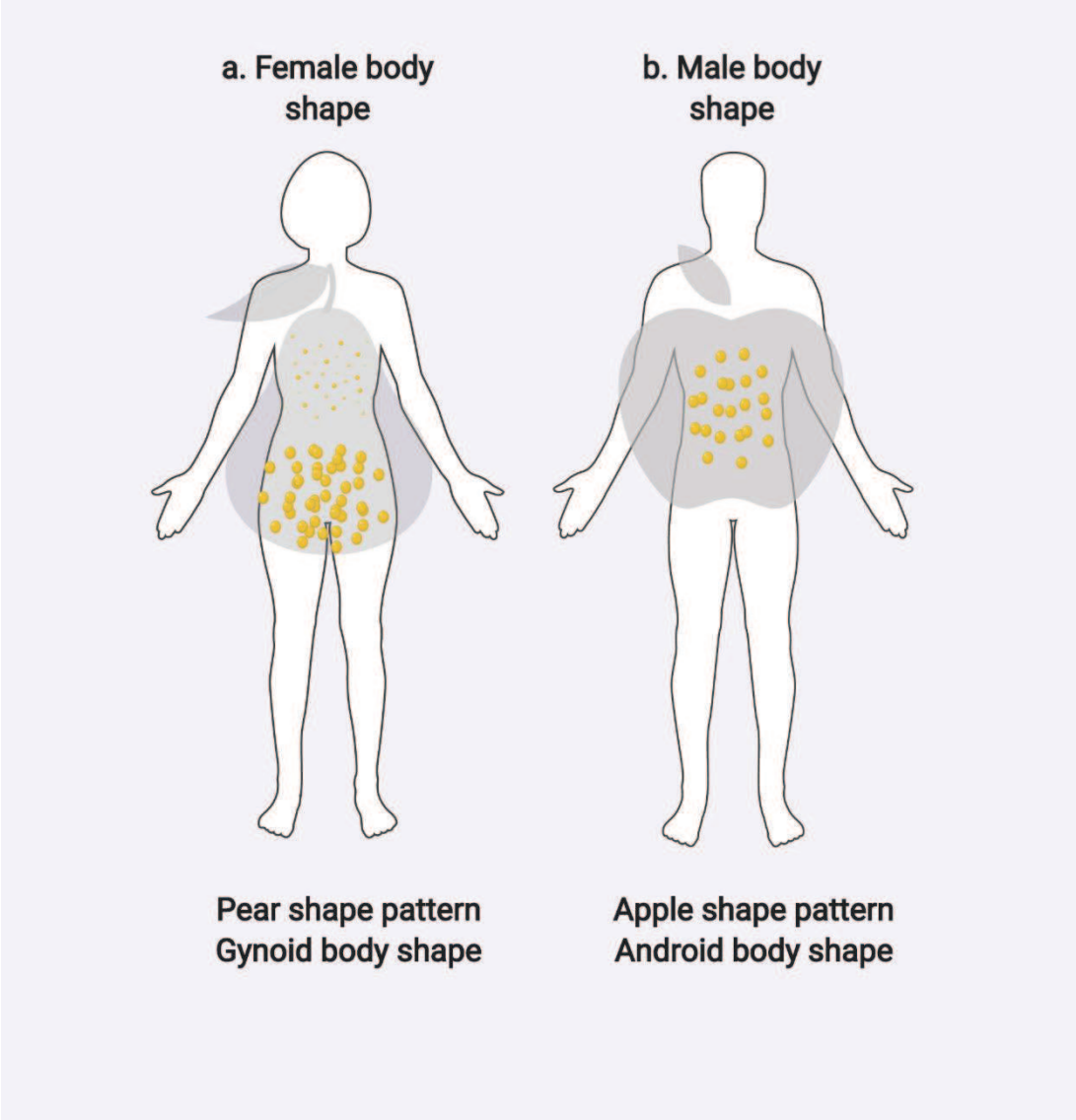


Figure 1-4: Gender differences in body fat distribution
a. Females body store fat on hips, thighs, and buttocks, in the subcutaneous area, giving them a pear shape, also known as gynoid obesity. **b.** Males accumulate fat mostly in the abdominal region in the visceral area, giving them an apple shape, also referred to as android obesity.

1.1.6 Assessment and diagnosis

Although the excess fat mass in obesity is associated with many diseases (Smith & Ravussin, 2002), studies have shown that the distribution and function of the fat mass are considered to be the major causal factor. Therefore, it is crucial to accurately identify people who are at risk and to estimate the level of the risk not only for the individual but also at the population and policy levels. Human body fat can be quantified in several ways, varying in complexity and ease of use. Several methods have been suggested to measure the degree of obesity, fat mass, and distribution. They can be categorised into two types: direct or indirect body fat estimation. Each method has advantages and disadvantages, and the method to be used will depend on the aims and circumstances of a particular exercise. Most of the time, combined methods are used to estimate the level and distribution of adiposity.

1.1.6.1 Indirect body fat measurements

Indirect methods provide estimates or indices of body composition based on results from direct or criterion methods. Indirect methods include anthropometry and bioelectrical impedance analysis (BIA). This type of measurements depends on biological interrelationships among direct or criterion measured body components and tissues and their distribution among normal individuals. As a result, indirect methods tend to have larger predictive errors than direct methods and are affected by sample specificity and disease conditions.

Body mass index (BMI)

Body Mass Index (BMI) is the most popular tool to estimate obesity and overweight in adults. Both the World Health Organization (WHO) and the National Institutes of Health (NIH) uses BMI for the classification of overweight and obesity. It has the advantage that a subject's height and weight are easy, non-invasive, and inexpensive to measure, which makes it a good measure of general adiposity in epidemiological studies. The BMI is calculated using the ratio of weight divided by the square of height (Kg/m^2). Table 1-1 shows the classification of overweight and obesity by BMI based on the WHO guidelines (World Health Organization, 2017). The healthy range for adults is between 18.5 and 24.9. A BMI range between 25 and 29.9 is defined as overweight, and obesity is defined as BMI of 30 and higher.

Obesity is further classified into three categories: class I (30-34.9), class II (35-39.9), and class III (≥ 40). Since extreme obesity is spreading worldwide, researchers have further divided class III obesity into two categories: super obesity (50-59) and super-super obesity (≥ 60) (Laghi, 2018). Large scale prospective studies have shown that high adiposity measured by body mass index (BMI) is associated with increased risk of all-cause mortality (Gonzalez *et al.*, 2011; Aune *et al.*, 2016).

Although BMI is one of the most commonly used anthropometric measurements to assess adiposity, it has its limitations. Body mass index uses total body weight and does not distinguish between lean and fat mass, providing no information about body fat distribution (Adab, Pallan & Whincup, 2018). Additionally, the cut-off points used to diagnose overweight and obesity are assumed to be independent of factors such as age, sex, race/ethnicity, and body composition all of which are known to affect body fat (Zhu & Leung, 2015; Zhu *et al.*, 2017; Park *et al.*, 2015).

Table 1-1: Classification of overweight and obese adults by BMI using the WHO guidelines (World Health Organization, 2017)

Category	**BMI (Kg/m^2)**
Underweight	< 18.5
Normal	18.5 – 24.9
Overweight	25 – 29.9
Obesity class I	30 – 34.9
Obesity class II	35 – 39.9
Obesity class III	≥ 40

Waist circumference (WC)

Waist circumference (WC) is a non-direct, simple, inexpensive, and yet effective tool for assessing central obesity. Studies have shown that it correlates well with abdominal obesity assessed by imaging methods (Harrington *et al.*, 2013), and is highly associated with type 2 diabetes (T2DM) (Wahrenberg *et al.*, 2005; Feller, Boeing & Pischon, 2010), cardiovascular diseases (CVD)(Yusuf *et al.*, 2005; De Koning *et al.*, 2007), and mortality (Cerhan *et al.*, 2014). Different measurement locations have been proposed in the literature for measuring WC (Ross *et al.*, 2008; Seimon *et al.*, 2018). Ross *et al.* did a systematic review of 120 studies (236 samples) to determine whether measurement protocol influenced the relationship of WC with morbidity of CVD and T2DM and with mortality from all causes and from CVD. The result of the study suggested that WC measurement protocol has no substantial influence on the association between WC and all-cause of mortality, CVD, and diabetes at the population level (Ross *et al.*, 2008). Although measuring WC at some of these positions require more skills, using bony structures as a stable landmark that are not affected by changes in weight is easier and more accurate than using the umbilicus or minimal circumference (Ma *et al.*, 2013). Both the World Health Organization (WHO) and the National Institutes of Health (NIH) use bony structures in measuring WC. WHO recommend using the midpoint between the lowest rib and the iliac crest while the NIH protocol recommend using the highest point at the iliac crest (Figure 1-5).

Selection of the most optimal cut points for WC values to predict cardiometabolic risk is sophisticated because they are likely to be influenced by age, sex, race/ethnicity, BMI, and other factors. Also, WC may over or under evaluate the risk for tall and short individuals with similar WC. In 2005, the International Diabetes Federation (IDF) proposed ethnic and sex values for WC (Table 1-2) (Zimmet, Alberti & Serrano Ríos, 2005).

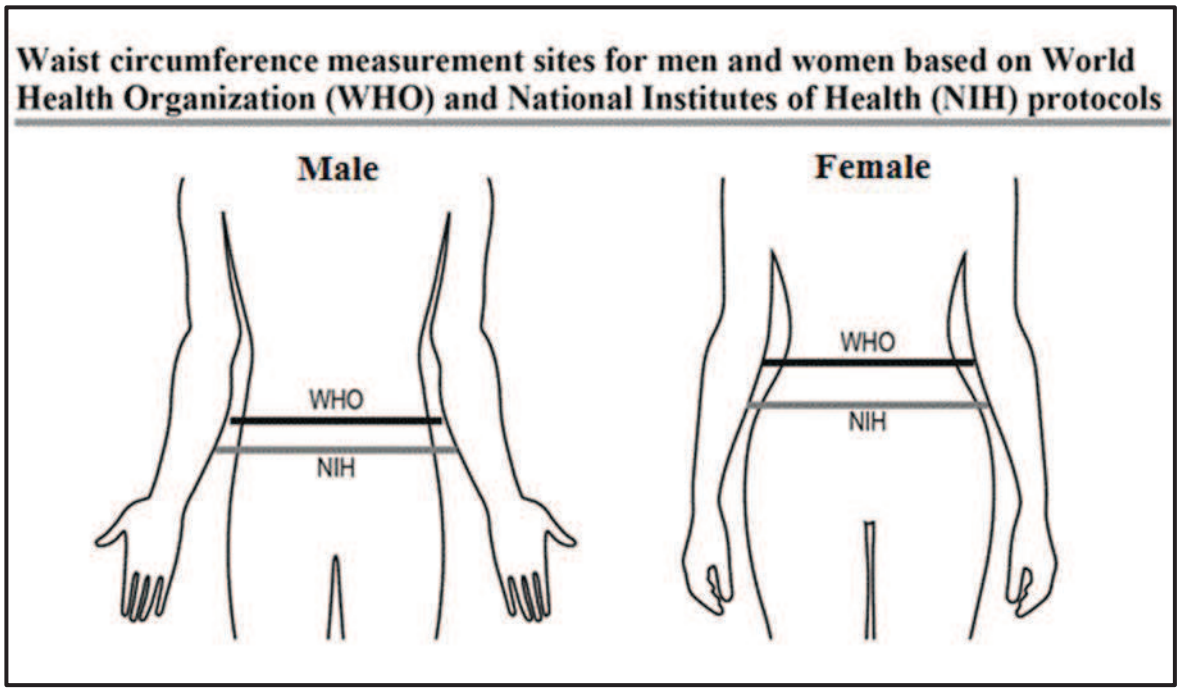


Figure 1-5: Waist circumference measurement sites for men and women per WHO and NIH. Following the WHO protocol, the measure is taken midway between the highest point of the iliac crest and the bottom of the ribcage. Following the NIH protocol, the measure is taken at the highest point of the iliac crest reproduced from (Patry-Parisien, Shields & Bryan, 2012) with permission.

Table 1-2: Ethnic-specific values for waist circumference guidelines proposed by the IDF (2007) in which sex and ethnic-group (not a country of residence) specific (Alberti, 2007)

Country/Ethnic Group Waist Circumference (WC)		
Europids	Male Female	≥ 94 cm ≥ 80 cm
South Asian	Male Female	≥ 90 cm ≥ 80 cm
Chinese	Male Female	≥ 90 cm ≥ 80 cm
Japanese	Male Female	≥ 90 cm ≥ 80 cm
Ethnic South and Central Americans	Use South Asian recommendations until more specific data are available	
Sub-Saharan Africans	Use European data until more specific data are available	
Eastern Mediterranean and Middle East (Arab) population	Use European data until more specific data are available	

Bioelectric Impedance Analysis (BIA)

Bioelectric Impedance Analysis (BIA) estimate body composition by measuring the resistance of the body as a conductor to a minimal alternating electrical current. It gives an estimation for total body water (TBW), fat-free mass (FFM), and fat mass (FM). BIA is widely used because it is affordable, portable, and easy to use. Besides, it provides instant results and is risk-free for patients. The BIA measurements depend on the tissue, water and electrolyte content which are affected by age, sex, ethnicity, disease state, level of fatness, physical activity, nutrition states, hydration levels, phase of the menstrual cycle, and underlying medical conditions (Dehghan & Merchant, 2008). In order to be reliable, BIA requires different equations to be used depending on ethnicity, age, gender, level of physical activity, and amount of body fat. While BIA measurements correlate with total abdominal fat (Browning *et al.*, 2012), it cannot be used for measuring VAT. BIA is widely used, and it is suitable for giving a general estimation for body composition, but there are still doubts about its accuracy and precision (Franco-Villoria *et al.*, 2016).

1.1.6.2 Direct body fat measurements

Direct body fat methods measure the body composition, such as its density, or describe amounts and distributions of adipose tissues, muscle, and skeletal. Direct body fat measurements include computed X-ray tomography (CT), magnetic resonance imaging (MRI), and dual-energy X-ray absorptiometry (DEXA).

Dual-Energy X-Ray Absorptiometry (DEXA) has a significant role in body fat assessment because it has higher accuracy and precision for the differentiation of lean and fat tissues (Duren *et al.*, 2008). It uses low-level x-rays that pass through different types of tissues at different rates providing estimates of fat mass, fat-free mass, and bone density. Since it requires very little radiation, this makes it suitable for repeated measures in clinical settings. Although DEXA has been shown to correlate strongly with VAT measured using CT (Micklesfield *et al.*, 2012) and MRI (Neeland *et al.*, 2016), there are certain manufacturers and models have been found to underestimate or overestimate body fat at low or high body fat percentage levels (Schoeller *et al.*, 2005).

On the other hand, Computed Tomography (CT) scan and Magnetic Resonance Imaging (MRI) can distinguish the various adipose tissue depots in the body (Ross, 2003) and also allow the measurement of fat in other non-adipose tissue compartments such as muscles and liver (ectopic fat deposits) (Wang, Chen & Eitzman, 2014). Both are considered as the gold-standard imaging techniques for body fat analysis (Seidell, Bakker & van der Kooy, 1990). The absence of radiation with MRI makes it more appropriate than CT, particularly when several measures are required overtime in the same individual or when populations such as children are being investigated. Although CT and MRI have the advantage of distinguishing subcutaneous and visceral fat, they have one limitation that is neither MRI nor CT can accommodate individuals with severe obesity. Due to their high cost, sophisticated equipment, and labor-intensive image analysis, the use of CT and MRI to assess adiposity are limited.

1.1.7 Treatment

Obesity is now considered a chronic disease that requires treatment. It is a complex problem with no single solution to apply to any individual. Also, there is convincing evidence showing that tackling obesity can improve or even reverse the co-morbidity associated with it. Therefore, the main goals for treating obesity is to provide a sustained weight loss, improve/remission of obesity-related comorbidities, as well as improve quality of life.

Several strategies for weight loss have been introduced, including dietary changes, exercise, and physical activity, behavioural therapy, pharmacotherapy, and bariatric surgeries. Each approach has its advantages and disadvantages. Choosing the right plan depends on age, sex, degree of obesity, comorbidity, and personal preference. Besides, losing weight may require choosing one or a combination of these treatments

Out of all the methods used to treat obesity and its comorbidity, bariatric surgeries are by far the most effective in obtaining substantial and durable weight loss leading to improvement and remission of obesity-associated diseases such as T2DM. Gloy V. L. *et al*. did a meta-analysis to quantify the overall effects of bariatric surgery compared with non-surgical treatment for obesity (Gloy *et al.*, 2013). The meta-analysis included 11 RCTs studies with 796 participants. The

results showed more body weight loss [mean difference −26 kg (95% CI= −31 to −21)] for the bariatric surgery treatment group compared with non-surgical treatment group and also improvements in metabolic parameters. Although bariatric surgery has shown positive outcomes regarding weight loss and reversing obesity-related comorbidities, the invasiveness, high financial cost, and long-term problems such as micronutrient deficiencies make it not a suitable option for all patients.

Since the cornerstone approach for treating obesity is a modification of diet and exercise, several weight loss programs have been introduced that mimic the results of bariatric surgeries in reversing co-morbidities such as T2DM by achieving similar weight loss. For example, the Look AHEAD (Action for Health in Diabetes) study was a randomised control trial (RCT) designed to assess the effects of an intensive lifestyle intervention (ILI) on clinically important health outcomes in overweight/obese individuals with type 2 diabetes (Wadden *et al.*, 2014).The study delivered a heavily supported programme combining physical activity and dietary advice, and achieved a mean weight loss of 8.6 kg. Remission was observed in 11.5% of participants after 1 year and 7.5% after 4 years. Although the study did not find significant differences in cardiovascular morbidity and mortality between study groups after 8 years of follow up, it did demonstrate significant differences in weight loss, maintenance, and improvements in many other aspects of diabetes morbidity (Salvia, 2017). Moreover, the Diabetes Remission Clinical Trial (DiRECT) was also a RCT conducted at primary care practices in the UK (Leslie *et al.*, 2016). The study uses an intensive weight management program within routine primary care practice to treat T2DM. Participants were randomly assigned (1:1) to either a structured weight-management program (intervention) or best-practice care in accordance with guidelines (control). The intervention group included withdrawal of diabetes and antihypertensive drugs, total diet replacement (825–853 kcal/day formula diet for 3–5 months), stepped food reintroduction (2–8 weeks), and structured support for long-term weight loss maintenance. The co-primary outcomes were a weight loss of at least 15 kg, and remission of diabetes, defined as Hemoglobin A1c (HbA1c) less than 6·5% (48 mmol/mol) after the withdrawal of diabetes drugs at baseline (remission was determined independently at 12 and 24 months) (Figure 1-6). At 12 months, 24% of participants in the intervention group achieved a weight loss of 15 kg or more;

half achieved 10 kg loss. Diabetes remission was achieved in 46% of participants in the intervention group compared to 4% in the control group at 12 months [odds ratio (OR) 19·7, 95% CI 7·8–49·8; $p < 0·0001$]. The remission was closely related to the degree of weight loss maintained at 12 months, with 86% of participants achieving at least 15 kg weight loss (Lean *et al.*, 2017). At 24 months, 11% of participants in the intervention group and 2% of participants in the control group had a weight loss of at least 15 kg (OR = 7·49, 95% CI= 2·05 to 27·32; $p = 0·0023$). Diabetes remission was achieved in 36% of participants in the intervention group and 3% of the control group (OR = 25·82, 95% CI = 8·25 to 80·84; $p < 0·0001$)(Lean *et al.*, 2019).

These data demonstrate the importance of weight loss in tackling the T2DM epidemic and suggests that substantial weight loss is an achievable target in at least some patients.

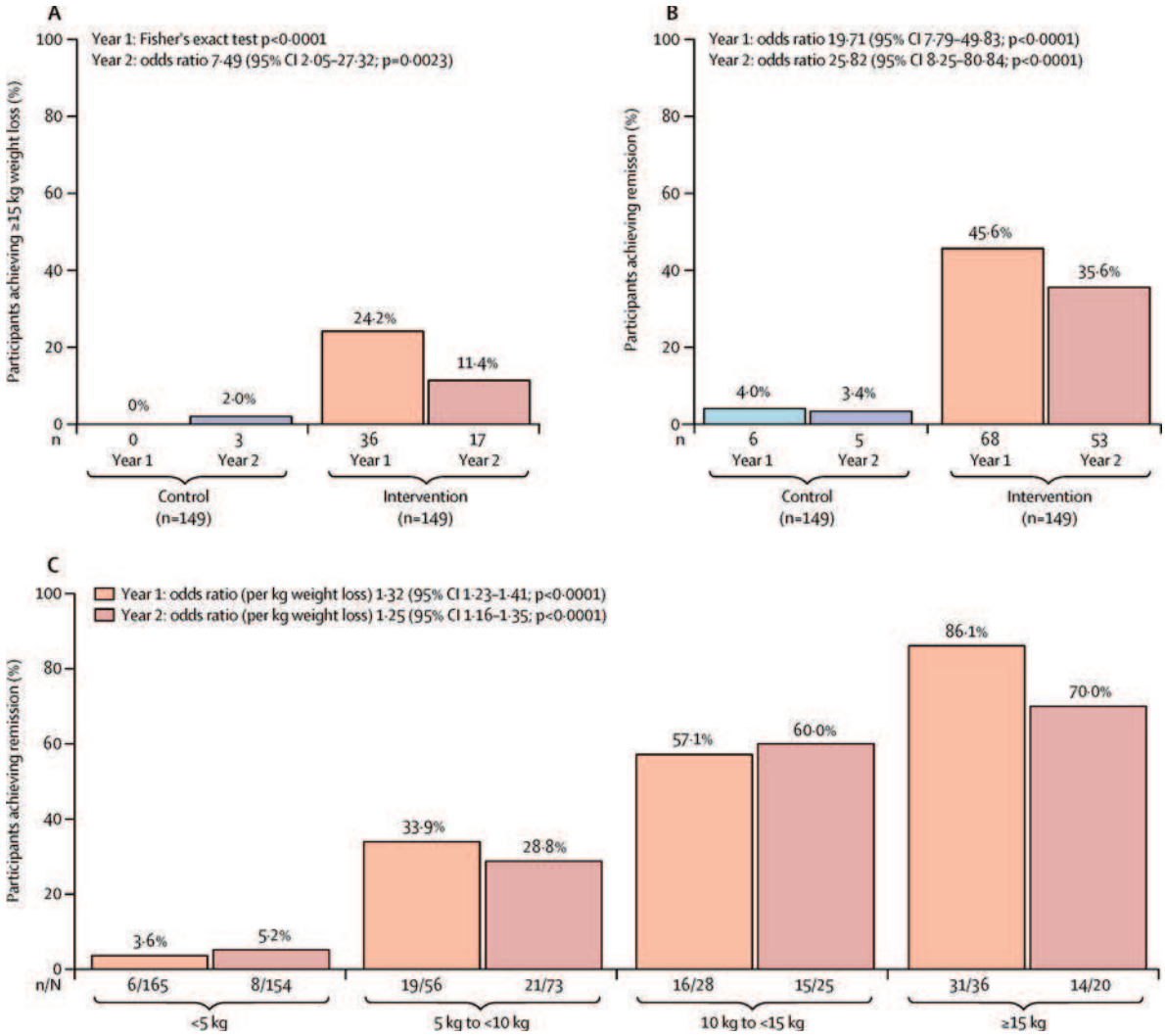


Figure 1-6: Diabetes remission in the Diabetes Remission Clinical Trial (DiRECT)
Primary outcomes and remission of type 2 diabetes in relation to weight loss at 12 and 24 months. Regression models adjusted for practice list size, study centre, and a random effect for practice. (A) The first co-primary outcome, the achievement of at least 15 kg weight loss, by the randomised group. (B) The second co-primary outcome, remission of type 2 diabetes (HbA1c <48 mmol/mol [6·5%] and off anti-diabetes drugs since baseline), by the randomised group. (C) Remission of type 2 diabetes in relation to weight loss achieved (both randomised groups combined) reproduced from (Lean *et al.*, 2019) with permission.

1.2 Obesity and T2DM

It is well established that obesity is a significant risk factor for developing T2DM, as well as being prevalent in those with T2DM. A meta-analysis published in 2010 showed that the relative risk (RR) for developing T2DM in patients with obesity was 7.19 (95% CI: 5.74, 9.00) and 2.99 (95% CI: 2.42, 3.72) in patients who were overweight (Abdullah *et al*. 2010). Given the fact that the prevalence of obesity is rising globally, it is not surprising to see that the incidence of T2DM also rises sharply with this obesity epidemic. The relation between body fat and the risk of T2DM is complicated and not fully understood. Moreover, not all people with obesity are at high risk for T2DM (Hinnouho *et al*. 2015).

It has been observed that around 30% of individuals with obesity maintain normal metabolic function "metabolically healthy obesity" (Freeman & Pennings, 2012; Hinnouho *et al.*, 2015; Bell, Kivimaki & Hamer, 2014), whereas some individuals with mildly overweight or even healthy weight suffer from severe metabolic abnormalities (Bradshaw, Monda & Stevens, 2012; St-Onge, Janssen & Heymsfield, 2004). Another extreme example is patients with lipodystrophy (a heterogeneous group of rare disorders characterised by the generalized or partial absence of adipose tissue). These patients suffer from severe insulin resistance (IR), hyperlipidemia, fatty liver, and diabetes despite the lack of adipose tissue (Bindlish, Presswala & Schwartz, 2015). Although the concept of metabolically healthy obesity is controversial (Iliodromiti *et al.*, 2018), all these observations show that there is much more than just total fat gain that can explain this relation. So far, three components have been identified that can explain the relationship between obesity and T2DM: body fat distribution, inflammation, and insulin resistance.

1.2.1 Body fat distribution

More than 60 years ago, Professor Jean Vague from the University of Marseille in France was the first to propose that body fat distribution was a better predictor for obesity complications than excess fat mass. He suggested that although females usually have twice the fat levels as males, they live longer with less morbidity from the metabolic complications of obesity (Vague, 1947). Vague used the term "android obesity" to define the pattern of fat distribution mostly characterised by an accumulation of adipose tissue over the trunk whereas he referred to the lower body fat pattern commonly found in women as "gynoid obesity" where adipose tissue accumulates mostly around the hips and thighs. He classified android type as high-risk obesity and gynoid type as low-risk obesity. These remarkable clinical observations did not receive immediate attention from the medical community.

1.2.1.1 Fat distribution and T2DM

Several extensive imaging studies have shown the importance of fat distribution in assessing metabolic abnormalities. In that regard, a large epidemiological study using data from the Framingham Heart Study (FHS) showed that in 3093 participants who underwent CT scan to assess SAT and VAT and without diabetes, the odds ratio (OR) for insulin resistance per 1 SD increase in SAT was 2.5 (95% CI: 2.2 – 2.7; $P < 0.0001$), whereas the OR for insulin resistance per 1 SD increase in VAT was 3.5 (95% CI: 3.1 – 3.9; $P < 0.0001$) after adjusting for age, sex, smoking, alcohol, menopausal status, and hormone replacement therapy. These results demonstrate that both SAT and VAT were correlated with insulin resistance, but the correlation with VAT was stronger (Preis *et al.*, 2010). In contrast, McLaughlin *et al.*, demonstrated that after adjustment for BMI, VAT was associated with insulin resistance while SAT was protective against insulin resistance - a 1 SD increase in SAT mass decreased the odds of insulin resistance by 48%, whereas a 1 SD increase in VAT mass increased the odds of insulin resistance by 80%. The study included 115 healthy, overweight, or moderately obese adults, and CT scans were used to quantify body fat distribution. Moreover, a cross-sectional study using data from the Dallas Heart Study (DHS) showed that among the 954 participants with obesity, VAT was associated with an adverse metabolic, dyslipidemic, and

atherogenic obesity phenotype while, SAT was associated with a more benign phenotype (Neeland *et al.*, 2013).

The relation of body fat distribution and IR was further supported by the surgical removal of visceral adipose tissues in both animals and humans (Gabriely *et al.*, 2002; Thörne *et al.*, 2002). By contrast, surgical removal of SAT with liposuction showed no significant improvement in obesity-associated metabolic abnormalities (Klein *et al.*, 2004).

1.2.2 Inflammatory Hypothesis of Obesity

Obesity is associated with a particular type of inflammation referred to as chronic low-grade systematic inflammation. This type of inflammation is characterised by increased infiltration of immune cells into adipose tissue and a modest increase in circulating pro-inflammatory cytokines with the absence of clinical signs. Despite its much lower intensity compared to acute inflammation, the stimulus of chronic inflammation persists, causing a profound effect on the body metabolic pathways. The trigger of this type of inflammation in obesity is not precisely clear, but it is thought to be related to the storage of excess amount of fat causing hypertrophy and necrosis of adipocytes leading to a significant alteration in the immune cells population in adipose tissue (Neels & Olefsky, 2006).

Obesity-induced systemic inflammation is believed to originate mainly in adipose tissue (Mraz & Haluzik, 2014). Adipose tissue inflammation is characterised by a significant increase in macrophages and, to a lesser extent, other immune cells (Wensveen *et al.*, 2015). Macrophages are the largest subpopulation of adipose tissue immune cells. Their function is to maintain adipose tissue homeostasis by patrolling for pathogens, eliminating dead cells, and resolving inflammation. They can change their phenotype according to the changing environment, from anti-inflammatory to pro-inflammatory type (Boutens & Stienstra, 2016). Adipose tissue macrophages (ATM) are found in both lean and obese (subjects), but they differ in number, localisation, and inflammatory status (Figure 1-7). In lean subjects, ATM makes up approximately 5 – 10% of the total number of cells and are distributed throughout the adipose tissue (AT), expressing fewer inflammatory properties. Whereas in subjects with obesity, ATM can be as high as 50% of the total number of cells mostly located around dead adipocytes forming crown-like

structures (CLS), while displaying profound pro-inflammatory features (Weisberg *et al.*, 2003; Lumeng *et al.*, 2007). The increasing number of macrophages is because of the production of inflammatory adipokines such as monocyte chemoattractant protein-1 (MCP-1) by the resident macrophages attracting more blood monocytes into the stromal vascular fraction of adipose tissue (Boutens & Stienstra, 2016). The physiological role of recruited macrophages is probably to clear dead adipocytes (Rutering *et al.*, 2017). Macrophages recruitment result in a pro-inflammatory state leading to secretion of many cytokines including tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1ß (IL-1ß) and recruit more immune cells in adipose tissue. All these cytokines contribute to local and systematic low-grade inflammation.

C-reactive protein (CRP) is a nonspecific acute-phase reactant and a sensitive marker of systemic inflammation that is synthesised by the liver in response to IL-6 stimuli. The high concentration of CRP has been linked to many disease states, including T2DM (Wang *et al.*, 2012). Extensive cross-sectional studies showed that CRP is positively associated with higher BMI, especially in patients with metabolic syndrome (Aronson *et al.*, 2004; Ramdas *et al.*, 2016). This was supported by a systematic review done by Selvin *et al.* to test if weight loss interventions are directly associated with a decline in CRP levels (Selvin, Paynter & Erlinger, 2015). The study included weight loss intervention studies (surgical, dietary, and exercise) from 1966 to 2006. They found that weight loss was associated with reduced CRP level (for each 1 kg, the mean change in CRP was – 0.13 mg/L). Also, a large cross-sectional study including 2410 patients with vascular diseases who had ultrasonography to analyse subcutaneous and visceral fat mass, showed that visceral fat is associated with increased plasma concentration of CRP (Faber *et al.*, 2010).

1.2.2.1 Inflammation and T2DM

Low-grade chronic systematic inflammation found in obesity has been implicated in the pathogeneses of several diseases, including T2DM (Akash, Rehman & Chen, 2013; Kohlgruber & Lynch, 2015). Researchers discovered that people with T2DM have elevated levels of inflammatory cytokines. These inflammatory cytokines contribute to the development of T2DM by altering insulin-signalling pathways and action (Chen *et al.*, 2015). This was confirmed by data from epidemiological

studies demonstrating that increased levels of markers and mediators of inflammation correlated with the incidence of T2DM (Bertoni *et al.*, 2010; Spranger *et al.*, 2003; Herder, Carstensen & Ouwens, 2013). Furthermore, prospective studies have also confirmed and extended these findings by showing that participants who developed T2DM during the follow-up period had high levels of inflammatory markers at baseline compared to subjects who did not develop the disease (Freeman *et al.*, 2002; Hu *et al.*, 2004; Kolb & Mandrup-Poulsen, 2005; Wang *et al.*, 2013a).

The emerging role of inflammation in the pathogeneses of T2DM has led to a growing interest in targeting inflammation to improve, treat, and prevent the disease. Increasing evidence has suggested the possibility of targeting inflammation as a promising approach for the treatment of T2DM (Pollack *et al.*, 2016).

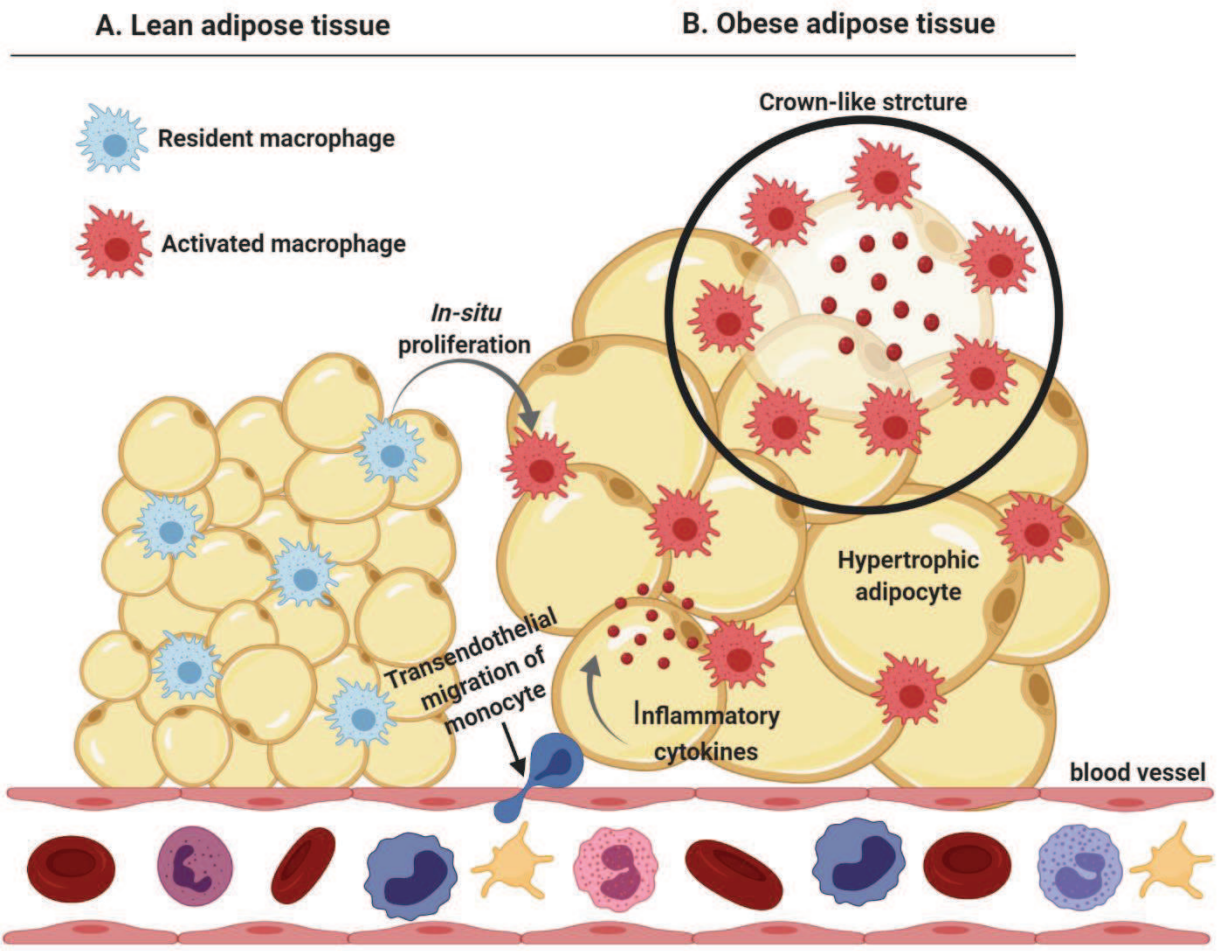


Figure 1-7: Functional differences between lean and obese adipose tissue.
In lean adipose tissue (AT) promote anti-inflammatory polarisation of macrophages. Adipose tissue (AT) expansion during weight gain leads to recruitment of macrophages through a variety of signals. These macrophages predominantly localised around dead adipocytes forming crown-like structures (CLSs) around dead adipocytes.

1.2.3 Insulin resistance

Insulin resistance (IR) is a pre-diabetes condition, characterised by failure of target organs, primarily the liver, muscle, and adipose tissue, to respond normally to insulin resulting in a compensatory increase in beta-cell insulin production and hyperinsulinemia. When increased insulin secretion is no longer enough to prevent hyperglycaemia, the subject progresses from insulin resistance to T2DM. The progression from IR to T2DM takes many years to occur. Several prospective human studies have highlighted the importance of insulin resistance in the pathogenesis of T2DM, where they showed that IR could be used as a predictor for the future development of T2DM. For instance, in the Whitehall II study, they followed up 6538 British civil servants free of diabetes mellitus at baseline for 13 years, those who developed diabetes had decreased baseline insulin sensitivity at 13 years before diagnosis and a significant reduction in insulin sensitivity during the past 5 years compared with those who didn't develop diabetes (Tabák *et al.*, 2009). Despite the high number of studies on IR, its pathogenesis remains elusive.

Obesity is a known risk factor for the development of insulin resistance. Several studies showed that insulin resistance (IR) is strongly associated with the metabolic disturbance that occurs in obesity, and probably it precedes their onset (Hardya, Czecha & Corvera, 2014). Although obesity and excessive insulin levels are associated conditions, whether obesity causes high levels of insulin or vice versa is not clear. However, it has been postulated that the association between obesity and insulin resistance is likely a cause and effect relationship (Kahn, Hull & Utzschneider, 2006). Others proposed that IR associated with obesity is a defence mechanism that protects critical tissues from nutrient excess induced metabolic dysfunction (Hoehn *et al.*, 2009; Nolan *et al.*, 2015). This was supported by human and animal studies, which indicated that weight loss or gain correlates closely with increasing or decreasing insulin sensitivity respectively (Yang *et al.*, 2015; Clamp *et al.*, 2017). For example, in the Prevention of Diabetes and Obesity in South Asians (PODOSA) Trial the authors investigated the effect of a lifestyle intervention on cardiometabolic biomarkers in 151 South Asian participants who were at risk for developing T2DM (Welsh *et al.*, 2016). After 3 years, there was an adjusted mean reduction of 1·44 kg (95% CI 0.18 to 2.71) in weight and 1.59 cm (95% CI 0.08 to 3.09) in waist circumference in the intervention, compared with control and every 1kg weight reduction during follow-up was associated with a

decrease in Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) (−4.5%, $p < 0.001$). Other studies have suggested that high concentrations of circulating insulin (hyperinsulinemia) can lead to increased storage of fat leading to obesity and obesity-related conditions such dyslipidaemia, hypertension, and atherosclerosis (Castro *et al.*, 2014). The relationship between obesity and IR is seen across all ethnic groups and is evident across the full range of body weights (Kodama *et al.*, 2013).

The presence of excess adipose tissue mass alone is not always responsible for the obesity-associated insulin resistance. In humans, the expansion of visceral adipose tissue depots is associated with IR, and many would argue that expansion of subcutaneous adipose tissue is associated with decreased risk for developing IR (Hocking *et al.*, 2013; Shimizu, Yoshida & Minamino, 2015). McLaughlin *et al.* confirm this argument by studying 115 healthy adults who were overweight or moderately obese (McLaughlin *et al.*, 2011). The results showed that VAT was higher in participants with insulin resistance and SAT and thigh fat were significantly lower compared to participants who were insulin sensitive. In logistic regression analysis, each SD increase in VAT increased the odds of being IR by 80%, whereas each increase in SAT decreased the odds by 48%; each increase in thigh fat decreased the odds by 59% and retained significance after adjusting for other depots.

Furthermore, this observation was also described in animals. Moitra and co-workers, who had developed transgenic fatless mice, found that fatless mice developed T2DM despite the absence of fat tissue in the body and became hyperinsulinemia and hyperglycaemic at one and four weeks of age, respectively (Moitra *et al.*, 1998). Also, in adipose-specific GLUT4–knockout mice, disruption of insulin-stimulated glucose uptake by adipocyte was enough to cause peripheral insulin resistance and glucose intolerance but without changes in adipose tissue mass (Boucher, Kleinridders & Kahn, 2014).

1.2.3.1 Measurement of Insulin resistance

Glucose clamp technique has been considered as the gold standard method in the literature for measuring insulin resistance. It measures the amount of glucose necessary to compensate for increased insulin levels without causing

hypoglycaemia. However, the method is time consuming, expensive, labour intensive, technically difficult to perform and suitable only for studies with a small number of subjects.

Homeostasis model assessment for insulin resistance (HOMA-IR), which was proposed in 1985 (Matthews *et al.*, 1985), is a relatively simple mathematical index for assessing insulin resistance. It is based on the relationship of basal glucose and insulin levels which reflect the balance between hepatic glucose output and insulin secretion and is maintained by a feedback loop between liver and β cells(Wallace, Levy & Matthews, 2004). The major advantage of HOMA-IR is that it requires only one draw of blood from a fasting patient. It does not require extensive technical expertise and constitutes a much lower cost compared with euglycemic hyperinsulinemia clamp. Thus, HOMA-IR is more practical for use in large scale epidemiological studies and for clinical situations. In 1998, Levy, Mathews and Herman developed a computerized model for HOMA-IR, which is commonly referred to as HOMA2 (Levy JC, Matthews DR, 1998) that is available for use by researchers online at: http://www.dtu.ox.ac.uk/index.htm?maindoc=/publications/

1.2.4 Adipose Tissue Expandability and Ectopic Fat Accumulation

One theory that might explain the association between obesity and T2DM is the defect in adipose tissue expandability and ectopic fat accumulation (Figure 1-8), which includes all the three components mentioned above.

Normally excess calories are well handled by SAT adipocytes. In prolonged positive energy balance conditions, excess energy is stored in SAT either by increasing: the number of adipocytes (hyperplasia) or the size of pre-existing adipocytes (hypertrophy). Although adipocytes can change their diameter 20-fold and their volume several thousand-fold (Guengerich *et al.*, 2001), there is a limit to this expansion. The maximum volume an adipocyte can reach to maintain their normal function is called the critical cell size, which is different between the sexes, and it is genetically determined (De Ferranti & Mozaffarian, 2008). When the adipocytes become overburdened by the great excess of calories and their ability to expand becomes limited, an imbalance occurs leading to the release of non-

esterified fatty acids (NEFAs) and triglycerides (TAGs) into the circulation instead of being stored inside the adipocyte (Campbell P. J., Carlson & Nurjhan, 1994). Higher concentrations of circulating NEFAs and TAGs start to accumulate ectopically in non-adipose tissues such as skeletal muscle, liver, heart, pancreas, and VAT that normally contain only small amounts. These tissues are more susceptible to the toxic effects of this accumulation (lipotoxicity) as they are not designed to store large amounts of lipids (Schaffer, 2003). Numerous harmful effects have been associated with the unhealthy enlarged adipocytes, including inflammation, fibrosis, hypoxia, altered adipokines secretion, and mitochondrial dysfunction.

The relationship between ectopic fat accumulation and metabolic abnormalities has been confirmed in many studies showing the presence of ectopic fat is associated with insulin resistance and T2DM. (Franssens *et al.*, 2016; Targher, Marchesini & Byrne, 2016; Gaborit *et al.*, 2015). The deposition of excess FFAs interferes with insulin signalling in several ways. In humans, increased plasma FFAs lead to intramyocellular lipid accumulation which plays a critical role in the pathogenesis of insulin resistance and type 2 diabetes (Shulman, 2000). Intracellular FFAs or their metabolites activate a serine/threonine kinase cascade that ultimately results in reduced insulin receptor substrate-1 tyrosine phosphorylation, reduced insulin receptor substrate-1- associated phosphatidylinositol 3-kinase activity and failure to promote translocation of the GLUT4 glucose transporter to the plasma membrane in response to insulin stimulation (Ragheb *et al.*, 2010). Moreover, hypertrophic adipocyte and ectopic fat accumulation is associated with an infiltration of macrophages into the adipose tissue (Surmi & Hasty, 2008). This promotes the increase secretion of several cytokines like IL-6, IL-8, and MCP-1 which have been shown to reduce both IRS-1 and GLUT4 expression and induce insulin resistance (Gustafson *et al.*, 2009).

In addition, other studies showed that adipocyte hyperplasia protective against metabolic abnormalities (Hoffstedt *et al.*, 2010; Kim *et al.*, 2014). This was supported by studies done on individuals with metabolically healthy obesity where a higher ratio of small to large adipocytes were found in their subcutaneous abdominal adipose tissue and with the alterations in adipose tissue immune cells (Weyer *et al.*, 2000; Klöting *et al.*, 2010; Cotillard *et al.*, 2014).

Moreover, a randomised control trial done using suction lipectomy to determine if surgical removal of fat is restored after one year in non-obese women, and lead to an anatomical redistribution of fat (Hernandez *et al.*, 2011). The results showed that after one year, fat was restored and redistributed from the thigh to the abdominal area suggesting that peripheral fat storage may protect from increase fat in the central region.

Taken together, these studies showed the significant role of SAT adipocyte expandability and its role in accommodating excess TAGs and defect in this role is likely to be an essential contributor to the development of metabolic dysfunction associated with obesity.

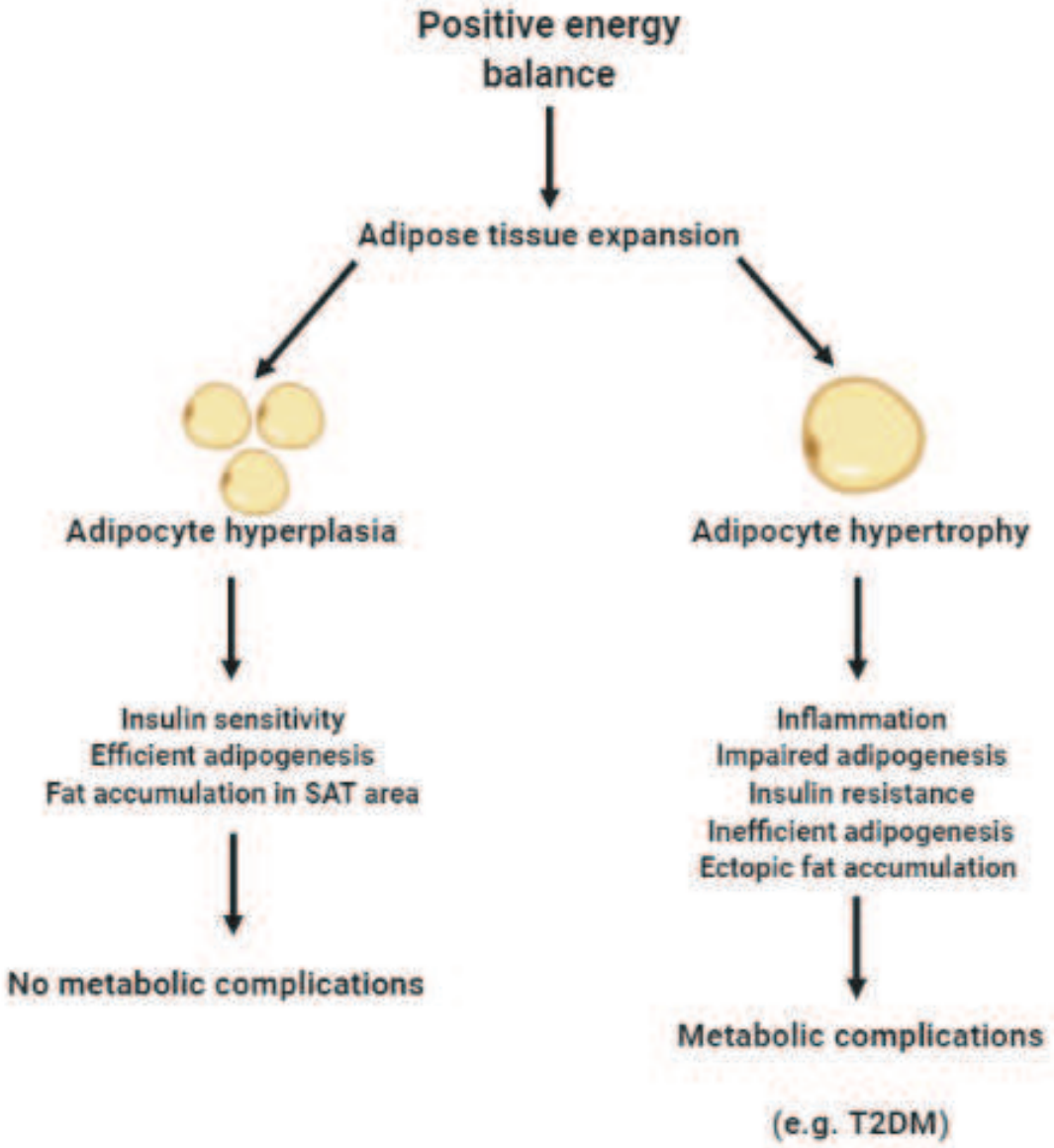


Figure 1-8: Adipose tissue expandability and ectopic fat accumulation.
During chronic excessive energy intake and low physical activity, high nutrient flux into adipose tissue. Adipocytes respond by increasing their size (hypertrophy) or number (hyperplasia). Limited expandability of adipose tissue number through hyperplasia produce hyperplastic adipocyte, which becomes dysfunctional leading to inflammation, insulin resistance, and ectopic fat accumulation outside the SAT. All these factors contribute to the development of T2DM.

1.3 Circulating microRNAs and T2DM

1.3.1 MicroRNA

MicroRNAs (miRNAs) are a class of small, single-stranded, non-coding RNAs (20–25 nucleotides long) found in the genomes of eukaryotic organisms. These appear not to be fragments of cellular debris but play a significant role in physiological and pathological mechanisms in our body (Deiuliis, 2015). MicroRNA regulate post-transcriptional processes through silencing target messenger RNAs (mRNAs) either by inhibiting translation or by degrading the target mRNA (Filipowicz, Bhattacharyya & Sonenberg, 2008). They were initially discovered in *Caenorhabditis elegans* in 1993 (Lee, Feinbaum & Ambros, 1993). Later in 2000, the second highly conserved microRNA let-7 was discovered (Reinhart *et al.*, 2000) and since then, many new microRNAs have been identified. According to the latest estimates, 2603 mature miRNAs have been discovered in humans (http://www.mirbase.org). Each microRNA has the potential to target and regulate several genes (Slack, Rupaimoole & Slack, 2017). Therefore, their involvement in biological processes is not surprising, and their dysfunction can be a sign of various diseases (e.g., cancer, cardiovascular diseases, and diabetes). Moreover, a study in mice showed that deletion of either Drosha or Dicer causes the loss of most miRNAs, leading to severe pathology (Bernstein *et al.*, 2003) which emphasise the role of miRNAs in the development of diseases.

1.3.2 Biogenesis of MicroRNA

MicroRNA biogenesis is a complex, highly regulated molecular mechanism that involves multiple steps (Figure 1-9). The process of miRNA formation starts in the nucleus with the transcription of miRNA by RNA polymerase II or III from chromosomal DNA (intergenic or intragenic region) to produce hairpin-shaped primary miRNA transcripts (pri-miRNA). After that, pri-miRNA is processed by the Microprocessor complex, which consists of the RNase III enzyme Drosha and DGCR8 (DiGeorge syndrome critical region gene 8), to produce a double-stranded stem-loop structure called precursor miRNA (pre-miRNA). Then, the pre-miRNA is actively transported outside the nucleus by Exportin 5 (XPO5) into the cytoplasm, where it is further cleaved by the enzyme Dicer, which is another ribonuclease III enzyme, to produce a 22-nucleotide double-stranded miRNA duplex consist of the

mature miRNA and a complementary strand. Finally, the mature strand of the dicer duplex is loaded onto Argonaute proteins, forming RNA-induced silencing complex (RISC). The mature miRNA guides the RISC complex to complementary sequences within the 3' untranslated region (UTR) of target mRNA(s), leading to translational repression and/or transcript degradation (De Guire *et al.*, 2013; Regazzi, Guay & Regazzi, 2013; Sebastiani *et al.*, 2017). The other strand (complementary strand) is thought to be degraded normally in the cytoplasm (Ender & Meister, 2010).

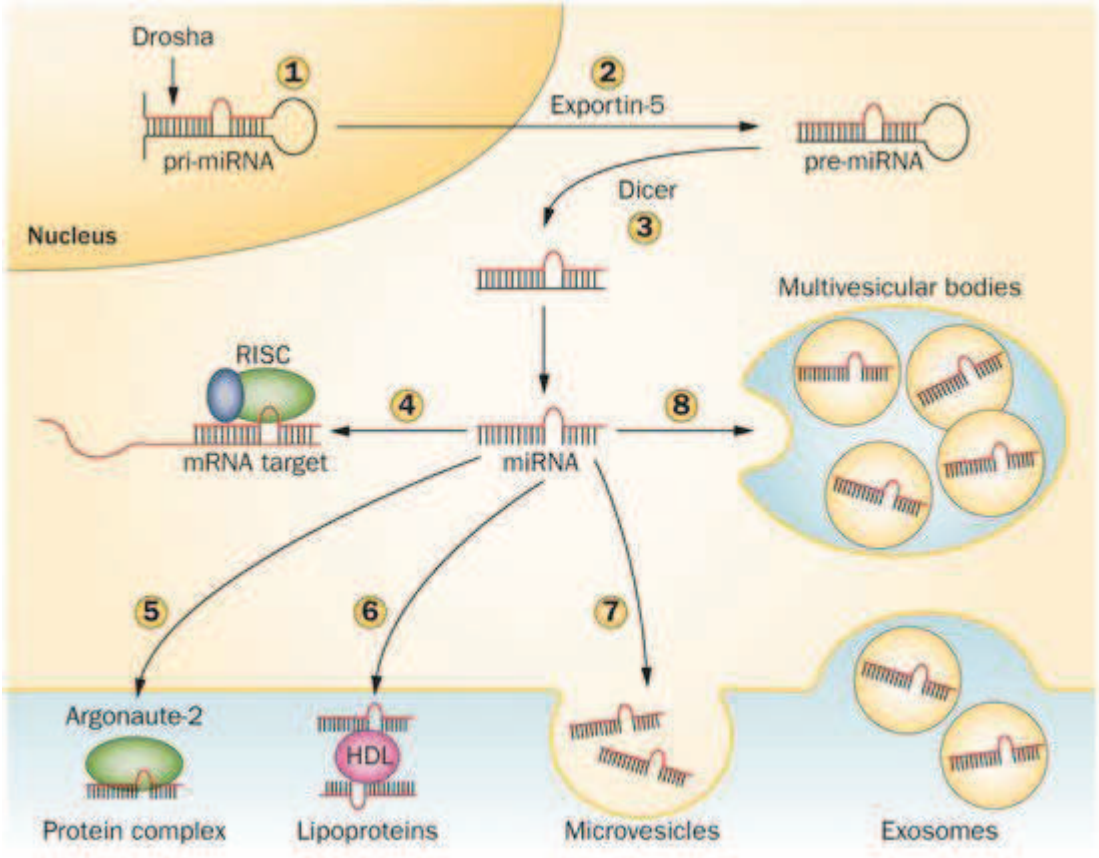


Figure 1-9: Biogenesis and release of miRNAs.
1. Pre-miRNAs are generated in the nucleus by the ribonuclease III enzyme Drosha after cleavage of pro-miRNAs. **2.** The pre-miRNAs are then transported in the cytoplasm through a process involving Exportin-5 and the GTP-binding protein Ran. **3.** The pre-miRNAs are further cleaved by Dicer to yield 21–23 nucleotide duplexes. **4.** One strand of the miRNA duplex can either associate to the RISC complex and guide translational repression of target mRNAs or be released by the cells. In the latter case, the mature miRNA binds to RNA-binding proteins such as **5.** Argonaute-2, or **6.** lipoproteins. Alternatively, the miRNAs can be loaded in **7.** microvesicles formed by plasma membrane blebbing, or in **8.** exosomes that are released in the extracellular space upon exocytic fusion of multivesicular bodies with the plasma membrane. Reproduced from (Regazzi, 2013) with permission.

1.3.3 Circulating MicroRNA

MicroRNAs were thought to be exclusively intracellular until recent studies that showed miRNAs are also extracellular, being present in a cell-free circulating form in the bloodstream (plasma or serum) and other body fluids, such as milk, urine, and saliva (Mitchell *et al.*, 2008). The detection of intact miRNAs in body fluids was surprising because usually RNAs in the extracellular environment are degraded by the high RNase activity. However, the discovery of many circulating miRNA in extracellular fluids suggests that they are protected from degradation through a protective mechanism. The mechanisms for the release of these miRNAs in the extracellular environment is not clear. MiRNAs circulate in different forms which can be roughly subdivided into vesicle-associated (exosomes, microvesicles, and apoptotic bodies) (Valadi *et al.*, 2007; Zernecke *et al.*, 2009; Sohel, 2016) and non-vesicle associated, with RNA-binding proteins (Argonaute 2)(Arroyo *et al.*, 2011) or high density lipoprotein complexes (HDL), Figure 1-9 (Vickers *et al.*, 2014).

An essential feature of circulating miRNA is their remarkable stability which interested researchers to use them as biological biomarkers for disease. Studies have shown that circulating miRNAs remain stable even if they were exposed to severe conditions such as multiple freeze-thaw cycles, prolonged storage time, high or low pH, and even boiling (Gilad *et al.*, 2008; Sohel, 2016).

The discovery of circulating miRNAs was considered a major scientific breakthrough and since their discovery, a substantial body of evidence was introduced showing the potential use of these circulating miRNAs as diagnostic biomarkers for several diseases (De Guire *et al.*, 2013; DiStefano & Gerhard, 2016; Mitchell *et al.*, 2008; Regazzi, Guay & Regazzi, 2013; Creemers, Tijsen & Pinto, 2012). For example, one of the most widely studied miRNAs to date is mir-122. It is highly expressed in liver tissue and has been considered as a promising biomarker of hepatocellular injury, hepatic biology, and liver diseases (Jopling, 2012; Pirola *et al.*, 2015; Musaddaq *et al.*, 2019).

1.3.4 Circulating miRNAs as biomarkers for T2DM

In recent years, the role of circulating miRNA in the pathophysiology of diabetes has been appreciated but it still not been fully explored. Several circulating miRNAs have been found to be associated with the risk factors involved in T2DM pathogenesis, such as insulin resistance, ß-cell dysfunction, and obesity.

In 2010, Zampetaki and colleagues were the first groups to identify several circulating miRNAs that were related to T2DM (Zampetaki *et al.*, 2010). They did a prospective study that included > 800 blood samples randomly selected from the Bruneck population (Bolzano Province, Italy). Five miRNAs (mir-15a, mir-28-3p, mir-29b, mir-126, and mir-223) were identified to be dysregulated in either pre-diabetes or T2DM and non-healthy controls.

In 2011, Kong L *et al.* found seven circulating miRNAs (miR-9, miR-29a, miR-30d, miR-34a, miR-124a, miR-146a, and miR-375) that were elevated in the serum of patients with T2DM compared to patients with pre-diabetes and individuals with normal glucose tolerance (Kong *et al.*, 2011).

In 2012, Karolina *et al.* studied circulating miRNAs present in the blood and exosomes of 265 patients with different health conditions associated with the metabolic syndrome (MetS) (Karolina *et al.*, 2012). There was an upregulation of miR-27a, miR-150, miR-192, miR-320a, and miR-375 in both patients with T2DM and MetS. Also, there was a strong correlation between high fasting blood glucose and increased levels of miR-27a and miR-320a.

In 2013, Pescador *et al.* investigated the expression of serum miRNA to determine whether miRNA expression was deregulated and if any observed deregulation was specific to either obesity or diabetes or both. They selected 69 participants and divided them into four groups: normal, healthy controls (n = 20), patients with T2DM (n = 13), patients with obesity (n = 20), and patients with T2DM and obesity (n = 16). The results demonstrated that the levels of circulating miR-15b, miR-138 and miR-376a in serum could be used to distinguish patients who have both obesity and T2DM from patients who only have obesity or only have T2DM (Pescador *et al.*, 2013).

In 2014, a cross-sectional analysis was done by Ortega *et al*. were they identified 10 circulating miRNAs (mir-140-5p, mir-142-3p, mir-222, mir-423-5p, mir-125b, mir-192, mir-195, mir-130b, mir-532-5p, and mir-126) that were dysregulated in the plasma of 48 patients with T2DM compared to 45 individuals with normal glucose tolerance (Ortega *et al.*, 2014). They also did a randomised, double-blinded, and placebo-controlled 3-month trial of metformin treatment on 35 patients with T2DM. The results showed three circulating miRNAs (mir-140, mir-222, and mir-192) of the ten identified circulating miRNAs shifted their concentration after treatment with metformin suggesting their potential use for T2DM management.

In 2015, a meta-analysis was done by Zhu & Leung to identify potential miRNA biomarkers of type 2 diabetes. The meta-analysis identified 40 significantly dysregulated miRNAs in T2DM both cellular and circulating miRNAs. The authors reported eight circulating miRNAs (miR-103, miR-107, miR-132, miR-144, miR-142-3p, miR-29a, miR-34a, and miR-375) as potential blood biomarkers (Zhu & Leung, 2015).

In 2016, a more extensive cohort study was done by Wang *et al*. consisting of 68 patients with T2DM, 68 patients with T2DM-associated microvascular complications, and 68 controls. They identified five circulating miRNAs (mir-661, mir-571, mir- 770-5p, mir-892b, and mir-1303) were significantly upregulated in patients with T2DM. Moreover, levels of these five miRNAs were markedly higher in patients with complications than those without complications. (Wang *et al.*, 2016).

In 2017, a systematic review was published on the relationship between miRNAs and T2DM (He *et al.*, 2017). The review included 59 independent studies, including a total of 2671 patients with T2DM and 2573 healthy controls. The samples included adipose tissue, islet, skeletal muscle, whole blood, PBMC (peripheral blood mononuclear cell), and serum and plasma. The authors identified 158 dysregulated miRNAs associated with T2DM in all seven tissues included, and they revealed that several pathways related to T2DM may be targeted by these miRNAs. Out of the 158 miRNAs identified, 31 miRNAs were circulating in plasma or serum.

In 2018, Ma *et al*. investigated the relationship of circulating miRNAs with insulin sensitivity, as measured by the gold standard hyperinsulinemic-euglycemic clamp technique. The study included 81 patients without diabetes, sedentary, and weight-stable across a wide range of insulin sensitivities. The results showed that circulating mir-16, mir-107, mir-33, mir-150, and mir-222 were associated with insulin sensitivity and metabolic risk factors (Ma, Fu and Garvey, 2018).

To date, the list of circulating miRNAs involved in the pathogenesis of T2DM is still expanding. However, individual studies are generally of very small size by modern epidemiological standards, reflecting difficulty in the measurement of miRNA at scale (Chapter 3.1.2). Although existing studies provide many novel insights into the potential aetiology of T2DM, they will be prone to small study bias. The critical question is whether changes in the expression of these miRNAs are specific for T2DM, which is an essential factor for a circulating miRNA to be a therapeutic target or a clinical biomarker.

1.4 Aim and objectives of this thesis

This thesis aims to investigate the relationship between obesity, inflammation, and type two diabetes (T2DM) and gain a better understanding of how these three relate. I also aimed to identify putative circulating miRNAs as novel biomarkers for metabolic dysfunction. To fulfil these aims, I will used three studies with different designs and different population phenotypes data sets:

1. Using the **C**arotid **A**therosclerosis: **ME**tformin for insulin **R**esist**A**nce (CAMERA) study (Preiss *et al.*, 2014) which is a randomised, placebo-controlled double-blinded trial. Participants were without diabetes, had large waist circumferences and coronary heart disease (CHD). Plasma samples will be used for:

    a) To develop a reliable and reproducible protocol for optimal extraction, quantification, and analysis of miRNA expression in human plasma samples that can be implemented in a large-scale study.
    b) Investigation in a cross-sectional analysis of the association of the expression of targeted circulating miRNAs with:

        i. Anthropometric biomarkers including weight, BMI, waist, and body fat.

        ii. Insulin sensitivity biomarkers including fasting blood glucose (FBG), insulin, Homeostasis model assessment of insulin resistance (HOMA-IR), HbA1c, and adiponectin.

        iii. Liver enzymes including gamma glutamyl transferase (GGT) and alanine aminotransferase (ALT).

        iv. Inflammatory biomarkers including CRP and white blood cells (WBC) count.

    c) Exploration of the effect of randomisation to metformin on the expression of targeted circulating miRNAs.

2. Using the UK Biobank, a large-scale prospective epidemiological study, I will investigate in a cross-sectional analysis:

    a) Different patterns of body fat distribution (anthropometrics measurements, bio-impedance measurements, DEXA measurements, and MRI measurements) in both sexes and their association with T2DM.

    b) The association between the body fat distribution measurements and WBC count.

    c) The association of WBC count with T2DM.

    d) Investigate whether WBC count is a link between adiposity and the development of T2DM.

3. Using the **S**urgi**C**al **O**besity **T**reatment Study (SCOTS) a prospective cohort study of patients undergoing bariatric surgical procedures in Scotland, I will evaluate the feasibility of the proposed study The REduction of Adipose Levels of Inflammation when Treating obesitY (REALITY). To do that I will:

    a) Asses patient's recruitment, their willingness to participate and give both adipose tissue and blood samples.

    b) Determine the optimal method for collecting adipose tissue (RNA*Later* vs snap freezing). I will compare between the two methods in terms of the amount of total RNA extracted, the expression of housekeeping genes (mir-103, mir-331, And RNU44), and the expression of the selected miRNAs (mir-222. Mir-221, mir-192, mir-193b, mir-144, and mir-155).

    c) Examine the two types of controls to normalise the expression of circulating miRNAs.

    d) Measure the expression of the selected six circulating miRNAs in plasma samples.

e) Measure adipocyte diameter using semiautomated method.

2 GENERAL METHODS

2.1 Introduction

In this chapter, all the general methods, equipment, kits, and reagents used to produce this thesis are described. All analyses were carried out in the laboratory facilities of the Institute of Cardiovascular and Medical Sciences (ICAMS) at the University of Glasgow by the author, unless otherwise acknowledged.

2.1.1 Studies used in this thesis

2.1.1.1 The CAMERA Trial

The **C**arotid **A**therosclerosis: **ME**tformin for insulin **R**esist**A**nce (CAMERA) study (Preiss *et al.*, 2014) was a randomised, placebo-controlled double-blinded trial done at the Glasgow Clinical Research Centre (Glasgow, UK) between the years 2009 and 2012 prior to design of this thesis. Use of this study is therefore post-hoc. The study was designed to assess the cardiovascular benefits of metformin in individuals without diabetes and with coronary heart diseases. I used baseline and 18-month plasma samples from 154 participants. Details of the study are found in chapter 3 and 4.

2.1.1.2 UK biobank

UK Biobank is a unique large scale prospective epidemiological study, open to data access requests from bona fide researchers worldwide. The aim of the study was to improve, prevent, diagnose and treat a wide range of illnesses including cancer, heart diseases, and diabetes, providing unparalleled power and phenotyping in a single observational study. Baseline assessment recruited more than 502,000 people between the years 2006 and 2010 (Sudlow *et al.*, 2015). The imaging study began in May 2014 and aims to recall 100,000 participants for detailed imaging scans of vital organs (brain, heart, abdomen, bones, carotid artery, and body composition) as well as a repeat of the baseline measurements. The study included Body MRI scan, and DEXA scan of the whole body, in specific participant subsets. I used all the baseline and imaging study data in our study. Details of the study are found in chapter 5.

2.1.1.3 The SCOTS study

Surgi**C**al **O**besity **T**reatment Study (SCOTS) is a prospective cohort study of patients undergoing bariatric surgical procedures in Scotland. The study recruited 450 patients to assess medium term outcomes and complications of bariatric surgery. The patients are followed-up for 10 years post-bariatric surgery using multiple data collection methods. Five patients who were due to have bariatric surgery were included in our study (Logue *et al.*, 2015). Details of the study are found in chapter 6.

2.2 MiRNA Isolation and Quantification

2.2.1 MiRNA Extraction from plasma samples

Extraction of miRNA was performed using the miRNeasy Mini Kit (Qiagen, Hilden, Germany Cat. No. 217004) which is designed for purification of total RNA, including miRNA and other small molecules, from cultured cells and both human and animal tissues. This kit combines phenol/guanidine-based lysis of samples and the silica membrane-based purification of total RNA.

2.2.1.1 Extraction

All work was done under a fume hood and on top of ice. 300 µL of plasma sample was separated into two 1.5 mL Eppendorf tubes (150 µL each tube). Five volumes (750 µL) of QIAzol Lysis Reagent (Qiagen, Hilden, Germany Cat. No.79306) was added to each tube and mixed by vortex until all precipitate disappeared. Tubes were then placed in the fume hood at room temperature for 5 minutes to ensure complete dissociation of nucleoprotein complexes. Chloroform (150 µL) was added next to all tubes containing the homogenate, mixed by vortex and allowed to stand for 3 minutes at room temperature. Following centrifugation for 15 minutes (8000 G, 4°C), the upper clear aqueous phase was separated into two new 1.5 mL Eppendorf tubes (250 µL each), making a total of 4 tubes for each plasma sample. 1.5 volume of 100% ethanol was added to each tube. One RNeasy Mini spin column was prepared for each sample by placing it into 2 mL collecting tube. All four tubes of the same sample were added to the same column. One by one, the samples were mixed by pipetting, collected (up to 700 µL) into the column, and centrifuged (8000 G, 4 minutes, at room temperature), with the flow-through being discarded. This was repeated three more times for the remainder of the samples. Buffers in the kit were prepared as instructed by the manufacturer. RNeasy Mini spin column was washed with 700 µL of RWT buffer and centrifuged (8000 G, 2 minutes, at room temperature). Flow-through was discarded. The column was washed again with 700 µL RPE buffer and centrifuged (8000 G, 2 minutes, at room temperature). Flow-through was discarded. 500 µL of 75% ethanol was added onto the RNeasy Mini spin column and centrifuged (8000 G, 2 minutes, at room temperature). The spin column was put into a new 2 mL collection tube and dried by centrifugation (8000 G, 5 minutes, at room

temperature). After that, the spin column was transferred onto a new 1.5 mL collection tube. 20 µL of RNase-free water was pipetted directly onto the spin column and centrifuged (8000 G, 2 minutes, at room temperature). Using the same flow-through, the previous step was repeated twice more to concentrate the RNA. The sample was kept on ice until nanodrop RNA quantification.

2.2.2 MiRNA extraction from Adipose tissue

MiRNAs were extracted from adipose tissue using the QIAGEN RNeasy Lipid tissue mini kit (QIAGEN, Crawley, UK, Cat. No. 74804).

2.2.2.1 Homogenization of Adipose Tissue Using the Tissuelyser

Adipose tissues were homogenized using the Tissuelyser (QIAGEN, Germany, Cat.No.85220). Approximately, 100 mg of adipose tissue was disruptive by rapid agitation in the presence of 5 mm mean diameter Stainless steel beads (QIAGEN, Cat.No.69989) and lysing buffer (1 mL of QIAzol).

2.2.2.2 Extraction

Following homogenization, 200 µL of chloroform was added. Mixture mixed thoroughly by vortex and samples spun at 12,000 g for 15 minutes at 4℃. The aqueous layer (approximately 600 µL) was removed and placed in a fresh RNAse free tube. RNA was precipitated by addition of 70% ethanol and transferred to a RNeasy mini spin column. If necessary, samples were treated with DNase (See section DNase Treatment of RNA). Columns then underwent a series of washes to remove impurities before being eluted in 50 µL of RNase free H2O. RNA was stored at -80℃.

2.2.2.3 DNase Treatment of RNA

Further DNA removal was necessary before analysis of miRNA by TaqMan qRT-PCR to remove DNA contaminants that can be detected during qRT-PCR and potentially cause experimental artefacts. DNase treatment was performed by incubation of samples in on-column DNase digestion set (QIAGEN, Cat.No.79254) according to manufacturer's protocol. For each sample 10µl of DNase I stock solution was mixed with 70µl Buffer RDD, applied to column and allowed to incubate at room

temperature for 15 minutes. This step was performed during the first column wash of the miRNEasy protocol. Following DNAse treatment, the miRNeasy wash protocol was continued.

2.2.3 Total RNA Quantification using Nanodrop

The concentration of RNA in each sample was measured using the Nanodrop 1000, micro-volume ultraviolet-visible spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts v3.7) with the ND-100 software per the manufacturer's instructions. 2 µL of nuclease free water was carefully pipetted onto the end of a fibre optic cable/receiving fibre and used as a blank. Subsequently, 2 µL of each sample was measured in a similar method to the water blank. Concentration measurements were recorded, along with the ratio of sample absorbance (260/280, 260/230) to check for nucleic acid purity as well as protein and phenol contamination. Measurements were taken twice and stored at $-80℃$.

2.2.4 MiRNA Reverse Transcription

MiRNA reverse transcription (RT) was done to generate complementary DNA (cDNA) from an RNA using the enzyme Reverse transcriptase. Taqman MicroRNA Reverse Transcription Kit was used (Applied Biosystems, Foster City, California, no.4366596) as per manufacturer's protocol. Primers for each miRNA (RT, 5X) were used to prepare master mix.

Samples Preparation

A total of 9 ng miRNAs is required. The dilution was made using nuclease free water (Appendix 1). A fixed volume of 5 µL from the total RNA was used for the RT process.

Master Mix Preparation

One master mix was prepared for each miRNA primer. Each master mix contained dNTP (100 mM), multiscribe, 10X RT buffer, RNase inhibitor, nuclease free water and the specific miRNA primers allocated (5X, RT) (Appendix 1). The master mix was mixed and kept on ice.

Protocol

A 96-well plate was prepared, marked and kept on ice. 4.5 µL of diluted RNA samples were added to each well as allocated and the plate was spun. 3 µL of the master mix was added to each well and the plate was spun. The plate was sealed with adhesive sealing sheets and ran on a Polymerase Chain Reaction (PCR) block for RT reaction. Thermal cycle conditions were: 16℃ for 30 mins, 42℃ for 30 mins, 85℃ for 5 mins, 4℃ for 30 mins and then held at 12℃. The plate was kept in −20℃ until used for QPCR.

2.2.5 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the 2X Universal TaqMan Master Mix (Applied Biosystems, Foster City, California) and TaqMan primers for each miRNA (TM, 20X). Samples were running in duplicate including the positive and negative QC.

Plate Preparation

The 96-well cDNA plate was taken out from −20℃ and kept on ice until thaw. 7.5 µL RNase free water was added into each well and spun at 4℃ to bring everything down.

Master Mix Preparation

Calculations of the amount of master mix required is shown in (Appendix 2). One tube was prepared for each miRNA primer. To each tube, 2X TaqMan master mix, 20X primers/probes, and RNase free water was added.

Protocol

An MicroAmp optical 384-well reaction plate was prepared, marked, and kept on ice.1.4 µL of the cDNA product was pipetted on the side of the well and centrifuged. After that, 8.6 µL of master mix was pipetted into each well, centrifuged and sealed with an optical adhesive lid.

Quantitative real-time PCR (qRT-PCR) was performed using the QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, California V1.3). The Thermal cycling conditions were 95℃ for 10 mins., followed by 40 cycles of 95℃ for 15 s and 60℃ for 60 s. For each miRNA, four runs of qRT-PCR were done including quality control (QC) sample and negative control sample (both in duplicate). Data were analysed with the QuantStudio 7 software (Applied Biosystems, Foster City, California V1.3) to determine the threshold cycle (Ct).

2.3 Normalisation and Control for qRT-PCR Data

To produce reliable qRT-PCR data, corrections of variation must be made to miRNAs expression analysis, to adjust for reaction efficiency. This is an important step that needs to be done before interpreting qRT-PCR results (Hellemans & Vandesompele, 2011). There are two sources of variation, biological and technical variation. Our aim was to measure the biological differences and reduce the technical effect, which can be introduced at any steps from sample collection to miRNA amplification.

The optimal method for normalisation is to use a theoretical constantly expressed housekeeping gene, because they represent endogenous controls that are affected by the same source of variability as the target gene. This method has been widely used for measuring the expression of mRNA and miRNA extracted from tissues and cells. However, using this method on plasma/serum samples is challenging because very few studies are available. Endogenous controls are frequently selected based on empirical observations, with little biological insight. Different studies reported different miRNA reference genes (Moldovan *et al.*, 2014; Shaffer, Schlumpberger & Lader, 2012). For choosing an appropriate endogenous control, it is important to first check whether the selected miRNA expression is stable between the samples before using it as a normaliser. Also, one makes sure that the chosen miRNA is not affected by diseases or clinical interventions, such as medications, which may vary between patients or between samples in an intervention study. Use of an exogenous control means one does not have to worry about biological effects, but the introduction of the exogenous control is subject to its own potential for technical errors.

To verify if the qPCR experiment passes initial quality control, technical replicates of the controls and samples must be within 0.5 cycles (1 cycle represent a doubling of material). This is achieved by calculating the standard deviation (SD) of triplicates measurement of the same sample. The SD should not exceed 0.25 (Institute of research in immunology and cancer University of Montreal, n.d.)(Institute of research in immunology and cancer University of Montreal, n.d.) (http://genomique.iric.ca/resources/files/Understanding_qPCR_results).
Moreover, a random plasma sample was used as a quality control sample to try to control for variation between runs.

Mean and standard deviation (SD) were calculated for endogenous control, exogenous control, and QC sample. In addition, the coefficient of variation (CV) for both intra and inter assay were calculated for all the three controls. Although calculating CV is not generally used for evaluating precision of qRT-PCR results, this was our attempt to quantify error.

2.3.1 Plasma samples

For measuring miRNAs in plasma, I evaluated both types of controls: endogenous control and exogenous control.

2.3.1.1 Exogenous Control

A miRNeasy Serum/Plasma spike-in control (Qiagen, Hilden, Germany Cat. No. 219610) was used. It is a Caenorhabditis elegans (*C. elegans)* mir-39 miRNA mimic and is supplied lyophilized at 10 *p*mol per tube (Appendix 3). Spike-in controls can be added to the sample in two ways: 1) before extraction, or 2) before transcription of RNA. Although the latter avoid differences in template quality and provide more stable results, I choose to spike-in the exogenous control before extraction to provide a more reliable estimate of the whole technical variability that maybe introduce at different steps. Also, the synthetic miRNA was added (spiked-in) after the addition of the denaturing agents (Qlazol) to avoid its degradation by plasma RNase. A trial run was conducted using 8 random normal samples to test the stability of mir-39 and its reliability as a control.

2.3.1.2 Endogenous Control

Mir-520d-5p was tested as a possible endogenous control since it was reported to be an excellent housekeeping control candidate for studies of plasma miRNA due to its consistent results and a very narrow standard deviation (Rice *et al.,* 2014). A trial run was conducted using the same 8 random normal samples used in testing mir-39, to test its potential use as an endogenous control gene.

2.3.2 Adipose samples

Three housekeeping miRNAs were tested (mir-103, mir-331, and RNU44) which have been shown to be a stable miRNA endogenous control in adipose tissue (Neville *et al.*, 2011; Kristensen *et al.*, 2017).

2.4 Circulating Adiponectin

The plasma adiponectin concentrations were measured by Enzyme Linked Immunosorbent Assay (ELISA) Kit (Quantikine Human Adiponectin/Acrp30 Immunoassay, Cat. No. SRP300). The assay employs the Quantitative sandwich enzyme immunoassay technique with a 96 well polystyrene microplate coated with a monoclonal antibody specific for human adiponectin globular domain (Figure 2-1).

The kit is designed to measure the amount of adiponectin in serum or plasma samples. The assay sensitivity is 2ng/mL without dilution of samples. Intra- and Inter coefficient of variation is 4.1% and 4% respectively at adiponectin concentration = 7 µg/ml.

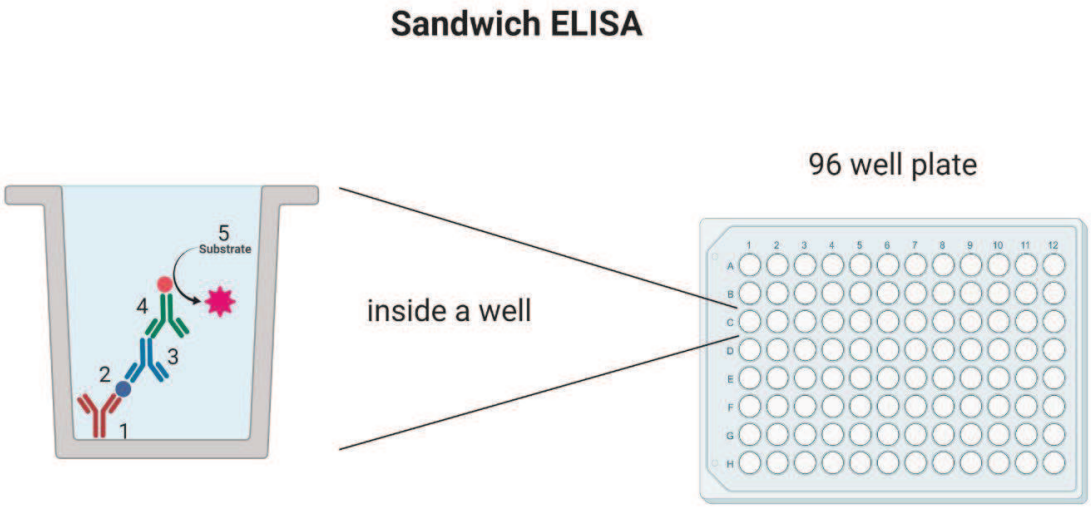


Figure 2-1: Sandwich ELISA reaction
1.The capture antibodies are bound to the surface of each well. **2.** The antigen-containing sample is added to the plate and captured by the bounded antibodies. **3.** Specific antibodies are added and bind to the antigen hence the word sandwich. **4.** Enzyme-linked secondary antibodies are added and bind specifically to the antibodies. **5.** A substrate is added to be converted by the enzyme into a colour or fluorescent.

2.4.1 Adiponectin ELISA Method

All samples were 100-fold diluted. Human adiponectin standards, substrate, and washing buffer were prepared according to the manufacture instructions. Initially, 100 µL of assay diluent was added to each well. 50 µL of serially diluted human adiponectin standards and prepared quality control 1 and 2 (Cat. No. QC36, R&D System) were added in duplicate to the designated wells. Sequentially 50 µl of the unknown samples were added to the remaining wells of the microplate. The plate was covered with adhesive strip and incubated at room temperature for 2 hours. After that, plate was washed 4 times using 400 µL of wash buffer per well and 200 µL of Human Adiponectin Conjugate was added to each well and incubated for 2 hours. Then, the plate was washed and 200 µL of substrate was added and incubated for 30 minutes at room temperature protected from light. The reaction was stopped by adding 50 µl of the stop solution to each well.

The optical density was read at 450 nm with wavelength correction set to 540 nm using SpectraMax 190 absorbance microplate reader (Molecular Devices, LLC). The concentrations of unknown samples were determined using a standard curve which was constructed by plotting the mean absorbance for each standard against their concentration using a log/log point analysis as shown in (Figure 2-2).

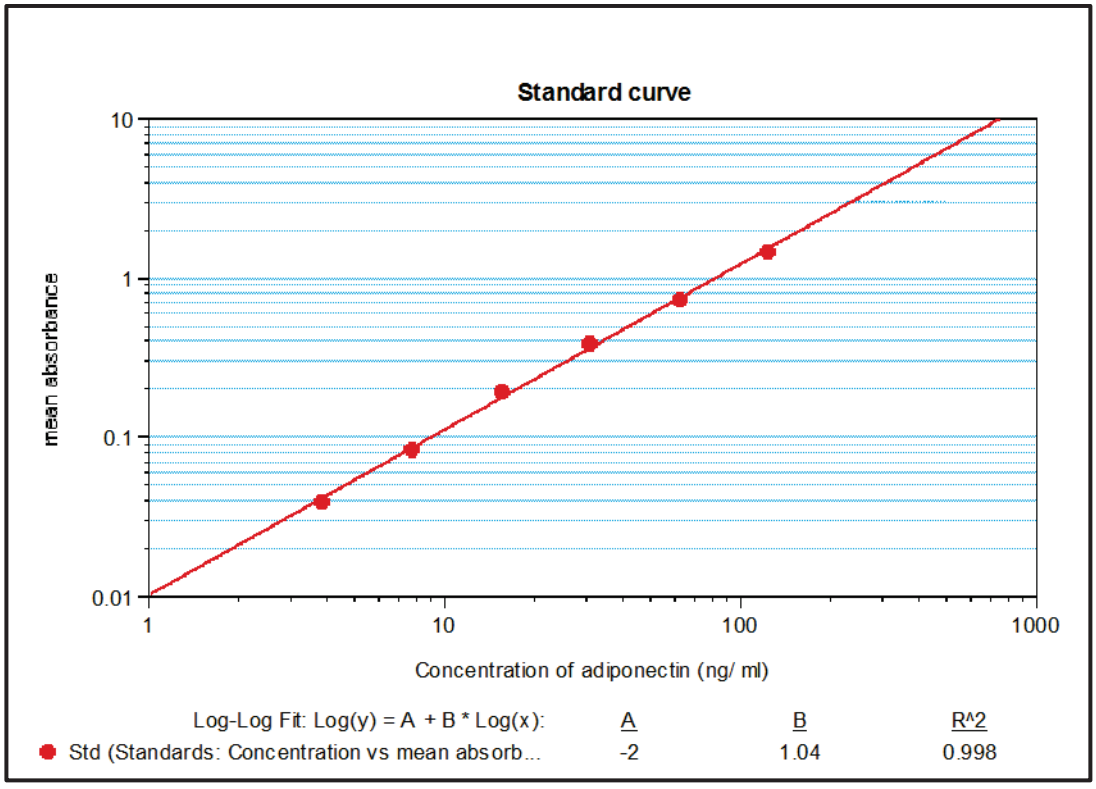


Figure 2-2: Adiponectin standard curve
Standard curve for adiponectin is constructed by plotting the mean absorbance for each standard against their concentration using a log/log point analysis.

2.5 Statistical Analysis

2.5.1 Cohort demographics and classical risk factors

All continuous classical risk factor variables were tested for normality by visual inspection of their histograms and normal Q-Q plots. Descriptive statistics were expressed as mean ± SD for normally distributed variables, and median [and inter-quartile range] for not normally distributed variables. Note that throughout the thesis, use of a "±" denotes a standard deviation after a mean, and use of a [range] after a number denotes a median and interquartile range. Non-parametric variables were normalized by log transformation to use parametric tests. Categorical variables were summarized as number (%). Comparison between mean values of continuous variable were evaluated using Student's two tailed *t* test. All the statistical tests were performed using IBM SPSS (Statistical Package for Social Sciences) windows version 22 (SPSS Inc. Chicago, USA) and Microsoft excel 2016. The criterion for statistical significance was $p < 0.05$. As this is a hypothesis generating exploratory study, I did not employ any formal correction for multiple comparisons.

2.5.2 RT-qPCR Data Analysis

The Ct represents the number of cycles required to attain a threshold concentration for the miRNA of interest. Each PCR cycle increases the concentration 2-fold, and therefore the Ct value as a concentration is naturally expressed as log 2 and are therefore normally distributed.

The mean Ct and standard deviation (SD) between the triplicate measures for controls and samples were calculated. The technical triplicates must be within 0.5 cycles (1 cycle represent a doubling of material). The triplicate is valid when the SD is smaller than 0.25. If the SD is over 0.25, measurements were repeated.

To measure the precision and repeatability of our RT-qPCR results, the coefficient of variation (CV) for both intra and inter assay were calculated for exogenous control (mir-39 spiked-in control), endogenous control (mir-520d-5p, mir-103, mir-331-3p, and RNU44). Although calculating CV is not used for evaluating precision of qRT-PCR results, this was our attempt to quantify error. Intra-assay CVs of <10%

and Inter-assay CVs of < 15% are generally considered acceptable in most quality control settings outside of routine clinical biochemistry.

2.5.3 Cross-Sectional Analysis

Pearson correlation (r) was used to investigate the association of the six miRNAs expressions with markers of metabolic health using baseline samples.

2.5.4 Relative Quantification method (RQ)

The relative quantification (RQ) method was used to compare paired observations at different timepoints. It allows the quantification of the change in gene expression level of a target gene across two samples, in our case baseline and 18-month samples. The results were expressed as fold change of expression level in the 18 months sample compared to the baseline sample. This is done by first, calculating the differences in Ct between the target miRNA with that of the spiked-in external control (mir-39) from the same sample (ΔCt). After that, the ΔCt was then used to calculate the ΔΔCt (ΔCt baseline - ΔCt after 18 months). Finally, RQ was calculated using the formula ($2^{-\Delta\Delta Ct}$). RQ value is generally considered significant when there is a minimum of two-fold change in the established literature (Marabita *et al.*, 2016).

3 CROSS-SECTIONAL ASSOCIATIONS OF CIRCULATING MIRNAS EXPRESSION WITH MARKERS OF METABOLIC HEALTH: CAMERA TRIAL

3.1 Introduction

Insulin resistance (IR) is a critical feature and major contributor to the developing of Type 2 diabetes (T2DM)(Ma, Fu & Garvey, 2018b). Although the cause of IR is still unknown, it is closely related to obesity, particularly central obesity (Westphal, 2008). Both central obesity and IR are signs of metabolic disturbance that proceed T2DM. Waist circumference (WC) is a convenient index for measuring abdominal obesity (Grundy *et al.*, 2013). Besides, it is more strongly correlated with intra-abdominal fat content and has been used as a predictor for the prevalence or incidence of T2DM (Qiao & Nyamdorj, 2010; Siren, Eriksson & Vanhanen, 2012).

Recently, circulating miRNAs have been proposed as new disease biomarkers that can help in the diagnoses, prognosis, and monitoring of several diseases. This was supported by a strong body of evidence showing that dysregulation of their expression was associated with several diseases (De Guire *et al.*, 2013; DiStefano & Gerhard, 2016; Mitchell *et al.*, 2008; Regazzi, Guay & Regazzi, 2013; Creemers, Tijsen & Pinto, 2012). Also, these circulating miRNAs are surprisingly stable and can resist aggressive physiological conditions such as extreme variations in pH, boiling, multiple freeze-thaw cycles, and extended storage, despite the presence of blood RNases.

A meta-analysis published in 2015 (Villard *et al.*, 2015) were the authors investigated circulating miRNAs for diagnosing obesity and T2DM in human. A total of 26 studies were included. These studies compared the expression of circulating miRNAs between two conditions (obese vs. controls, diabetes or pre-diabetes vs. controls) or the effect of lifestyle interventions (exercise, metformin treatment or bypass surgery) on their expression. Six studies compared miRNAs levels between subjects with obesity vs. subjects who were lean, eight studies compared patients with pre-diabetes vs. subjects who were control, and 14 studies compared subjects with diabetes vs. subjects who were BMI-matched and without diabetes. A total of 10 miRNAs were found to be altered in blood of patients suffering from T2DM (increased: mir-320a, mir-142-3p, mir-222, mir-29a, mir-27a, mir-375; decreased: mir-197, mir-20b, mir-17, mir-652) and 7 miRNAs in blood of subjects with obesity were identified (increased: mir-142-3p, mir-140-5p, mir-222; decreased:mir-21-5p, mir-221-3p, mir-125-5p, mir-103-5p). Both patients

with obesity or T2DM had elevated concentrations of mir-142-3p and mir-222. This study suggested that a specific profile of circulating miRNAs could become a valuable biomarker to identify those who are at risk for developing T2DM.

3.1.1 The CAMERA Trial

The **C**arotid **A**therosclerosis: **ME**tformin for insulin **R**esist**A**nce (CAMERA) study (Preiss *et al.*, 2014) was a randomised, placebo-controlled double-blinded trial done at the Glasgow Clinical Research Centre (Glasgow, UK) between the years 2009 and 2012. The study was designed to assess the cardiovascular benefits of metformin in individuals without diabetes. The primary outcome was carotid intima media thickness (cIMT), and the study was powered to detect that metformin had no effect on cIMT and little or no effect on several surrogate markers of cardiovascular disease.

Caucasian ethnicity participants were recruited aged 35-75 on statins with proven coronary heart disease (previous acute coronary syndrome, coronary artery bypass surgery, or angiographically proven coronary heart disease), large waist circumferences (in agreement with the International Diabetes Foundation guidelines, ≥ 94 cm in males, ≥ 80 cm in females) (Alberti, Zimmet & Shaw, 2006), and prescribed a statin (type and dose were not adjusted).

Exclusion criteria were diabetes or participants with either HbA1c ≥ 7% or fasting blood glucose (FBG) ≥ 7 mmol/L (participant with HbA1c of 6 - 6.9 and FBG < 7 mmol/L at the screening had an oral glucose tolerance test; those with post-challenge glucose of ≥ 11.1 mmol/L were excluded). Moreover, pregnancy or lactation, premenopausal women not taking hormonal replacement (daily oral contraception or regular injectable hormonal contraception were excluded from the study. Patients who had acute coronary syndrome within the previous 3 months uncontrolled angina, class 3 or 4 heart failure (according to the New York heart association functional classification), hepatic impairment, renal impairment (estimated glomerular filtration rate < 45 mL/min per 1.73 m2 at screening), hypersensitivity to metformin, acute illness (dehydration, severe infection, shock, acute cardiac failure), and suspected tissue hypoxia were also excluded.

Written informed consent was provided from each participant. The study was approved by the Medicines and Healthcare Products Regulatory Agency and West Glasgow Research Ethics Committee and done in accordance with the principles of the Declaration of Helsinki and good clinical practice guidelines. A total of 173 participants were randomly assigned to metformin or placebo (1:1) on the CAMERA website and followed for 18 months (Figure 3-1). The randomisation sequence was generated independently by the Robertson Centre for biostatistics. All patients, investigator, trial staff, and statisticians were masked to treatment allocation.

For the first week, participants were taken one tablet (850 mg metformin or placebo). Later, the dose was increased to 2 tablets daily (one with the morning meal and one with the evening meal). A total of 29 participants reduce their tablet intake to 1 per day (19 in the metformin group and 10 in the placebo group). After that, 15 participants were excluded from the study after primary outcome analysis (11 in the metformin group and 4 in the placebo group) leading to a total number of 158 participants (75 in the metformin group and 83 in the placebo group). Finally, 4 participants were excluded from our study because they didn't have both baseline and 18-month plasma samples.

3.1.1.1 Power calculation

Assuming the minimum relevant difference to detect between metformin and placebo groups is 1dCt (a 2-fold change) with a dCt SD of 1.5 in each group, a sample size of 81 and 73 in each group gives 98% power with an alpha of 0.05. For dCt of 0.8 and 0.7 respectively, the power would be 91% and 82%.

Samples from the CAMERA trial were used to conduct this study for several reasons. First, it included a large sample of participants (154). Second, participants were subjected to a strict inclusion and exclusion criteria. Third, well standardized approach to sample collection, processing, and storage. Finally, although the participants without diabetes, they were high risk participants (previous CHD and high WC) who will be good candidates for exploring the expression of circulating miRNAs.

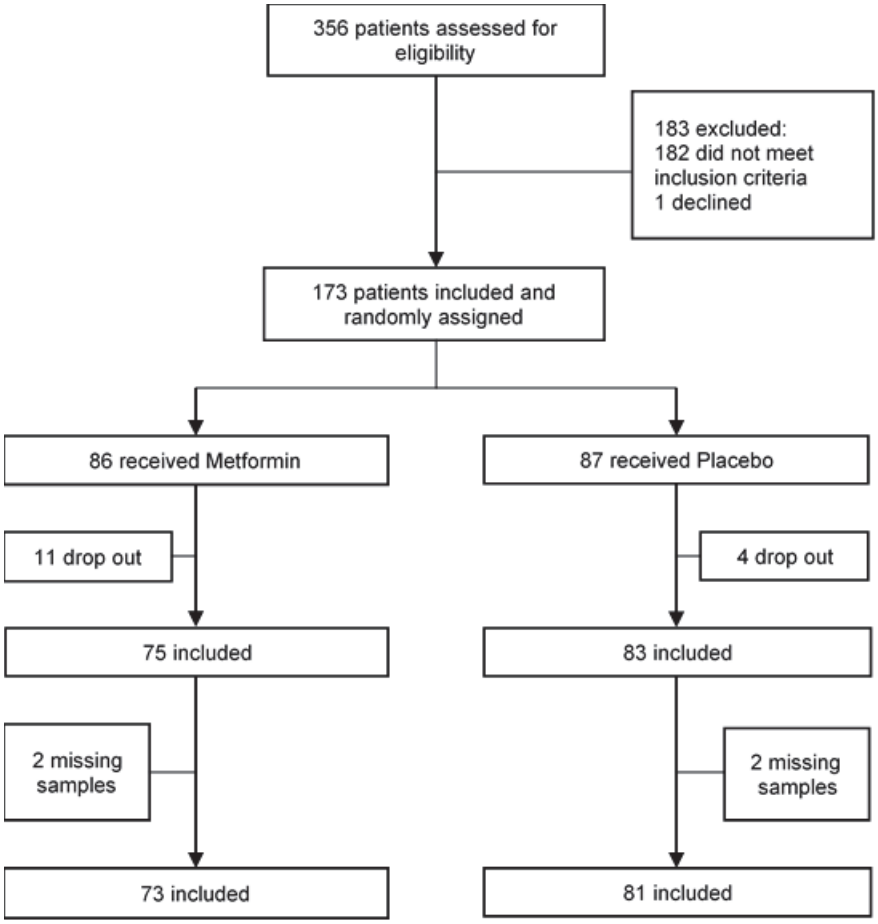


Figure 3-1: CAMERA trial profile
Illustration of baseline inclusion of participant blood samples in the miRNA study

3.1.2 MiRNA biomarker candidate selection to include in our study- A hypothesis-driven approach

Large numbers of miRNAs were identified to be differentially expressed in metabolic diseases. Although there are high number of publications reporting different circulating miRNAs associated with cardiometabolic diseases, only a few of these miRNAs associations were reproduced across several studies. The reasons for this may be: 1) miRNA profiling is a multi-step process and there is no standardised protocol, each study uses different sample types, different collection methods and different methodologies, and thus come to different conclusions, 2) no gold standard control to normalize the expression of these circulating miRNAs, and finally, 3) most studies used a very small sample size which increases the risk of type 2 error.

In order to make an evidence based informed decision as to which miRNAs to include in this study, A hypothesis-driven approach based on previously available evidence and not an exploratory approach was used. The reason for this because it is more focused, more efficient, require less time, and cheaper which makes it more suitable for large studies. This was done by performing a pseudo-systematic literature search of articles published in English from 2000 to 2016 using PubMed database. The key words for searching the database were: “miRNA”, “human”, “blood”, “insulin resistance”, “diabetes”, “adipose tissue”, and “obesity”. The yielded article abstracts were reviewed, focusing on studies of miRNA in blood samples. A candidate list of biomarkers was drawn up. A pragmatic decision was made to include six miRNAs, mir-222, mir-221, mir-144, mir-155, mir-192 and mir-193b (Table 3-1) subjectively based on:

1) greatest existing evidence that change in glycaemic control or BMI would influence their expression

2) frequency of citation in the literature

3) traded off with feasibility of technical completion

Table 3-1: Summaries of studies aid in the selection of specific miRNAs to include in our studies

Reference	Type of Study	Groups	Sample	miRNAs Tested	Findings
(Karolina *et al.*, 2011)	1. Animal model	T2DM rat model	Pancreas, adipose, skeletal muscle, liver and blood	mir-192, mir-29a, mir-30d, mir-320a mir-144, mir-146a, mir-150 and mir-182	**mir-144** had the highest up-regulation upon T2D in most tissues
	2. Cross-sectional	50 Asian males (IGF and T2DM)	Blood	mir-192, mir-29a, mir-30d, mir-320a mir-144, mir-146a, mir-150 and mir-182	**mir-144** expression with increasing glycaemic status and correlate with down-regulation of insulin receptor substrate 1 (IRS1)
(Corral *et al.*, 2013)	Cross-sectional	20 T2DM and 20 NC	PBMC (peripheral blood mononuclear cells)	mir-155 mir-146a	**mir-155** decreased compared to controls and significant correlated with HbA1c, Glucose and BMI.
(Coleman *et al.*, 2013)	Cross-sectional	37 subjects undergoing coronary artery bypass grafting divided into: no diabetes (ND), diabetes patients not on metformin (DMMet) diabetes patients on metformin (DMMet+)	Internal mammary artery (IMA)	mir-222 and mir-221	**mir-222, mir-221** in DMMet group and were inversely correlated with metformin dose
(Ortega *et al.*, 2013)	1. Initial study	32 white men 6 patients with class 3 obesity (3 men and 3 women)	Plasma	799 miRNAs	108 miRNAs were detected
	2. Validation study	80 white men	Plasma	18 miRNAs associated with obesity	in morbid obesity; **mir-140-5p, mir-142-3p, mir-222 mir-532–5p, mir-125b, mir-130b, mir-221, mir-15a, mir-423-5p, mir-520c-3p**
	3. Longitudinal	22 patients (after surgery-induced weight loss) 9 white obese patients (5 men and 4 women) after diet induced weight loss	Plasma	mir-140-5p, mir-142-3p, mir-222 mir-532–5p, mir-125b, mir-130b, mir-221, mir-15a, mir-423-5p, mir-520c-3p	After surgery induced weight loss: **mir-140-5p, mir-122, mir-193a-5p, and mir-16-1** **mir-221, mir-199a-3p**

Reference	Type of Study	Groups	Sample	miRNAs Tested	Findings
(Ortega *et al.*, 2014)	1. Pilot study	12 age-matched men (6 T2DM vs 6 NGT) for profiling miRNAs in association with T2D	Plasma	discovery study to identify circulating miRNAs associated with T2DM (16 miRNAs)	**mir-140- 5p, mir-142-3p, mir-222 mir-423-5p, mir-125b, mir-130b, mi-192, and mir-126**
	2. cross-sectional	93 subjects (45 with [NGT] and 48 T2DM) Most relevant circulating miRNAs for T2D	Plasma	mir-140-5p, mir-142-3p, mir-222, mir-423-5p, mir-192, mir-125b, mir-195, mir-130b, mir-532-5p, mir-126	**mir-140- 5p, mir-142-3p, mir-222 mir-423-5p, mir-125b, mir-130b, mi-192, and mir-126**
	3. RCT	18 placebos and 17 metformin-treated T2D patients followed for 3 months	Plasma	mir-140-5p, mir-142-3p, mir-222, mir-423-5p, mir-192, mir-125b, mir-195, mir-130b, mir-532-5p, mir-126	**mir-140-5p, mir-222 mir-142- 3p, mir-192**
	4. Interventional clinical trail	7 healthy volunteers before and after a 6-h hyperinsulinemic- euglycemic clamp and insulin plus intra-lipid/heparin infusion	Plasma	mir-140-5p, mir-142-3p, mir-222, mir-423-5p, mir-192, mir-125b, mir-195, mir-130b, mir-532-5p, mir-126	**mir-222** after clamp **mir-140-5p, mir-222, mir-195** after intra lipid/heparin mixture
(Ortega *et al.*, 2015)	1. Pilot	Human cells line, baseline and after inflammation	Human primary mature adipocytes, macrophage-like cell line THP-1 with their supernatants (SN)	754 common mature miRNAs	**mir-221**, **mir-222**, and **mir-155** in inflamed adipocytes and in their SNs. **mir-193b** in SNs.
	2. Longitudinal	9 Human subcutaneous AT before and after bariatric surgery-induced weight loss (2 years)	Subcutaneous human adipose tissue	18 miRNAs	After weight loss: **mir-146b, mir-376c, mir-411** inside isolated cells **mir-221, mir-222, mir-155, mir-223, mir-19a/b** inside isolated cells and/or SNs **mir-146a, mir-155, mir-19a** in SNs

Reference	Type of Study	Groups	Sample	miRNAs Tested	Findings
(Párrizas *et al.*, 2015)	1. Initial screening	17 controls, prediabetes (10 IFG, 9 IGT), and 10 T2DM	Serum	142 miRNAs	**mir-15b, mir-125a-5p, mir-128, mir-191** in IGF **mir-150, mir-192, mir-193b** in both IFG and IGT
	2. Cross-sectional	29 controls, 22 prediabetes (22 IFG and 21 IGT), and newly diagnosed diabetes subjects.	Serum	mir-150, mir-192, mir-193b	**mir-192, mir-193b** in prediabetes compared to T2DM and correlated with serum TAG, HOMA-IR, and FLI
	3. Second cohort	12 controls and 6 prediabetes subjects undergoing a therapeutic exercise intervention for 16 weeks. 6-week-old male mice (glucose intolerance model)	Serum	mir-192 and mir-193b	**mir-192, mir-193b** at baseline **miR-192, miR-193b** after intervention.
(Zhu & Leung, 2015)	meta-analysis	38 controlled studies to compare miRNA expression profiles of T2DM and control samples published between 1993 and March 2014	Blood, liver, pancreas, muscle, adipose, and Glomeruli	343 dysregulated miRNAs in humans and animals 190 dysregulated miRNAs in humans	40 miRNAs are significantly dysregulated in T2DM. mir-29a, mir-34a, mir-375, mir-103, mir-107, mir-132, mir-142-3p, mir-144 are potential circulating biomarkers of T2DM. mir-199a-3p and mir-223 are potential tissue biomarkers of T2DM
(Li *et al.*, 2016)	Cross-sectional	70 patients with Atherosclerosis (AS) 55 healthy controls	serum 17 atherosclerotic lesions	mir-29a, mir-26b, mir-21, mir-25, mir-155, mir-145, mir-126	**mir-155** in the serum and atherosclerotic lesions of AS

miRNAs in red: upregulated, miRNAs in blue: downregulated
T2DM: type two diabetes mellitus, **NC:** normal controls, **IFG:** impaired fasting glucose, **IGT:** impaired glucose intolerance, **NGT:** normal glucose tolerance

3.2 Hypothesis

The expression levels of the chosen circulating miRNAs (mir-221, mir-222, mir-192, mir-193b, mir-144, and mir-155) are associated with markers of metabolic disturbance including anthropometric measurements, glycaemic markers, and inflammatory markers.

3.3 Aim

1) To develop a reliable and reproducible protocol for optimal extraction, quantification, normalisation, and analysis of miRNA expression in human plasma samples that can be implemented in a large-scale study.

2) Investigate cross-sectional associations of the six selected circulating miRNAs using baseline plasma samples from the CAMERA trial with:

    a) Anthropometric biomarkers including weight, BMI, waist, and body fat.

    b) Insulin sensitivity biomarkers including fasting blood glucose (FBG), insulin, Homeostasis model assessment of insulin resistance (HOMA-IR), HbA1c, and adiponectin.

    c) Liver enzymes including gamma glutamyl transferase (GGT) and alanine aminotransferase (ALT).

    d) Inflammatory biomarkers including CRP and white blood cells (WBC) count.

3) Explore the potential usage of the selected circulating miRNAs as biomarkers for metabolic dysfunction.

3.4 Methods

3.4.1 Samples

All the baseline Ethylenediaminetetraacetic acid (EDTA) plasma samples of the CAMERA trial (154 samples) were used in this study.

3.4.2 Micro RNA Isolation and Quantification

Details on RNA extraction and miRNA quantification can be found in the general method chapter 2. To summarise, plasma samples were defrosted, and then the total RNA was extracted and purified. This was achieved using a commercially available kit which uses a column-based extraction method (Chapter 2.2.1). Once the RNA was extracted from the plasma, reverse transcription was performed on the RNA using specific primers for the chosen miRNAs (mir-222, mir-221, mir-192, mir-193b, mir-155, and mir-144) to generate miRNA Complementary DNA (cDNA). The cDNA was then used in qRT-PCR experiments to measure the expression of the chosen miRNAs (Chapter2.2.5).

3.4.3 Normalisation and quality control for qRT-PCR results

The two methods for miRNA normalisation (endogenous and exogenous controls) were tested in our study samples. Moreover, a random plasma sample was used as a quality control (QC) sample to try to control for variation between runs.

3.4.3.1 Endogenous Control

A trial run was conducted using eight random plasma samples to test the possibility of using mir-520d-5p as a reliable endogenous control gene.

3.4.3.2 Exogenous Control

A trial run was conducted using the same eight random normal samples used for testing endogenous control to assess the efficiency of mir-39 as an exogenous control in our studied samples.

3.4.3.3 Quality Control Sample (CQ)

To evaluate the repeatability and precision of the RT-qPCR results, a quality control sample (QC) sample was included in each run (4 runs per miRNA). Inter-assay variation of the QC sample for each miRNA was calculated.

3.4.4 Measurement of Insulin Sensitivity and Insulin Resistance

Fasting glucose (mmol/L) and fasting insulin (μIU/ml) values were used to calculate insulin sensitivity based on the homeostasis model assessment (HOMA-IR) available online (University of Oxford, 2014) at

http://www.dtu.ox.ac.uk/index.htm?maindoc=/publications/

The cut-off values for HOMA-IR varies according to ethnicity (Esteghamati *et al.*, 2010; Gayoso-Diz *et al.*, 2013; Bermúdez *et al.*, 2014; Lee *et al.*, 2016). These normal HOMA-IR values ranges from 1.7-2.0 in healthy human adults. In this study I chose to use HOMA-IR ≥ 2 to diagnose insulin resistance.

3.4.5 Statistical Analysis

The selected miRNAs were normalised to cel-mir-39 and expressed as delta (dCt). This removes artefact due to differences in extraction efficiency between samples. All continuous classical risk factor variables were tested for normality by visual inspection of their histograms and normal Q-Q plots. Descriptive statistics were expressed as mean ± SD for normally distributed variables, and median ± inter-quartile range for not normally distributed variables. Non-parametric variables were normalised by log transformation to use parametric tests. Categorical variables were summarised as number (%). Comparison between mean values of a continuous variable between two groups (males and females) and (HOMA-IR ≤ 2 and HOMA-IR > 2) were evaluated using *Student's* two-tailed *t*-test for continues variables and chi-square test for categorical parameters. Pearson correlation (r) was used to investigate the association of the six miRNAs expressions with markers of metabolic health using baseline samples. Multiple linear regression analysis was used to determine whether the metabolic variables of interest are an independent predictor for the expression of the selected circulating miRNAs. Linearity checked by checking residuals were normally distributed and no

Multicollinearity between the independent variables. I identified confounders were interested in adjusting for a priori, namely age, sex and body fat because they are independent risk factors for insulin resistance. The exposure variable was the metabolic biomarker, and the expression of circulating miRNA the outcome variable. Because dCt values are on a log2 scale, back transformation of the beta coefficient yields the ratio of the geometric means (i.e. a percentage change) in the miRNA for a unit change in the exposure.

3.5 RESULTS

3.5.1 Normalisation and quality control for qRT-PCR results

3.5.1.1 Endogenous Control

The results of the pilot of quality control procedures showed low and inconsistent expression of mir-520d-5p between the samples (Ct > 40). Therefore, mir-520d-5p was not used as an endogenous control gene.

3.5.1.2 Exogenous Control

The result of the quality control pilot showed low variation between triplicates (SD = 0.15, CV = 0.6) and between the samples (SD = 0.2, CV = 0.8) (Table 3-2). After that, I measured mir-39 for the entire study (Table 3-3). Although the inter-assay CV was low (CV = 3.54) and acceptable (< 15%), it still shows more variation between runs compared to intra-assay (SD > 0.25).

3.5.1.3 Quality control (QC) sample

Table 3-4 shows the results of the quality control sample for each miRNA. The QC sample was stable and showed no variation between runs (CV < 1%). These results ensure that the variation detected between samples is not because of analytical variation but due to real biological differences.

Table 3-2: mir-39 Intra and inter-assay CV% for the trial run

Intra-Assay		Inter-Assay	
Mean	25.2	**Mean**	25.1
SD	0.15	**SD**	0.2
CV	0.6	**CV**	0.8
CV: Coefficient of Variation, **SD:** Standard Deviation			

Table 3-3: mir-39 Intra and inter-assay CV% for the entire study

Intra-Assay		Inter-Assay	
Mean	24.93	**Mean**	24.93
SD	0.11	**SD**	0.88
CV	0.45	**CV**	3.54
CV: Coefficient of Variation, **SD:** Standard Deviation			

Table 3-4: Inter-assay CV for QC sample

miRNA	Mean	SD	Inter- Assay CV
mir-221	28.44	0.23	0.81
mir-222	29.56	0.23	0.77
mir-192	33.52	0.18	0.54
mir-193	35.57	0.21	0.60
mir-144	35.43	0.21	0.60
mir-155	33.08	0.23	0.69
CV: Coefficient of Variation, **SD:** Standard Deviation			

3.5.2 General characteristics of the study population grouped by sex

Since sex is an important factor that affects the risk of metabolic diseases, I grouped the study population by gender (males n= 119 and females n= 35) (Table 3-5). The mean age of males (64 ± 8) was not different from the females (64 ± 8).

As expected, there were differences between sexes in anthropometrics measurements, with males having higher weight and WC while females had more fat mass (p < 0.001). There was a difference in biochemical biomarkers between the two groups. Males had higher glucose and (males = 5.3 mmol/L ± 0.5 and females = 5 mmol/L ± 0.6, p = < 0.001), and Alanine aminotransferase (ALT) (males median 26 U/L [interquartile range (IQR) 21-34] and females median 25.8 U/L [15-29], p = < 0.05). Females had higher adiponectin (males = 5.1 µg/mL [3.8-7.2] and females = 8.6 µg/mL [5.5-11.8], p = < 0.001) and total cholesterol (TC) (females median = 4.8 mmol/L [4.1-4.9] and males median = 4.2 mmol/L [3.5-4.6], p = < 0.01) compared to males. Moreover, inflammatory markers measured by C-reactive protein (CRP) and WBC were nominally higher in females than males, but both were not statistically significant (p > 0.05).

Fewer studies focus on the sex difference suggested that miRNAs may be differentially expressed between males and females (Wang *et al.*, 2013; Sharma and Eghbali, 2014; Ameling *et al.*, 2015). In this study, only two of the six circulating miRNAs measured showed sex differences in their expression. Mir-193b and mir-144 were more expressed in males (dCt = 9.1 ± 1.5 and 10 ± 1.3 respectively) compared to females (dCt = 9.7 ± 1.4 and 10.6 ± 1.3 respectively) (p < 0.05).

Table 3-5: Characteristics of the study population grouped by Sex

	Male n = 119 Mean (SD)	Female n = 35 Mean (SD)	*P*-value
Demographic Characteristics			
Age (years)	64 (8)	63 (8)	0.438
Smoking history			
Current	18 (15%)	14 (40%)	**0.001**
Former	60 (50%)	18 (18%)	
Never	41 (35%)	3 (9%)	
Blood pressure (mm Hg)	140/80 (19.1/11.5)	140/77 (19.9/7.2)	0.942/0.088
Anthropometric Characteristics			
Body weight (kg)	91.0 (11.3)	75.4 (13.3)	**< 0.001**
Body mass index (kg/m^2) *	30.0 (27.0 - 32.4)	29.2 (26.1 - 34.7)	0.527
Waist circumference (cm)	106.3 (8.3)	98.1 (10.2)	**< 0.001**
Body fat (kg) *	30.7 (27.8 - 33.0)	42.6 (38.5 - 46.1)	**< 0.001**
Biochemical Characteristics (Fasting)			
Glucose (mmol/L)	5.3 (0.5)	5.0 (0.6)	**0.003**
Insulin (pmol/L) *	10.1 (7.2 - 14.3)	9.5 (7.3 - 12.9)	0.501
HOMA-IR*	2.4 (1.6 - 3.4)	2.2 (1.6 - 2.8)	0.207
HbA1c (mmol/mol)	38.6 (36 - 41)	38.1 (37 - 40)	0.433
Adiponectin (µg/mL) *	5.1(3.8 - 7.2)	8.6(5.5 - 11.8)	**< 0.001**
Triglyceride (mmol/L) *	1.4 (1.1 - 1.9)	1.7 (1.1 - 2.3)	0.152
Total Cholesterol (mmol/L) *	4.2 (3.5 - 4.6)	4.6 (4.1 - 4.9)	**0.003**
HDL (mmol/L) *	1.1 (1.0 - 1.4)	1.1 (1.0 - 1.3)	0.466
Alanine aminotransferase (U/L) *	26.0 (21 - 34)	25.8 (15 - 29)	**0.028**
γ-glutamyl transferase (U/L) *	37.6 (23 - 58)	30.0 (21 - 43)	0.182
Inflammatory markers (Fasting)			
High sensitivity CRP (mg/L) *	1.7 (0.8 - 3.3)	2.5 (1 - 4.6)	0.114
WBC (x10^9 /L) *	6.5 (5.7 - 8.0)	7.0 (5.9 - 7.5)	0.698
Circulating miRNA Expression			
mir-222 (dCt)	3.93 (1.7)	4.03 (1.5)	0.926
mir-221 (dCt)	1.70 (1.6)	2.00 (1.8)	0.689
mir-144 (dCt)	10.00 (1.3)	10.60 (1.3)	**0.049**
mir-155 (dCt)	8.00 (1.2)	8.20 (1.3)	0.389
mir-192 (dCt)	8.80 (1.6)	9.20 (1.5)	0.166
mir-193b (dCt)	9.10 (1.5)	9.70 (1.4)	**0.032**

Categorical variables were summarised as number (%).
Variables not normally distributed were summarised as median (inter-quartile range).
HOMA-IR: Homeostatic model assessment for insulin resistance, **HDL:** high density lipoprotein, **CRP:** high sensitivity C reactive protein, **WBC:** white blood cells, **mir:** micro RNA, **dCt:** delta cycle threshold.

3.5.3 General characteristics of the study population grouped by HOMA-IR

The studied population was also classified as being either insulin sensitive (IS) or insulin resistance (IR) based on HOMA-IR. The HOMA-IR cut-off point chosen to make this distinction was HOMA-IR ≤ 2 for insulin sensitive (IS) and HOMA-IR >2 for insulin resistance (IR). Table 3-6 summarises the general characteristics of the two groups (IS, n = 59 and IR, n = 95).

There were no differences (p < 0.05) in age, sex, smoking history, and blood pressure between the two groups. As expected, there was a difference in all the anthropometric measurements between the two groups (p < 0.001*)* with the insulin resistance group (IR) having higher anthropometric values, e.g., Weight (IR = 90.5 kg ± 13.2 and IS = 82.4 kg ± 12.4, p = < 0.001). Total adiponectin concentration was lower in IR group compared to IS counterparts [IR = 5.0 µg/ml (3.5-7.5) and IS = 6.2 µg/ml (5.0-10.3), p < 0.05]. In this study, there was no difference in all lipids profile measured between the two groups (TAG, HDL, and TC). When comparing inflammatory markers between the two groups, there was no difference in CRP while WBC concentration was slightly higher in IS sensitive group (IS = 6.9 mg/L (6.1-8.6) compared to IR = 6.4 mg/L (5.6-7.5), p < 0.05).

Out of all the circulating miRNAs included in this study, mir-144 was the only miRNAs whose expression was different between the two groups. Mir-144 expression was higher in the IS group compared to the IR group (p < 0.05*)*.

Table 3-6: Characteristics of the Study population group according to their insulin sensitivity

	IS (HOMA < 2) n = 59 Mean (SD)	IR (HOMA > 2) n = 95 Mean (SD)	*P-value*
Demographic Characteristics			
Age (years)	64 (9)	63 (8)	0.683
Gender			
Male	43 (73%)	76 (80%)	0.305
Female	16 (27%)	19 (20%)	
Smoking history			
Current	14 (24%)	18 (19%)	0.436
Former	26 (44%)	52 (55%)	
Never	19 (32%)	25 (26%)	
Blood pressure (mm Hg)	140 / 80 (20 / 18)	139 / 79 (19 / 11)	0.738/0.449
Anthropometric Characteristics			
Body weight (kg)	82.4 (12.4)	90.5 (13.2)	**< 0.001**
Body mass index (kg/m^2) *	28.8 (26.2 -31.4)	30.8 (27.9 -33.6)	**< 0.001**
Waist circumference (cm)	101 (8.6)	107 (9.6)	**< 0.001**
Body fat (kg) *	25.2 (21.1-30.3)	29.3 (24.7-35.0)	**< 0.001**
Biochemical Characteristics (Fasting)			
Glucose (mmol/L)	5.0 (0.53)	5.4 (0.53)	**0.001**
Insulin (pmol/L) *	6.8 (5.3-7.7)	12.4 (10.3-16.3)	**0.001**
HbA1c (mmol/mol) *	38.1 (36-39)	38.7 (37-41)	0.267
Adiponectin (µg/mL) *	6.2 (5.0-10.3)	5.0 (3.5-7.5)	**0.01**
Triglyceride (mmol/L) *	1.7 (1.2-2.2)	1.4 (1.1-1.9)	0.382
Total Cholesterol (mmol/L) *	4.4 (3.7-4.7)	4.2 (3.6-4.7)	0.349
HDL (mmol/L) *	1.1 (1.0-1.4)	1.1 (1.0-1.3)	0.976
Alanine aminotransferase (U/L) *	22.0 (18-31)	26.0 (20-35)	0.338
γ-glutamyl transferase (U/L) *	29.0 (20-50)	38.0 (26-59)	0.072
Inflammatory markers (Fasting)			
High sensitivity CRP (mg/L) *	1.7 (0.8-3.5)	2.0 (0.93-3.5)	0.245
WBC (x10^9 /L) *	6.9 (6.1-8.6)	6.4 (5.6-7.5)	**0.022**
Circulating miRNA Expression			
mir-222 (dCt)	3.94 (1.3)	3.96 (1.9)	0.826
mir-221 (dCt)	1.83 (1.6)	1.72 (1.6)	0.793
mir-144 (dCt)	10.42 (1.4)	9.98 (1.3)	**0.049**
mir-155 (dCt)	8.13 (1.1)	7.95 (1.3)	0.476
mir-192 (dCt)	9.16 (1.6)	8.80 (1.6)	0.192
mir-193b (dCt)	9.37 (1.4)	9.11 (1.6)	0.368

#: Categorical variables were summarised as number (%).
***:** Continuous variables not normally distributed were summarised as median (inter-quartile range).
HOMA-IR: Homeostatic model assessment for insulin resistance, **HDL:** high density lipoprotein, **CRP:** high sensitivity C reactive protein, **WBC:** white blood cells, **mir:** micro RNA, **dCt:** delta cycle threshold.

3.5.4 Correlation between Circulating miRNAs Expression and Markers of Metabolic Disturbance

To determine the association of the selected circulating miRNAs (mir-222, mir-221, mir-192, mir-193b, mir-144, and mir155) with markers of metabolic disturbance in a high-risk population for developing T2DM, a cross-sectional analysis using Pearson correlation was done. Results are shown in Table 3-7 (for anthropometrics measurements), Table 3-8 (for markers of insulin sensitivity), Table 3-9 (for liver enzymes), and Table 3-10 (for inflammatory markers).

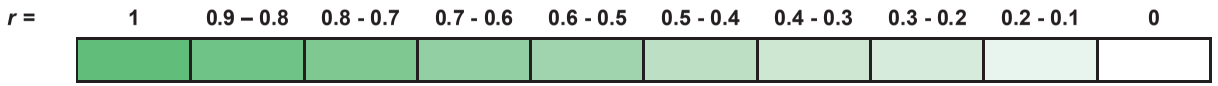
3.5.4.1 Circulating miRNAs Expression and Anthropometrics Variables

Several circulating miRNAs have been identified in obesity and found to be strongly associated with adiposity measurements such as BMI, total fat mass, and waist circumference. The relationship between the expression of the candidate miRNAs and some of the adiposity biomarkers (weight, BMI, body fat, and waist) was tested and results are shown in Table 3-7. There was no association between these anthropometric markers and the expression of the chosen circulating miRNAs.

Table 3-7: Pearson correlation of anthropometric variables with circulating miRNAs expression at baseline

	mir-221	mir-222	mir-192	mir-193b	mir-155	mir-144	Weight	BMI	TBF	WC
mir-221	1									
mir-222	0.619**	1								
mir-192	0.725**	0.487**	1							
mir-193b	0.602**	0.495**	0.801**	1						
mir-155	0.739**	0.596**	0.698**	0.611**	1					
mir-144	0.494**	0.405**	0.561**	0.492**	0.562**	1				
Weight	- 0.124	- 0.048	- 0.111	- 0.06	- 0.073	- 0.136	1			
BMI	- 0.066	- 0.035	- 0.032	0.048	- 0.004	- 0.077	0.715**	1		
BF	- 0.085	- 0.076	0.024	0.117	- 0.029	- 0.003	0.609**	0.892**	1	
WC	- 0.073	- 0.038	0.018	0.033	- 0.03	- 0.112	0.861**	0.746**	0.655**	1

dCt: delta cycle threshold, **BMI:** body mass index, **TBF:** total body fat, **WC:** waist circumference, ***r:*** Pearson Correlation Coefficient, ** $p < 0.01$



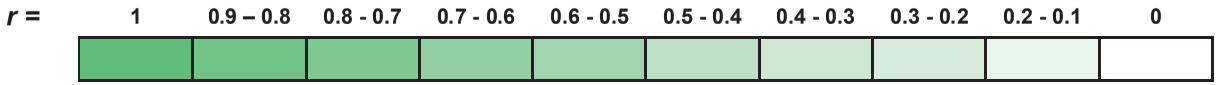
3.5.4.2 Circulating miRNAs with markers of insulin sensitivity

Table 3-8 demonstrates the correlation between miRNAs expression and markers of insulin sensitivity. Four miRNAs (mir-192, mir-193b, mir-155, and mir-144) were positively correlated with glucose concentration with mir-144 showing the strongest correlation with glucose (r = 0.251, p < 0.01). Out of all the six miRNAs included in our study, mir-144 was the only miRNA that showed positive correlation with insulin and HOMA-IR (r = 0.184, p < 0.05, and r = 0.207, p < 0.01, respectively). Total adiponectin concentration showed negative correlation with the expression of mir-221, mir-192 and mir-193b (r = 0.205, 0.179, and 0.189 respectively, p < 0.05). The expression of mir-221 and mir-222 were positively correlated with only HbA1c (r = 0.202, p < 0.05 and r = 0.245, p < 0.01, respectively). Other miRNAs (mir-193b, and mir-155) also showed positive associations with HbA1c (r = 0.17 and 0.175, respectively, p < 0.05).

Table 3-8: Pearson correlation of insulin sensitivity markers vs. circulating miRNAs expression at baseline

	dCt mir-221	dCt mir-222	dCt mir-192	dCt mir-193b	dCt mir-155	dCt mir-144	INS	FBG	HOMA-IR	HbA1C	Adipo
dCt mir-221	1										
dCt mir-222	0.619**	1									
dCt mir-192	0.725**	0.487**	1								
dCt mir-193b	0.602**	0.495**	0.801**	1							
dCt mir-155	0.739**	0.596**	0.698**	0.611**	1						
dCt mir-144	0.494**	0.405**	0.561**	0.492**	0.562**	1					
INS	- 0.052	- 0.073	- 0.115	- 0.124	- 0.096	0.184*	1				
FBG	- 0.135	- 0.033	- 0.187*	- 0.175*	- 0.165*	0.251**	0.207*	1			
HOMA-IR	- 0.072	- 0.086	- 0.138	- 0.152	- 0.121	0.207*	0.987**	0.338**	1		
HbA1C	- 0.202*	- 0.245**	- 0.147	- 0.17*	- 0.175*	0.141	0.228**	0.358**	0.267**	1	
Adipo	0.205*	0.102	0.179*	0.189*	0.152	0.019	- 0.269**	- 0.125	- 0.267**	- 0.162*	1

dCt: delta Ct, **INS:** insulin, **FBG:** fasting blood glucose, **HOMA-IR**: homeostatic model assessment for insulin resistance, **Adipo:** Adiponectin
* $p < 0.05$, ** $p < 0.01$



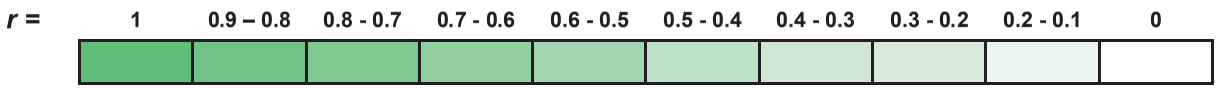
3.5.4.3 Circulating miRNAs and liver enzymes

Table 3-9 demonstrates the correlation between liver enzymes GGT and ALT with the expression of the selected miRNAs. All the six miRNAs expressions, except for mir-222, were positively correlated with liver enzymes with mir-192 and mir-193b showing the strongest association with GGT (r = 0.278 and 0.402 respectively, p < 0.01) and ALT (r = 0.399 and 0.616 respectively, p < 0.01).

Table 3-9: Pearson correlation of liver enzymes with circulating miRNAs expression at baseline

	dCt mir-221	dCt mir-222	dCt mir-192	dCt mir-193b	dCt mir-155	dCt mir-144	GGT	ALT
dCt mir-221	1							
dCt mir-222	0.619**	1						
dCt mir-192	0.725**	0.487**	1					
dCt mir-193b	0.602**	0.495**	0.801**	1				
dCt mir-155	0.739**	0.596**	0.698**	0.611**	1			
dCt mir-144	0.494**	0.405**	0.561**	0.492**	0.562**	1		
GGT	- 0.236**	- 0.103	- 0.278**	- 0.402**	0.225**	- 0.159*	1	
ALT	- 0.227**	- 0.143	- 0.399**	- 0.616**	- 0.138	- 0.156	0.342**	1

dCt: delta Ct, **GGT:** Gamma-Glutamyl Transferase, **ALT**: Alanine Aminotransferase
* $p < 0.05$, ** $p < 0.01$



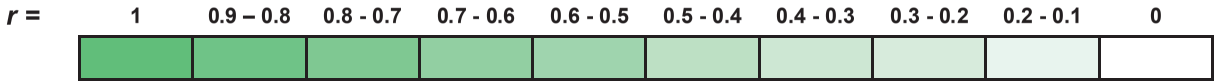
3.5.4.4 Circulating miRNAs and inflammatory biomarkers

In this study, CRP and WBC were used as markers for inflammation. Table 3-10 shows the correlation between the expression of the six selected circulating miRNAs and both CRP and WBC. None of the candidate miRNAs chosen were associated with CRP or WBC concentration in the current sample study.

Table 3-10: Pearson correlation of inflammatory markers with miRNAs expression at baseline

	dCt mir-221	dCt mir-222	dCt mir-192	dCt mir-193b	dCt mir-155	dCt mir-144	CRP	WBC
dCt mir-221	1							
dCt mir-222	0.619**	1						
dCt mir-192	0.725**	0.487**	1					
dCt mir-193b	0.602**	0.495**	0.801**	1				
dCt mir-155	0.739**	0.596**	0.698**	0.611**	1			
dCt mir-144	0.494**	0.405**	0.561**	0.492**	0.562**	1		
CRP	0.046	0.04	0.029	0.022	- 0.036	- 0.083	1	
WBC	- 0.135	- 0.127	0.011	- 0.027	- 0.017	- 0.044	0.111	1

dCt: delta Ct, **CRP:** C-reactive protein, **WBC:** white blood cells
** $p < 0.01$



3.5.5 Multiple linear regression analysis

Multiple linear regression analysis was used to determine whether the above associations between the expression of circulating miRNAs (dependent variable) and independent variables of interest remained significant after adjusting for other confounding independent variables, age, sex, and body fat. I elected to only show significant associations for clarity; miRNA that had no unadjusted association with metabolic markers were omitted from relevant tables.

Table 3-11 shows the association of the expression of circulating mir-144 with markers of insulin sensitivity (FBG, insulin, and HOMA-IR). In models adjusting for age, sex, and body fat, FBG showed the strongest association with the expression of circulating mir-144. For a 1mmol/L increase in fasting plasma glucose (FBG), the expression of circulating mir-144 was 35% (95% CI = 20% - 50%, $p < 0.01$) elevated. Similarly, higher HOMA-IR and fasting insulin were positively associated with the expression of circulating mir-144, for every unit increase in HOMA-IR, the expression of circulating mir-144 was 10.2% (95% CI = 2.5% - 17.2%, $p < 0.05$) higher and for every 1 pmol/L increase in insulin the expression of circulating mir-144 was 2% elevated. Adjustment for age, gender, and body fat did not attenuate these associations. The expression of the other circulating miRNAs showed variable associations with markers of insulin resistance and glycaemia, but none of the expression of the chosen circulating miRNAs was associated with markers of adiposity.

Table 3-11: Linear regression model showing the associations of insulin sensitivity markers vs. mir-144 expression at baseline. Effect estimates are % effect on miRNA dCt values 95% CI).

miRNA	Predictor	Model 1	Model 2	Model 3
		B (95% CI)	B (95% CI)	B (95% CI)
mir-144	FBG (per 1mmol/L)	35%** (20 – 50)	32%** (10 – 48)	32%* (9 – 49)
	Insulin (per 1pmol/L)	2%* (0.1 – 5)	2%* (0.1 – 4)	2%* (0.1 – 5)
	HOMA-IR (per 1 unit)	10%* (3 – 17)	10%* (2 – 16)	10%* (2 – 17)

Model 1: No adjustment
Model 2: adjustment for age and sex
Model 3: adjustment for age, sex and body fat
CI = confident interval, **B** = regression coefficient, **FBG** = fasting blood glucose.
* $p < 0.05$, ** $p < 0.01$

Additionally, the expression of circulating mir-192 and 193b showed strong associations with liver enzyme. In models adjusting for age, gender, and body fat, these association remined strong (Table 3-12). For every 10-unit increase in ALT, the expression of circulating mir-192 was 28% (95% CI = 16% - 38%, $p < 0.001$) elevated and the expression of circulating mir-193b was 40% (95% CI = 30% - 50%, $p < 0.001$). For every 10-unit increase in GGT, the expression of circulating mir-192 (95% CI = 3% - 10%, $p < 0.001$) was 10% higher and the expression of circulating mir-193b was 10% (95% CI = 6% - 10%, $p < 0.001$) higher.

In the previous section, total adiponectin was negatively correlated with the expression of circulating mir-221, mir-192, and mir-193b. Multiple linear regression models adjusting for age, gender, and body fat were done (Table 3-13). The expression of circulating mir-221 was inversely associated with adiponectin concentration. This association remained significant even after adjusting for age, sex, and body fat. In contrast, the expression of circulating mir-192 and mir-193 were no longer related to adiponectin in multivariable models after further adjustment for the same confounding factors.

Table 3-12: Linear regression model showing the associations of liver enzymes vs. mir-192 and 193b expression at baseline. Effect estimates are % effect on miRNA dCt values.

miRNA	Predictor	Model 1	Model 2	Model 3
		B (95% CI) p-value	B (95% CI) p-value	B (95% CI) p-value
mir-193b	GGT (per 10 U/L increase)	10%*** (6 – 10)	10%*** (5 – 10)	10%*** (5 – 10)
	ALT (per 10 U/L increase)	40%*** (30 – 50)	40%*** (30 – 50)	40%*** (30 – 50)
mir-192	GGT (per 10 U/L increase)	10%*** (0.1 – 10)	10%*** (0.1 – 10)	10%*** (0.1 – 10)
	ALT (per 10 U/L increase)	28%*** (16 – 38)	27%*** (30 – 50)	29%*** (19 – 40)

Model 1: No adjustment
Model 2: adjustment for age and sex
Model 3: adjustment for age, sex and body fat
*** $p < 0.001$
GGT: Gamma-Glutamyl Transferase, ALT: Alanine Aminotransferase

Table 3-13: Linear regression model showing the associations of circulating adiponectin vs. mir-221, mir-192, and mir-193b at baseline. Effect estimates are % effect on miRNA dCt values.

miRNA	Predictor	Model 1	Model 2	Model 3
		B (95% CI) p-value	B (95% CI) p-value	B (95% CI) p-value
mir-221	Adiponectin (per 1µg/mL)	5 %* (1 – 9)	5 %* (0.6 – 9)	5 %* (0.6 – 10)
mir-192		4 %* (0.4 – 8)	3 % (0.1 – 7)	4 % (0.1 – 8)
mir-193b		4 %* (0.7 – 8)	3 % (0.3 – 7)	3 % (0.3 – 7)

Model 1: No adjustment
Model 2: adjustment for age and sex
Model 3: adjustment for age, sex and body fat
* $p < 0.05$

3.6 DISCUSSION:

The present study was conducted to investigate cross-sectional associations of the expression of targeted circulating miRNAs (mir-222, mir-221, mir-192, mir-193b, mir-144, and mir-155) with metabolic health markers, using a large cross-sectional design. It also presents a framework for quality control of circulating miRNA measurement in large scale studies.

3.6.1 Analytical and pre-analytical Conditions for miRNAs expression

Despite their promising potential use in clinical application, there are several problems that prevent the use of circulating miRNAs as diagnostic tools for now. One of the problems is the lack of a standardised protocol; each study used different samples, different collection methods, and different profiling methodologies. Additionally, there is no proper control to normalise the circulating miRNAs expression. Finally, most studies published have been carried out using small numbers of participants. These factors can contribute to the variation seen in results among different studies. Before beginning this study, a specific protocol was planned to standardise both preanalytical and analytical variables as much as possible to make sure that the results are reliable.

For samples a decision was made to use samples from the CAMERA trial because it was a well-defined large sample of participants (154) who were subjected to a strict inclusion/exclusion criterion, and although the participants didn't have diabetes, they were high risk participants (previous CHD and high WC) who will be good candidates for exploring the expression of circulating miRNAs.

A hypothesis-driven approach was used to select the candidate circulating miRNAs to include in this study instead of exploratory approach because it is more focused, more efficient, require less time, and cheaper which makes it more suitable for large studies. This led to a decision to include six circulating miRNAs in this study based on previous exciting evidence that changing in weight, glycemia and inflammation affect their expression (mir-222, mir-221, mir-192, mir-193b, mir-155, and mir-144.

For starting material, I decided to use EDTA plasma samples from the CAMERA trail were decided to be used and not whole blood because cellular fraction (white and red blood cells) can add and influence the total amount of circulating miRNAs. Moreover, haemolysed samples were excluded because haemolysis can alter plasma miRNA biomarker levels by up to 50-fold (Pritchard *et al.*, 2012). Unnecessary freezing and thawing were avoided as much as possible despite the fact that circulating miRNAs are found to be stable in harsh conditions (Sohel, 2016). For total RNA extraction, miRNeasy Mini kit from Qiagen was used as it produces the highest yield of total RNA compared to another kit (Sourvinou, Markou & Lianidou, 2013). Quantification reverse transcription polymerase chain reaction (qRT-PCR) was used for the measurement of miRNAs expression cause it is one of the well-established methods for the quantification of circulating miRNAs and considered as the golden standard method (Sohel, 2016). Beside unlike microarray, it is more suitable to use for our approach (hypothesis-driven). The final challenge was choosing an appropriate control to normalise the expression of circulating miRNAs. A trial run was conducted to test the two types of controls, endogenous (mir-520d-5p) and exogenous (cel-mir-39) using eight random samples. The endogenous control results showed low and inconsistent expression although it was reported to be an excellent housekeeping control candidate for measuring plasma miRNA due to its consistent results and a very narrow standard deviation (Rice *et al.*, 2014). Alternatively, the exogenous (cel-miR-39) results, which is one of the widely accepted controls for circulating miRNA, showed generally acceptable variation between the triplicates (SD = 0.15, CV = 0.6%) and between the 8 samples (SD = 0.2, CV = 0.8%) (Table 3-2). These CVs should be interpreted as a guide due to the exponential nature of the Ct value. According to the results from conducting a trail, a decision was made to exclude mir-520d-5p and use mir-cel-39 as a control.

After that, the expression of mir-39 in the entire study samples (308 plasma samples) was measured (Table 3-3), and the results showed low variation between the duplicates (SD = 0.11%, CV = 0.45%) and more variation between the samples (SD = 0.88%, CV = 3.5%). Although the inter-assay CV between the samples is low (3.54%) and acceptable (< 15%), it still showed more variation between runs compared to intra-assay (SD > 0.25). One reason for this variation is batch effect, which represents a common source of variability (Marabita *et al.*, 2016), especially

when dealing with a large number of samples (308 extractions). Another reason could be the effect of RNases. Despite the presence of RNase activity in plasma, endogenous circulating miRNAs exist in a form that is protected from its effect, suggesting that they are found in a protective form. Some studies showed that circulating miRNAs remain stable even after boiling, high or low pH, prolonged storage time and multiple freeze-thaw cycles (Gilad *et al.*, 2008; Moldovan *et al.*, 2014; Shaffer, Schlumpberger & Lader, 2012). It is likely that separation of plasma from cells is the critical pre-analytical variable. By contrast, synthetic external miRNAs added directly to plasma were naked, sensitive, not stable, and can be degraded rapidly (Mitchell *et al.*, 2008). To assure that the test run was valid, and results were reliable, a decision was made to include quality control (QC) samples in each run. The QC sample results were stable and reproducible in all the miRNAs measured (Table 3-4).

3.6.2 The association between circulating miRNAs expression and markers of metabolic disturbance

3.6.2.1 Circulating miRNAs Expression and Anthropometrics Variables

In this study, none of the six selected circulating miRNAs were associated with anthropometric measurements (weight, BMI, waist, and body fat). It was expected to observe the association of circulating mir-222 and mir-221 with markers of adiposity since mir-222 has been reported to be highly expressed in obese patients while mir-221 was low (Ortega *et al.*, 2013). Surprisingly, the observation of similar expression was not seen in this study. The reason for this could be because the finding of Ortega group was in morbid obesity (mean BMI ≥ 40) while our study population mean BMI was 30.

3.6.2.2 Circulating miRNAs with markers of insulin sensitivity

The key finding of the present study was that among the six circulating miRNAs included, the expression of mir-144 was the only circulating miRNA to display a positive association with HOMA-IR and insulin. Also, among the six selected circulating miRNAs, mir-144 showed the highest association with glucose concentration. All these associations remained significant even after adjusting for age, gender, and body fat.

The level of circulating mir-144 has been found to be increased in patients with T2DM. A meta-analysis of controlled profiling studies showed that the expression of 40 miRNAs were dysregulated in T2DM in several tissues (blood, liver, pancreas, muscle, adipose tissue, and glomeruli) (Zhu & Leung, 2015). One of these 40 miRNAs were circulating mir-144, which was upregulated in human T2DM blood samples. Additionally, Yang *et al*. showed that the expressions of platelet and plasma mir-144 were altered in T2DM patients with (n = 56) or without (n = 58) ischemic stroke compared to healthy controls (n = 30) (Yang *et al.*, 2015). Moreover, mir-144 appear to contribute to the regulation of insulin signalling. This was supported by the findings of Karolina *et al.*, which showed that elevated circulating mir-144 correlate with the downregulation of insulin receptor substrate 1 (IRS1) at both mRNA and protein levels. Although the participants in this study did not have diabetes, all of them were high-risk patients for developing T2DM, and most of them were IR thus highlighting the potential plasma mir-144 as a key player in insulin resistance which could develop to diabetes over time. This study provides additional evidence for the role of plasma mir-144 in the pathogenesis of T2DM and the possibility of using it as a diagnostic biomarker.

Wang *et al*. reported a higher expression of circulating miR-144 seems to be ethnicity related; it was only associated with T2DM in native Sweden (Caucasian, n = 68) and not Iraqis patients (Arabs, n = 84) (Wang *et al.*, 2014). In this study, ethnicity was not a factor since all participants were Caucasians.

MiR-221 and miR-222 are two highly homologous miRNAs that have been studied extensively in human malignancies (Song *et al.*, 2017). Few studies have associated the alteration in their expression with diabetes. Ortega *et al*. reported that circulating mir-222 was highly expressed in Caucasian male patients with T2DM (n = 48) compared to patients with normal glucose tolerance (n = 45) and were associated with fasting glucose and HbA1c (Ortega *et al.*, 2014). Also, insulin administration rapidly altered the expression of circulating mir-222. Shi *et al*. also reported higher mir-222 expression in the omental adipose tissue of Chines women with gestational diabetes (n = 13) at the time of caesarean delivery compared with pregnant women with normal glucose tolerance (n = 13) (Shi *et al.*, 2014). The authors further used 3T3-L1 adipocytes to demonstrate that stimulation with a relatively high concentration of oestrogen increased mir-222 expression and seems

to negatively regulate adipose insulin sensitivity via suppression of oestrogen receptor α (ER-α) protein and insulin-sensitive membrane transporter glucose transporter 4 (GLUT4) protein. In another study done by Coleman *et al*. showed that the levels of mir-221 and mir-222, measured in whole tissue homogenates of the internal mammary artery (IMAs) taken from 73 patients undergoing coronary artery bypass surgery, were higher in patients with diabetes compared to normal controls and it remained significantly associated even after adjustment for age, body mass index, and serum lipids (Coleman *et al.*, 2013). Additionally, Meerson and colleagues reported a positive correlation between the expression of mir-221 in subcutaneous adipose sample and BMI, glucose, and insulin in 29 Pima Indian participants without diabetes (Meerson *et al.*, 2013).

Similar association with both mir-222 and mir-221 and glucose, insulin, and HOMA-IR were not observed in this study. Such disparity between the results could be explained by multiple reasons. Firstly, different population studied. The association found between mir-222 and mir-221 and markers of glycemia was in patients with diabetes, whereas the population in this study included patients without diabetes. Secondly, different ethnicity. For example, Meerson *et al*. study population were Pima Indian participants not suffering from diabetes, one of the highest prevalence of diabetes and obesity of any population in the world (Pearson, 2015), while the participants in this study were Caucasians. A previous study showed that racial/ethnic differences in circulating miRNAs expression between ethnic groups (Wang *et al.*, 2014). Thirdly, different samples (tissue vs. plasma). It is still not known whether miRNAs extracted from cellular or extracellular sample should be utilized interchangeably, and it remains unknown if expression from these distinct sources is suitable. In a large human cohort (2,391 participants) done by Shah *et al*. showed that whole blood and plasma miRNA expression is distinct and that miRNA expression from different human sources should not be used interchangeably as biomarkers of disease (Shah *et al.*, 2016). For example, in Coleman *et al*. study, cellular miRNAs (IMAs tissues) were used to measure the expression of miRNAs, which probably demonstrate local intracellular function, while circulating miRNAs (plasma samples) were used in this study which may demonstrate systemic function. Finally, the different methodology used to process samples and measure the expression of miRNAs, which is considered the major factor for the inconsistency between studies.

Meanwhile, the association found in this study between both mir-222 and mir-221 and HbA1c was similar to Ortega *et al.* findings (Ortega *et al.*, 2014). Glycated haemoglobin (HbA1c) has been considered as a good indicator of overall glycaemic control in 2-3 months. Studies have shown the possibility of using it as a surrogate marker for insulin resistance (Borai *et al.*, 2011; Saha & Schwarz, 2017) which can explain why there was an association between mir-222 and HbA1c and not glucose in the current study.

Adiponectin is an insulin-sensitising and anti-inflammatory adipocytokine, which is considered as a potential mediator between obesity, insulin resistance, and diabetes (Lindberg *et al.*, 2017; Weber *et al.*, 2017). It has been shown that mir-221 may regulate IR via effects on adiponectin receptor 1 (ADIPOR1). Meerson *et al.* displayed that mir-221 directly downregulates ADIPOR1 expression suggesting that it may be possible that obesity-associated upregulation of mir-221 can result in a decrease in ADIPOR1, which may, in turn, lead to the development of obesity-related metabolic consequences such as insulin resistance or T2DM (Meerson *et al.*, 2013). In another study done by Hwang *et al.*, showed that there was a negative correlation between mir-221 expression and ADIPOR1 (Adiponectin receptor 1) mRNA expression in breast tumours as well as in breast cancer cell lines (Hwang *et al.*, 2013). In the present study, total plasma adiponectin was negatively associated with the expression of circulating mir-221. In multiple linear regression models adjusting for age, gender, and body fat didn't change the association. The result demonstrates that the association between circulating adiponectin and the expression of circulating mir-221 is independent of obesity.

Upregulation of the expression of circulating mir-192 and mir-193b in the pre-diabetes state was previously reported by Párrizas *et al.* (Párrizas *et al.*, 2015). Their study included 92 participants (29 healthy control, 22 pre-diabetes with impaired fasting glucose (IFG), 21 prediabetes with impaired glucose tolerance (IGT), and 20 newly diagnosed T2DM). Out of the 176 circulating miRNAs they tested (most frequently detected in human serum/plasma), circulating levels of three miRNAs (mir-150, mir-192, and mir-193b) achieved high levels in both the IFG and IGT groups but were not altered in patients with T2DM. Interestingly, they also found that mir-192 and mir-193b decrease to normal in insulin resistant humans after therapeutic lifestyle intervention consisting of a monitored exercise

program and diet recommendations. Also, Jaeger *et al.* did a longitudinal study where they assessed the association of four circulating miRNAs, including mir-192 with incident T2DM (Jaeger *et al.*, 2018). The study included 213 patients from the Vorarlberg Institute for Vascular Investigation and Treatment (VIVIT) cohort who didn't have T2DM at baseline and followed for six years. Patients who developed diabetes (n = 35) had higher levels of mir-192 compared to those who did not (n = 178). In agreement with previous findings, the results of this study showed that both mir-192 and mir-193b were positively correlated with glucose in patients without diabetes but high-risk, and most of them were IR. In contrast to the findings of the present study, Ortega *et al.* reported decreased levels of mir-192 in the serum of patients with T2DM (Ortega *et al.*, 2014).

Furthermore, the expression of mir-155 was positively associated with glucose, HbA1c. In a study done by Lin *et al.*, showed that downregulation of mir-155 levels was found in serum from patients with T2DM (n = 30) compared to healthy controls and showed a negative correlation with HOMA-IR suggesting that mir-155 might be involved in glucose homeostasis and insulin action. (Lin *et al.*, 2016). Moreover, mir-155 expression in PBMCs (peripheral blood mononuclear cells) from patients with T2DM (n = 20) was decreased compared to normal controls (n =20) (Corral-Fernández *et al.*, 2013). A possible reason for these variations could be the different population studied and different samples used.

3.6.2.3 Circulating miRNAs and liver enzymes

In this study, the expression of both mir-192 and mir-193b were associated with liver enzymes (ALT and GGT) independent of age, sex, and body fat. Párrizas *et al.* also reported a good correlation between mir-192, mir-193b and FLI (Fatty Liver Index, a mathematical index calculated from blood TAG, BMI, waist circumference, and hepatic enzyme GGT and has been used as a substitution for hepatic steatosis). Jaeger *et al.* reported a positive correlation between serum levels of mir-192 and AST, ALT, and GGT using baseline samples.

3.6.2.4 Circulating miRNAs and inflammatory biomarkers

Increased levels of mir-155 have been associated with various inflammatory diseases, including rheumatoid arthritis (RA) (Alivernini *et al.*, 2018), and it is

considered as a potential regulator of inflammation. It plays a key role in the immune response, and studies have demonstrated its involvement in the development of atherosclerosis (Li *et al.*, 2016; Faccini *et al.*, 2017). C-reactive protein (CRP) is a sensitive, nonspecific marker for systemic inflammation and a powerful predictor for cardiovascular diseases (Koenig, 2013). A recent study included 132 patients who underwent coronary angiography to measure coronary flow, 66 patients with coronary slow flow and 66 patients with normal coronary flow showed that plasma mir-155 was positively correlated with CRP (Su, Yang & Li, 2018). In the present study, there was no association between mir-155 and both CRP and WBC although. A possible reason for these variations could be the different population studied and different samples used.

3.6.3 Circulating miRNAs as novel minimally invasive biomarkers

Data presented in this study showed significant associations between the selected circulating miRNAs and markers of metabolic health. These circulating miRNAs appears to be promising biomarkers for metabolic. Importantly, the miRNAs had specific associations with different metabolic biomarkers suggesting that they are not providing overlapping information. Particularly, mir-144 which showed a specific association with insulin and HOMA-IR. This is crucial information which should be incorporated into future study design. Further prospective evaluation of these circulating miRNAs, diabetes and other metabolic diseases is needed, to validate these findings and to further explore the exciting potential of circulating miRNAs to emerge as clinically useful novel biomarkers for metabolic diseases.

3.7 Conclusion

This study offers one of the first sets of sizeable data using circulating miRNAs as reproducible biomarkers, with acceptable quality control, in stored plasma samples. This provides a platform for further work on the possibility of using them as biomarkers in future.

The present study showed broadly expected cross-sectional associations of the six selected circulating miRNAs (mir-222, mir-221, mir-192, mir-193b, mir-144, and mir-155) with biomarkers of metabolic risk in a population with coronary heart disease but without diabetes. These data support the putative utilities of miRNAs as reproducible biomarkers; however, there is still a long way to go to prove their usefulness in clinical practice as diagnostic biomarkers. Inconsistency in results from different studies making it difficult to interpret and compare various investigations. Standardization of both preanalytical and analytical procedures must be implemented for the analysis of circulating miRNAs before their use as a diagnostic tool. Also, a large cohort of patients is needed in future studies.

3.7.1 Strength and limitation

One of the strong features of this study is the use of CAMERA trial samples. The CAMERA trail was a high-quality study with several strengths. Firstly, its design as a randomised placebo-controlled double-blind trial, which is considered as the 'gold standard' in evidence-based medicine for evaluating the effects of an intervention. Secondly, a well-defined large sample of participants (154) who were subjected to a strict inclusion/exclusion criterion. Finally, the well standardized approach to sample collection (from a single local centre) and sample handling (quickly processed and stored in $-80°C$). Besides, using a robust planned method to measure the expression of circulating miRNAs.

There were also limitations to our study. First, the lack of endogenous control to normalize miRNAs expression. As known, the optimal method for normalisation of gene expression is to use a constantly expressed housekeeping gene, because they represent endogenous controls that are affected by the same source of variability as the target gene. However, using this method on plasma/serum samples is challenging because very few studies are available and different studies reported

different miRNA reference genes (Moldovan *et al.*, 2014; Shaffer, Schlumpberger & Lader, 2012). Alternatively, I used a spiked-in exogenous (cel-miR-39) control to normalize our results. Although it is one of the widely accepted controls for circulating miRNA, it does not correct for variation and quality of the sample (Schwarzenbach, Calin & Pantel, 2016). Second, the study is a cross-sectional analysis, and this type of study does not suggest a cause and effect relation. To make the case for use of circulating miRNA as clinical biomarkers, prospective studies would be required to establish whether the dysregulation of the expression of the studied miRNAs are associated with incident type 2 diabetes.

4 THE EFFECT OF METFORMIN ON THE EXPRESSION OF CIRCULATING MIRNAS: CAMERA TRIAL

4.1 Introduction

Metformin (1,1-dimethylbiguanide), a first line oral glucose-lowering agent for patients with type 2 diabetes (T2DM), is derived from a natural product used in herbal medicine, biguanide derivate. It is considered as the drug of choice for the treatment of individuals with T2DM with BMI in the overweight and obese range in whom diet, and exercise treatments have failed. Both the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) official guidelines have recommended metformin as first-line oral therapy (Yang *et al.*, 2017). These recommendations were based on the results published by the UK Prospective Diabetes Study (UKPDS) (Turner, 1998). The study was initiated in 1977 as a multi-centre randomised control trial including newly diagnosed T2DM. The objective of the study was to determine whether improved glucose control in individuals with T2DM will prevent the complications of diabetes and whether there are differences between conventional treatment (diet) and drug treatments with three different drugs (sulfonylurea, metformin, or insulin). The study concluded that glucose control with metformin appears to decrease diabetes-related complications in patients who have a body mass index in the overweight range with less weight gain and hypoglycaemic attacks compared to the other two drugs. Specifically, the CAMERA trial reported no effect of metformin on cIMT, but it did induce a small weight loss of ~3kg (SD = 4·2) versus 0·0 kg (SD = 3·8) in the placebo group at 18 months. In addition, HbA1c, fasting insulin concentration, and HOMA- IR were reduced in the metformin group compared with placebo at 18 months.

Although It has been known that the antihyperglycemic effect of metformin is mainly due to decreases in hepatic glucose production, decreases in intestinal absorption of glucose, and improved insulin sensitivity by increasing peripheral glucose uptake and utilization, the molecular mechanisms of action remain unclear (Rena, Hardie & Pearson, 2017).

Beyond its effect on lowering blood glucose, a growing body of evidence suggested that metformin appears to be a potential drug with multiple therapeutic effects. Recent studies demonstrated that metformin could exert protective effects in cardiovascular diseases, pancreatic ß cell failure, cancer, and aging (Yang *et al.*, 2017). It has been used to treat other conditions where insulin resistance is

involved such as obesity (Seifarth, Schehler & Schneider, 2013), extreme insulin resistance with acanthosis nigricans (Akter *et al.*, 2016), polycystic ovarian syndrome (Study *et al.*, 2010).

Metformin has been reported to modify the expression of several miRNAs. This relationship was first studied using cancer cells in culture and murine tumour xenografts where they found that metformin inhibits tumorigenesis by upregulating DICER1, which is a key enzyme in microRNAs (miRNAs) processing (Blandino *et al.*, 2012).

Recent evidence suggests that metformin treatment affects the miRNA profile in patients with T2DM (Coleman *et al.*, 2013; Ortega *et al.*, 2014). In a recent study published in 2018, the authors measured the expression of 86 circulating miRNAs in plasma of 47 patients with T2DM before and after three months of metformin treatment (Demirsoy *et al.*, 2018). The patients received no diabetes treatment before the start of the study. The expression level of 13 circulating miRNAs (mir-let- 7e-5p, let-7f-5p, mir-21-5p, mir-24-3p,mir-26b-5p, mir-126-5p, mir-129-5p, mir-130b-3p, mir-146a- 5p, mir-148a-3p, mir-152-3p, mir-194-5p, mir-99a- 5p) were found to be downregulated in patients with T2DM following metformin treatment. This information was not published at the time of study design.

Having established the cross-sectional associations of the six selected circulating miRNAs with metabolic biomarkers, the next logical question was whether an intervention with metformin, which is widely acknowledged to improve metabolic health, would influence the expression of the six targeted circulating miRNAs. Beside, although metformin did not affect carotid intima-media thickness (CIMT) and other markers of cardiovascular diseases in the CAMERA study, there was a notable reduction in measurements of adiposity and glycaemia in metformin groups after 18 months, and from our previous analysis, the selected miRNAs showed variable associations with glycaemic markers but not adiposity markers.

4.2 Hypothesis

Randomisation to metformin will alter the expression of the selected circulating miRNAs (mir-221, mir-222, mir-192, mir-193b, mir-144, and mir-155). After the cross-sectional data I hypothesised specifically that mir-144 was the most likely candidate biomarker (associated with HOMA) to be influenced by metformin.

4.3 Aim

To explore the effect of randomisation to metformin on circulating miRNAs (mir-222, mir-221, mir-192, mir-193b, mir-155, and mir-144) using samples from the CAMERA RCT.

4.4 Methods

4.4.1 Samples

Samples from the CAMERA trial were used to conduct this study. A total of 154 participants without diabetes and have large waist and CHD, were included in this study. Participants were randomly assigned to metformin or placebo and followed for 18 months (metformin = 73, placebo = 81) (Figure 3-1). Two stored EDTA plasma samples were used for each participant; one sample was taken at baseline, and the other after 18 months of treatment. A total of 308 paired stored plasma samples were processed. Samples were thawed and immediately processed. The paired samples were processed simultaneously.

4.4.2 Total RNA extraction and miRNA quantification

Details on RNA extraction and miRNA quantification can be found in the general methods chapter. Samples from each participant (baseline and 18 months) were extracted within a single run to reduce the variability of measurements within individuals.

4.4.3 Statistical Analysis

The selected miRNAs (mir-221, mir-222, mir-144, mir-155, mir-192, and mir-193b) were normalized to cel-mir-39 and expressed as dCt. All continuous classical risk factor variables were tested for normality by visual inspection of their histograms and normal Q-Q plots. Descriptive statistics were expressed as mean ± SD for normally distributed variables, and median ± inter-quartile range for non-normally distributed variables. Non-parametric variables were normalised by log transformation to use parametric tests. Categorical variables were summarised as number (%). Comparison between mean values of continuous variables between two groups (placebo and metformin) were evaluated using *Student's* two-tailed *t*-test for continues variables and chi-square test for categorical parameters.

The relative quantification method (RQ) was used to compare the effect of metformin on the expression of the selected six miRNAs. It allows the quantification of the change in the gene expression level of a target gene across two samples, in our case baseline and 18-month samples. The results were

expressed as fold change of expression level in samples at 18 months compared to the baseline sample. This is done by first, calculating the differences in Ct between the target miRNA with that of the spiked-in external control (mir-39) from the same sample delta Ct (ΔCt) or dCt. After that, the ΔCt was then used to calculate the delta Ct (ΔΔCt) (ΔCt baseline - ΔCt after 18 months). Finally, RQ was calculated using the formula ($2^{-\Delta\Delta Ct}$). The RQ value was considered significant when there was a minimum of two-fold change. The RQ change between the two groups (placebo and metformin) were calculated for each miRNA and plotted using a line graph. To confirm and extend the results of the RQ analysis, a mixed design two-way repeated measures ANOVA analysis was done to determine the effect of time (baseline and 18 months) on the expression of the six targeted circulating miRNAs (the dependent variable) in the metformin group compared to the placebo group. The ΔCt for each miRNA at each time point was entered into the model, and the values were approximately normally distributed. The p-value from the interaction between randomisation group and time was reported as evidence against the null hypothesis that randomisation group had no effect on the miRNA. A p-value of < 0.05 was considered statistically significant evidence against the null hypothesis.

4.5 Results

4.5.1 Baseline characteristics

A total of 154 participants were studied, 73 in the metformin group and 81 in the placebo group. Table 4-1 summarises the baseline (before intervention) demographics, anthropometric measurements, biochemical characteristics, and miRNA expression of the two randomised groups (placebo and metformin).

As the table demonstrates, the average age of our study population was 63 years, with a mean BMI of 30 kg/m^2. Although all participants had no diabetes (mean glucose = 5.3), most of them were insulin resistance (mean HOMA-IR = 2.7). Among the six circulating miRNAs selected, mir-221 and mir-222 showed the highest expression in plasma while mir-144 showed the lowest expression.

Table 4-1: Baseline characteristics of the study population

		Placebo group (n = 81)	Metformin group (n = 73)
Demographic Characteristics			
Age (years)		64 (8)	63 (8)
Gender			
Male		61 (74%)	58 (80%)
Female		20 (25%)	15 (20%)
Smoking history			
	Current	16 (20%)	16 (22%)
	Former	45 (55%)	33 (45%)
	Never	20 (25%)	24 (33%)
Blood pressure (mm Hg)		140 / 79 (20 / 11)	140 / 79 (18 / 11)
Anthropometric Characteristics			
Body weight (kg)		87.5 (14.8)	87.3 (11.9)
Body mass index (kg/m^2) *		30.7 (27.1 - 33.1)	29.4 (27.5 - 32.3)
Waist circumference (cm)		104 (10)	105 (9)
Body fat (kg) *		29.4 (23.4 - 32.9)	27.0 (23.1 - 32.1)
Biochemical Characteristics (Fasting)			
Glucose (mmol/L)		5.2 (0.5)	5.3 (0.3)
Insulin (pmol/L) *		9.7 (7.5 - 14.4)	10.0 (7.2 - 13)
HOMA-IR*		2.3 (1.6 - 3.4)	2.3 (1.6 - 3.1)
HbA1c (mmol/mol) *		38.2 (36 - 40)	38.7 (37 - 41)
Adiponectin (µg/mL) *		5.6 (4.1 – 7.3)	5.8 (4.0 - 9.1)
Triglyceride (mmol/L) *		1.5 (1.1 - 1.9)	1.4 (1.1 - 2.0)
Total Cholesterol (mmol/L) *		4.3 (3.6 - 4.7)	4.2 (3.6 - 4.7)
HDL (mmol/L) *		1.1 (1.0 - 1.4)	1.1 (1.0 - 1.3)
Alanine aminotransferase (U/L) *		26.0 (18.5 - 33.5)	23.0 (19.5 - 35)
γ-glutamyl transferase (U/L) *		35.0 (21.5 - 54)	34.0 (22.5 - 64)
Inflammatory markers (fasting)			
High sensitivity C-reactive protein (mg/L) *		1.6 (0.9 - 3.2)	1.8 (0.8 - 3.9)
WBC (x10^9 /L) *		6.5 (5.7 - 8.0)	7.0 (5.9 - 7.5)
miRNA Expression			
mir-222 (dCt)		3.9 (1.6)	4.0 (1.8)
mir-221 (dCt)		1.7 (1.6)	1.8 (1.7)
mir-144 (dCt)		10.1 (1.4)	10.2 (1.3)
mir-155 (dCt)		8.1 (1.2)	7.9 (1.3)
mir-192 (dCt)		8.9 (1.4)	8.9 (1.8)
mir-193b (dCt)		9.3 (1.4)	9.2 (1.7)

***:** Variables are not normally distributed, median (inter-quartile range) were used.
HOMA-IR: Homeostatic model assessment for insulin resistance, **HDL:** high density lipoprotein, **CRP:** high sensitivity C reactive protein, **WBC:** white blood cells, **mir:** micro RNA, **dCt:** delta cycle threshold.

4.5.2 The Effect of Randomisation to Metformin

Table 4-2 shows the effect of metformin treatment over 18 months on anthropometric measurements, biochemical characteristics, inflammatory markers, and circulating miRNAs expression. Metformin led to a significant reduction in all anthropometric measurements (weight, BMI, WC, and body fat weight) in the available samples used in this study, consistent with previously reported data in the intention to treat analysis (Preiss *et al.*, 2014).

Mean weight loss after 18 months for the metformin group was 3.3 kg versus 0 kg in the placebo group ($p < 0.0001$). Also, metformin treatment improved measurements of insulin and insulin resistance (insulin and HOMA-IR). There were no differences in lipid measurements (TAG, TC, and HDL), circulating adiponectin, and liver enzymes (ALT and GGT) between the two groups after 18 months of metformin treatment. Likewise, metformin did not affect both inflammatory markers used in this study (CRP and WBC) ($p > 0.05$). Furthermore, the expression of the selected circulating miRNAs between the two groups (placebo and metformin) were compared. There was no difference between both groups after 18 months ($P > 0.05$).

Change in the expression of circulating miRNAs after 18 months between the two groups are represented as line graphs in Figure 4-1 for mir-221, Figure 4-2 for mir-222, Figure 4-3 for mir-192,

Figure 4-4 for mir-193b,

Figure 4-5 for mir-155, and Figure 4-6 for mir-144. For all the selected circulating miRNAs, there was an increase in their expression in time in both groups. Results showed no differences in the expression of circulating mir-221, mir-222, mir-192, and mir-193 between the placebo and metformin groups ($P > 0.05$). Although the expression of mir-144 and mir-155 were slightly reduced in the metformin group (Figure 4-6 and

Figure 4-5 respectively), the reduction was not significant ($P > 0.05$).

Table 4-2: Effect of randomisation to metformin treatment at 18 months on anthropometric and biochemical characteristics and circulating miRNAs expression.

	Placebo group (n = 81)	Metformin group (n = 73)	*P*-Value
Anthropometric Characteristics			
Body weight (kg)	87.5 (15.2)	84.0 (12.4)	**< 0.001**
Body mass index (kg/m^2) *	30.7 (27.1 - 33.0)	28.7 (26.1 - 31.3)	**< 0.001**
Waist circumference (cm)	104.4 (11.4)	101.8 (8.9)	**< 0.001**
Body fat (kg) *	28.2 (23.2 - 33.5)	25.1 (20.9 - 31.0)	**0.046**
Biochemical Characteristics (Fasting)			
Glucose (mmol/L)	5.4 (0.9)	5.2 (0.6)	0.062
Insulin (pmol/L) *	10.8 (7.6 - 17.6)	8.2 (6.3 – 12.4)	**0.031**
HOMA-IR*	2.6 (1.7 – 4.1)	1.9 (1.4 – 2.9)	**0.024**
HbA1c (mmol/mol) *	38 (36 - 40)	37 (36 - 39)	0.20
Adiponectin (µg/mL) *	4.8 (3.3 – 7.7)	6.1 (4.3 – 9.3)	0.201
Triglyceride (mmol/L) *	1.4 (1.1 - 1.8)	1.4 (1.1 – 1.9)	0.353
Total Cholesterol (mmol/L) *	4.3 (3.7 - 4.9)	4.1 (3.4 - 4.5)	0.430
HDL (mmol/L) *	1.1 (1.0 - 1.5)	1.1 (1.0 - 1.4)	0.065
Alanine aminotransferase (U/L) *	25.0 (19 - 34)	22 (17 - 30)	0.346
γ-glutamyl transferase (U/L) *	33.0 (21 – 54.5)	31.0 (18.5 – 47.5)	0.408
Inflammatory markers (fasting)			
High sensitivity C-reactive protein (mg/L) *	1.5 (0.7 - 2.9)	1.7 (0.8 - 3.3)	0.311
WBC (x10^9 /L) *	6.5 (5.5 - 8.0)	6.7 (5.8 - 8.3)	0.231
miRNA Expression			
mir-222 (dCt)	3.4 (1.7)	3.6 (1.7)	0.907
mir-221 (dCt)	1.4 (1.7)	1.4 (1.6)	0.507
mir-144 (dCt)	9.7 (1.4)	10.0 (1.5)	0.251
mir-155 (dCt)	7.4 (1.3)	7.4 (1.2)	0.972
mir-192 (dCt)	8.6 (1.6)	8.6 (1.5)	0.892
mir-193b (dCt)	9.1 (1.5)	9.0 (1.6)	0.694

***:** Variables are not normally distributed, median (inter-quartile range) were used.
HOMA-IR: Homeostatic model assessment for insulin resistance, **HDL:** high density lipoprotein, **CRP:** high sensitivity C reactive protein, **WBC:** white blood cells, **mir:** micro RNA, **dCt:** delta cycle threshold.

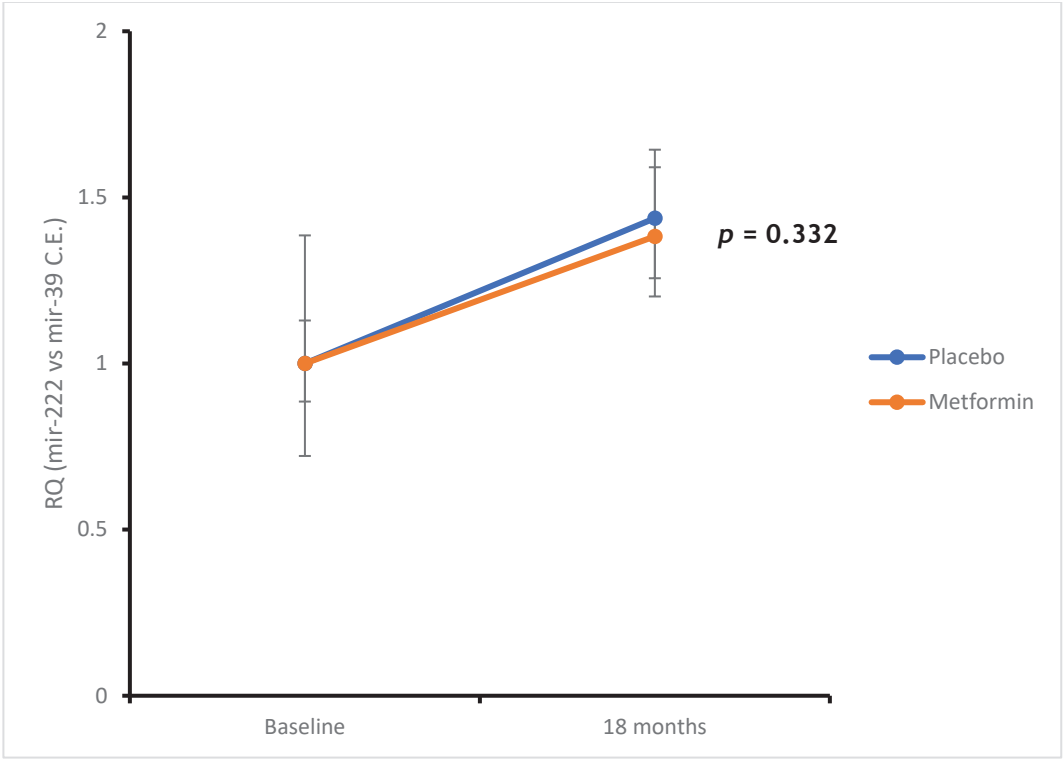


Figure 4-1: Line graph illustrating the RQ method for change for mir-222 between placebo and metformin groups over the 18 months.

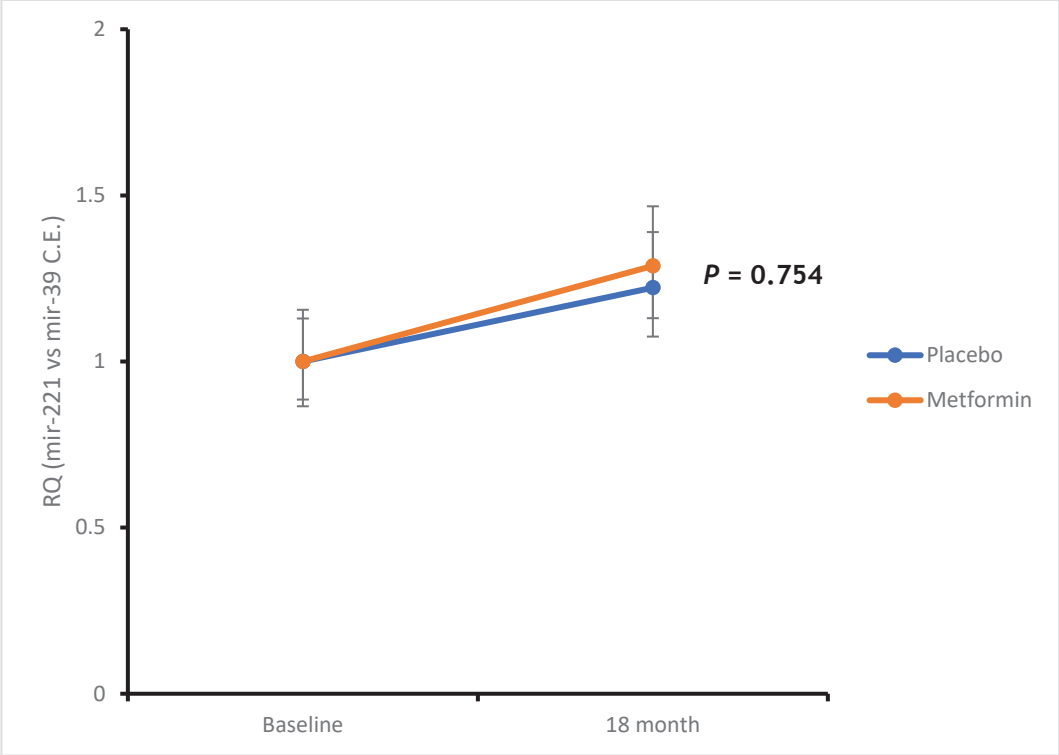


Figure 4-2: Line graph illustrating the RQ method for change for mir-221 between placebo and metformin groups over the 18 months.

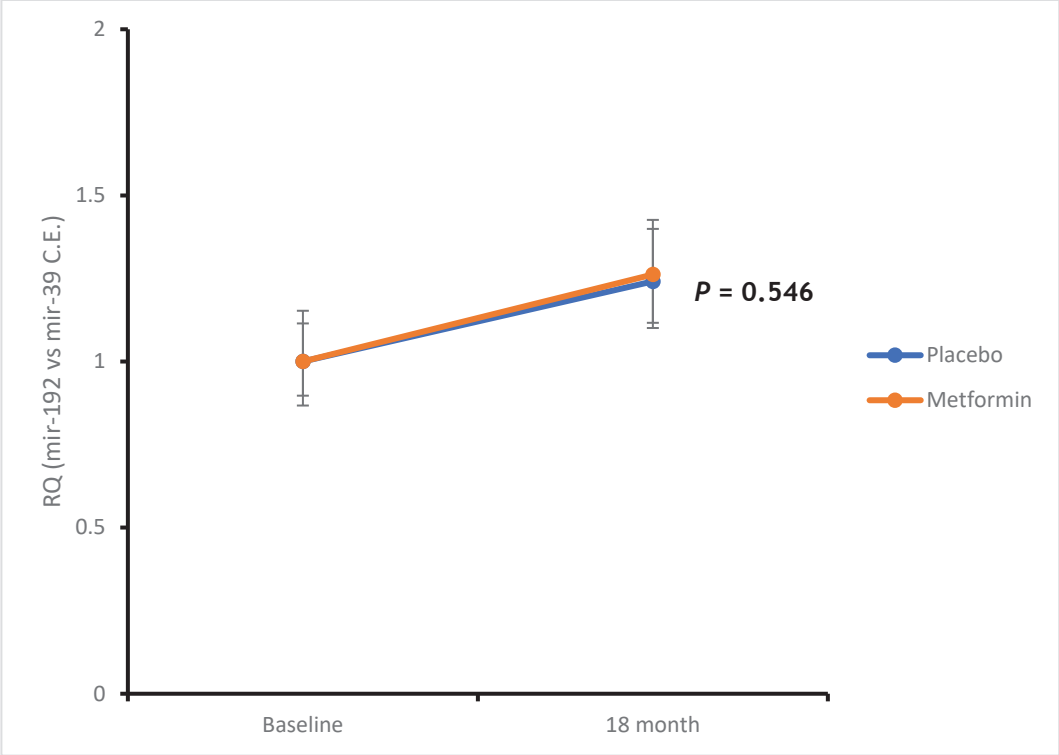


Figure 4-3: Line graph illustrating the RQ method for change for mir-192 between placebo and metformin groups over the 18 months.

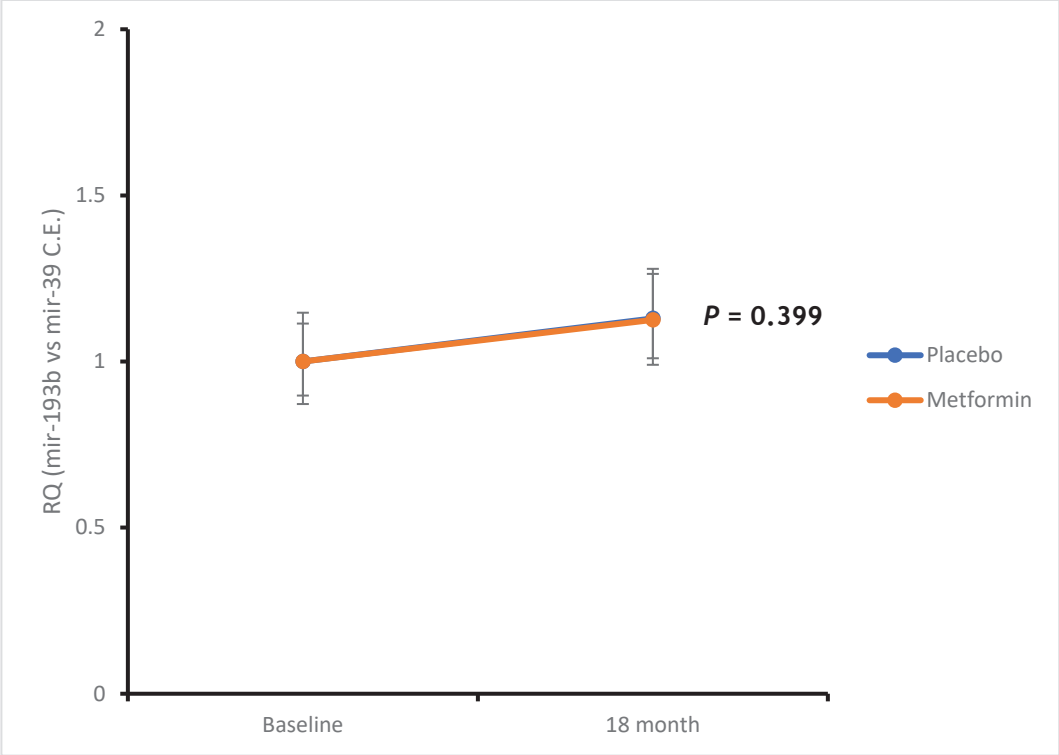


Figure 4-4: Line graph illustrating the RQ method for change for mir-193b between placebo and metformin groups over the 18 months.

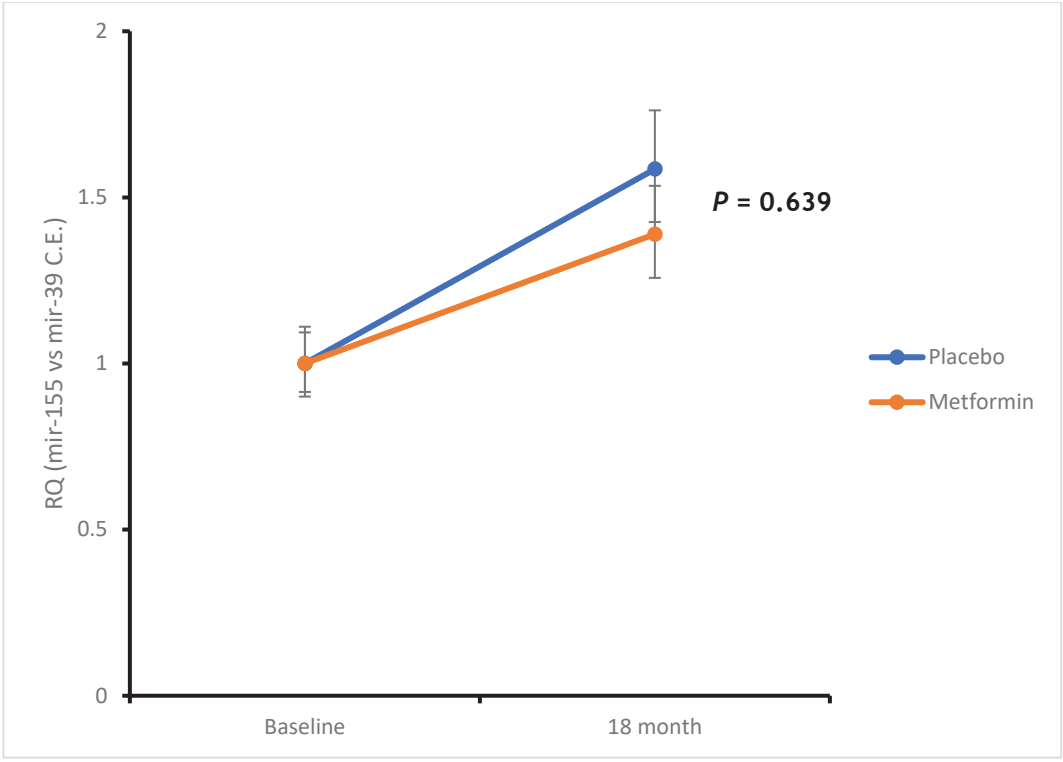


Figure 4-5: Line graph illustrating the RQ method for change for mir-155 between placebo and metformin groups over the 18 months.

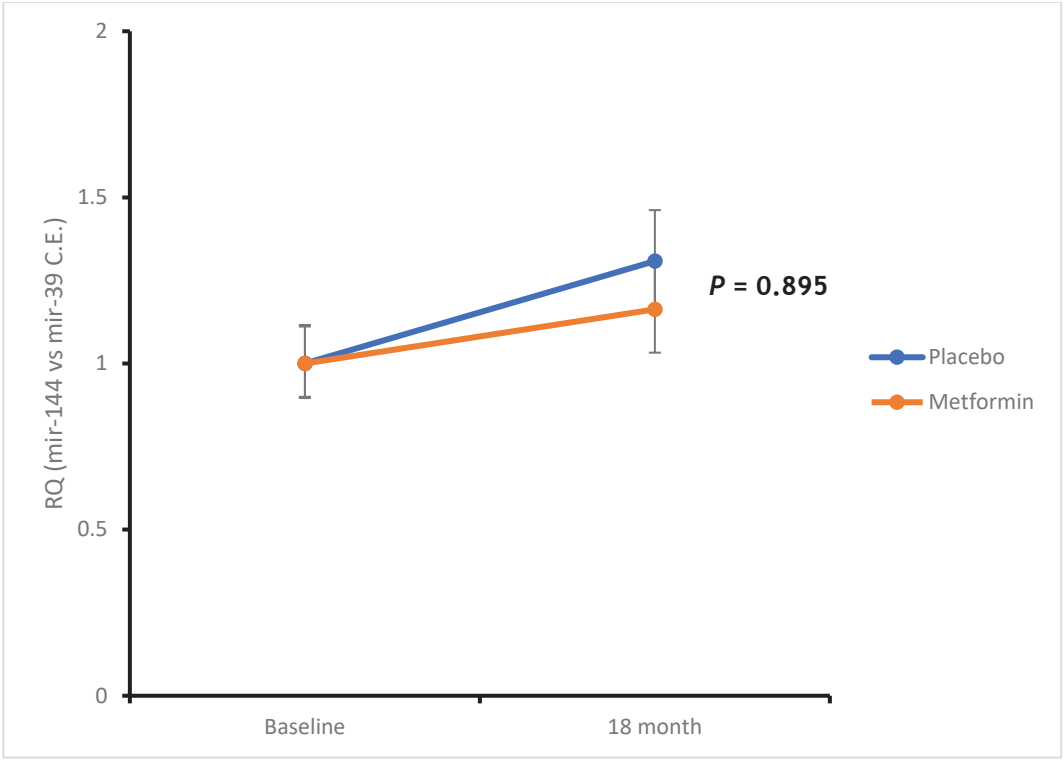


Figure 4-6: : Line graph illustrating the RQ method for change for mir-144 between placebo and metformin groups over the 18 months.

4.5.3 The effect of time and randomisation on the expression of circulating miRNAs

ANOVA analysis was also performed to determine the effect of time (baseline and 18 months) on the targeted circulating miRNAs expression and is it different between treatment groups (placebo and metformin). The results are shown in Table 4-3 (for mir-221), Table 4-4 for mir-222, Table 4-5 for mir-192, Table 4-6 for mir-193b, Table 4-8 for mir-155, and Table 4-7 for mir-144.

For all the targeted circulating miRNAs except mir-193b, there was an increase in their expression (lower ΔCt) over time. This increase was shown in both groups separately and combined (placebo and metformin). However, in line with the RQ plots there was no evidence that metformin randomisation had an effect different from placebo across time for any miRNA.

Table 4-3: mixed design two-way repeated measures ANOVA time by treatment analysis for mir-221.
Time p-value indicates evidence against the null hypothesis that time (in both treatment groups combined) has no effect on miRNA expression. Interaction p-value indicates evidence against the null hypothesis that metformin the same effect as placebo across time.

Randomised Treatment * Time				
Randomised Treatment	Time	Mean	95% Confidence Interval	
			Lower Bound	Upper Bound
Placebo	1	1.74	1.38	2.09
	2	1.45	1.09	1.81
Metformin	1	1.78	1.41	2.16
	2	1.42	1.04	1.80
Time 1 = baseline, 2 = 18 months				

Time $P = 0.014$
Interaction $P = 0.775$

Table 4-4: mixed design two-way repeated measures ANOVA time by treatment analysis for mir-222.
Time p-value indicates evidence against the null hypothesis that time (in both treatment groups combined) has no effect on miRNA expression. Interaction p-value indicates evidence against the null hypothesis that metformin the same effect as placebo across time.

Randomised Treatment * Time				
Randomised Treatment	Time	Mean	95% Confidence Interval	
			Lower Bound	Upper Bound
Placebo	1	3.89	3.52	4.26
	2	3.37	2.99	3.75
Metformin	1	4.02	3.63	4.41
	2	3.55	3.15	3.95
Time 1 = baseline, 2 = 18 months				

Time $P < 0.001$
Interaction $P = 0.509$

Table 4-5: mixed design two-way repeated measures ANOVA time by treatment analysis for mir-192.
Time p-value indicates evidence against the null hypothesis that time (in both treatment groups combined) has no effect on miRNA expression. Interaction p-value indicates evidence against the null hypothesis that metformin the same effect as placebo across time.

Randomised Treatment * Time				
Randomised Treatment	Time	Mean	95% Confidence Interval	
			Lower Bound	Upper Bound
Placebo	1	8.94	8.59	9.29
	2	8.63	8.29	8.96
Metformin	1	8.93	8.56	9.30
	2	8.59	8.24	8.95
Time 1 = baseline, 2 = 18 months				

Time $P = 0.007$
Interaction $P = 0.920$

Table 4-6: mixed design two-way repeated measures ANOVA time by treatment analysis for mir-193b.
Time p-value indicates evidence against the null hypothesis that time (in both treatment groups combined) has no effect on miRNA expression. Interaction p-value indicates evidence against the null hypothesis that metformin the same effect as placebo across time.

Randomised Treatment * Time				
Randomised Treatment	Time	Mean	95% Confidence Interval	
			Lower Bound	Upper Bound
Placebo	1	9.26	8.92	9.60
	2	9.08	8.75	9.42
Metformin	1	9.16	8.80	9.52
	2	8.99	8.64	9.34
Time 1 = baseline, 2 = 18 months				

Time $P = 0.152$
Interaction $P = 0.982$

Table 4-7: mixed design two-way repeated measures ANOVA time by treatment analysis for mir-155.
Time p-value indicates evidence against the null hypothesis that time (in both treatment groups combined) has no effect on miRNA expression. Interaction p-value indicates evidence against the null hypothesis that metformin the same effect as placebo across time.

Randomised Treatment * Time				
Randomised Treatment	Time	Mean	95% Confidence Interval	
			Lower Bound	Upper Bound
Placebo	1	8.11	7.84	8.38
	2	7.45	7.16	7.73
Metformin	1	7.91	7.63	8.20
	2	7.44	7.14	7.74
Time 1 = baseline, 2 = 18 months				

Time $P < 0.001$
Interaction $P = 0.370$

Table 4-8: mixed design two-way repeated measures ANOVA time by treatment analysis for mir-144.
Time p-value indicates evidence against the null hypothesis that time (in both treatment groups combined) has no effect on miRNA expression. Interaction p-value indicates evidence against the null hypothesis that metformin the same effect as placebo across time.

Randomised Treatment * Time				
Randomised Treatment	Time	Mean	95% Confidence Interval	
			Lower Bound	Upper Bound
Placebo	1	10.10	9.80	10.40
	2	9.72	9.40	10.03
Metformin	1	10.20	9.89	10.52
	2	9.99	9.65	10.32
Time 1 = baseline, 2 = 18 months				

Time $P = 0.009$
Interaction $P = 0.457$

4.6 Discussion

In the previous chapter, it was shown that circulating mir-144, mir-222, mir-221, mir-192, mir-193b, and mir-155 showed a broadly associations with markers of metabolic risk using baseline samples of the CAMERA trial. In this chapter, the aim was to explore the effect of metformin on the same circulating miRNAs using both the baseline samples and the 18-month plasma samples from the CAMERA trial. As far as I'm aware, this is the first large randomised study of circulating miRNAs ever conducted.

4.6.1 The Effect of Randomisation to Metformin

In this study, randomisation to metformin showed no effect on the expression of circulating mir-221, mir-222, mir-144, mir-155, mir-192, and mir-193b. This was true in analysis using both achieved ΔCt levels and using the RQ method traditionally employed in miRNA studies.

An effect of metformin on circulating mir-222, mir-221, and mir-192 expression has been reported in previous studies. Ortega *et al*. investigate the expression of 10 circulating miRNAs (including mir-222 and mir-192) in plasma of patients with T2DM before (baseline) and three months after treatment in a randomised, placebo-controlled which they previously showed their dysregulation in 93 patients with T2DM (Ortega *et al.*, 2014). The study consisted of 18 patients with T2DM in the placebo group and 17 patients with T2DM in the metformin group. The study reported that metformin lead to a marked decrease of circulating mir-222 (− 47%, $P = 0.03$), and increased mir-192 (49.5%, $P = 0.022$) in the metformin group and not placebo group. They also showed improvements in insulin resistance measured by hyperinsulinemic-euglycemic clamp Plus Intralipid/Heparin Infusion (− 28%, $P = 0.02$), fasting glucose and HbA1c (− 12.7%, − 9.5%, respectively $P = 0.0001$) in the metformin group. These changes are consistent with metabolic improvement in UKPDS (Holman *et al.*, 2008). Although participants in this study showed similar improvement in insulin resistance and not in glycaemia (Glucose and HbA1c) in the metformin group compared to placebo, there was no change in the expression of circulating mir-222 and mir-192. The discrepancy in the result may be due to differences in study population. The population in Ortega *et al*. study included participants with diabetes (FBG = 12 ± 3.4 mmol/L), whereas

participants in this study had no diabetes (FBG = 5.3 ± 0.5 mmol/L). Thus, the relationship between metformin and the selected circulating miRNAs found can be explained by an indirect effect of metformin on improving glycemia rather than a direct effect of metformin on these circulating miRNAs.

Furthermore, a cross-sectional study done by Coleman *et al*. included 37 participants undergoing coronary artery bypass surgery reported that subjects with diabetes on metformin (DMMet+, n = 10) exhibit lower levels of both mir-221 and mir-222 measured in whole tissue homogenates of the internal mammary arteries tissue (IMAs) compared to subjects with diabetes whose current medications do not include metformin (DMMet-, n = 9) and comparable to the subjects without diabetes (ND, n = 18) (Coleman *et al.*, 2013). They also found that there was an inverse correlation between the dose of metformin and the level of mir-221 and mir-222 (R^2 = 0.3577, p < 0.01; R^2 = 0.245, p < 0.05, respectively). Similar results were not observed in this study. The disagreement in results can be due to differences in study population (with diabetes vs. without diabetes) as was explained above. Also, different samples and different methodology used to measure the expression of miRNAs, which is considered the major factor for the inconsistency between studies.

4.6.2 The effect of time and randomisation on the expression of circulating miRNAs

For all the selected circulating miRNAs, there was an increase in their expression in time in both groups (placebo and metformin) as shown in Table 4-3 for mir-221, Table 4-4 for mir-222, Table 4-5 for mir-192, Table 4-6 for mir-193b, Table 4-8 for mir-155, and Table 4-7 for mir-144.

As previously discussed, circulating miRNAs are stable in plasma despite the presence of RNase (Glinge *et al.*, 2017; Moldovan *et al.*, 2014). Moreover, studies have shown that circulating miRNAs remain stable even after boiling, high or low pH, prolonged storage time and multiple freeze-thaw cycles (Gilad *et al.*, 2008; Moldovan *et al.*, 2014; Shaffer, Schlumpberger & Lader, 2012). The reason for the increase could be pre-analytical variation. Different sample processing that were used to obtain plasma may varied between the baseline samples and the 18-month samples which may influence the number of circulating miRNAs. It is more likely

that separation of plasma was the critical pre-analytical variable that causes this increase and since I used stored plasma samples, I don't have data to demonstrate this. Although this was a single centre trial with local blood separation performed rapidly, future studies should more clearly study the effects of pre-analytical variation on miRNA levels in plasma.

The results observed in CAMERA are therefore somewhat inconsistent with the published literature. Cross-sectional studies are prone to confounding by indication, and small RCTs are notoriously difficult to evaluate for quality indicators. This study substantially advances the evidence base because of its large size and more credible RCT design.

4.7 Conclusion

To the best of my knowledge, this study is one of the first studies to investigate the effect of a randomised intervention on miRNA expression using a large sample size. Randomisation to metformin failed to show any effect on the expression of circulating mir-222, mir-221, mir-192, mir-193b, mir-144, and mir-155 in population with coronary heart disease, and large waist but without diabetes. Inconsistency in results from different investigations could possibly be due to different clinical features and ethnicity of participants and different inclusion/exclusion criteria in different trials, which can lead to difficult interpretation and comparison between various investigations. Furthermore, miRNAs expression from different sources of samples (cellular and extracellular) should not be compared to each other and cannot be used interchangeably as biomarkers of disease. Besides, metformin, although first line drug and widely used, it has a relatively moderate metabolic effect. There are other glucose-lowering drugs which are proven to have better metabolic benefits such as GLP-1 Agonists (Hinnen, 2017). It has been found that GLP1 agonists can modulate the expression of different microRNAs (Capuani *et al.*, 2018). It might be useful, to explore the effect of GLP-1 Agonists on the expression of the selected circulating miRNAs in an RCT setting.

4.7.1 Strength and Limitations

The CAMERA trial was a high-quality study with several strengths. First, its design as a randomised placebo-controlled double-blind trial, which is considered as the 'gold standard' in evidence-based medicine for evaluating the effects of an intervention. Second, a well-defined large sample of participants (154) which were subjected to a strict inclusion/exclusion criterion. Finally, the well-standardised approach to sample collection (from a single local centre) and sample handling (quickly processed and stored in − 80℃). The major weakness in this study is data normalisation, which is a major challenge in data analysis because there is no universal/well-accepted control for normalisation of circulating miRNA data. Other potential weaknesses were mentioned in the trial itself. First, fewer participants than needed completed the trial (158 completed vs. 180 needed according to our sample size calculation) and extra four participants were excluded in this study because they did not have both samples

baseline and 18 months (Total participants included = 154). Second, several participants discontinued or reduced treatment. Participants were followed up for 18 months rather than 24 months. The study drug had a very moderate effect on metabolic profile in the selected patients. All these factors might have reduced our ability to detect a meaningful effect.

5 THE ASSOCIATION OF BODY FAT DISTRIBUTION WITH INFLAMMATION AND T2DM IN THE UK BIOBANK STUDY

5.1 Introduction

The fact that most individuals with T2DM have overweight or obesity highlights the strong association between excess body fat and the development of T2DM. Although there is convincing evidence that links the two together, the actual mechanism not fully understood because metabolic effects of obesity are not always proportional to the amount of total body fat mass.

Obesity has been evaluated mainly based on BMI, but recently strong evidence showed that the distribution of body fat might be critical to the development of T2DM (Neeland *et al.*, 2012; Wander *et al.*, 2013; Verboven *et al.*, 2018). For instance, substantial weight loss in patients with type 2 diabetes can lead to a recovery of glycemic control and is related to loss of ectopic fat in the liver and pancreas. The causal link between fat distribution and diabetes is not entirely clear but thought to be because of a combination of insulin resistance, systemic inflammation, and lipotoxicity, direct toxic effects from triglyceride accumulating ectopically within organs instead of subcutaneous adipose tissue (SAT).

Visceral adipose tissue (VAT), as a marker of adipose tissue dysfunction, plays a great role in metabolic dysfunction. Previous studies have considered VAT accumulation as a strong risk factor for developing T2DM (Sattar & Gill, 2014; Nordström *et al.*, 2016; Janochova, Haluzik & Buzga, 2019). Moreover, excess visceral fat contributes to the increases in systemic inflammation by secreting more pro-inflammatory molecules (Osborn & Olefsky, 2012).

It is still an ongoing debate whether dysfunctional adipose tissue (AT) leading to ectopic fat accumulation, inflammation or interaction between both, is a cause or rather a consequence of insulin resistance (IR) in obesity leading to T2DM. Therefore, this study was designed to investigate the relationship between fat location and T2DM and to identify if inflammation plays a role in this relation using data from the UK Biobank study.

5.1.1 UK Biobank

UK Biobank is a large scale prospective epidemiological study established by the Welcome Trust, Medical Research Council, Department of Health, Scottish

Government, and the Northwest Regional Development Agency and it was supported by the National Health Service (NHS). Also, it received funding from the Welsh Government, British Heart Foundation, Cancer Research UK, and Diabetes UK. The aim of the study was to improve, prevent, diagnose, and treat a wide range of illnesses, including cancer, heart diseases, and diabetes. It was approved by the North West - Haydock Research Ethics Committee in the UK (REC reference: 11/ NW/03820). The National Health Service (NHS) records were used to identify people to participate in the study. The invitation letters were sent to people who were 40-69 years old and living nearby one of the 22 assessment centres (Figure 5-1) covering a variety of different socioeconomic and ethnic groups from all around the UK. This ensured a reliable detection of the associations between exposure and health outcomes. All participants gave written informed consent before enrolment following the principles of the Declaration of Helsinki. The study was conducted in stages. It started with two pilot studies. Afterward, the main recruitment for baseline assessment began. Later, several further assessments have been carried out for more phenotyping assessments (for example, the imaging study). Finally, follow-up data on health outcomes are collected mainly through linkage with routinely available national health datasets.

5.1.2 Baseline assessment

Baseline assessment recruited more than 502,000 people between the years 2006 and 2010. It was done using a touchscreen questionnaire, a verbal interview with a trained member of staff, a series of physical examination, and a consent to access participants medical records. Participants also provided biological samples (blood, urine, and saliva) for future analysis.

5.1.2.1 Imaging study

The imaging study began in May 2014 and aims to recall 100,000 participants for detailed imaging scans of vital organs (brain, heart, abdomen, bones, carotid artery, and body composition) as well as a repeat of the baseline measurements. The study included Cardiac MRI scan, Body MRI scan, Brain MRI scan, 12-lead Electrocardiogram (ECG), Carotid ultrasound, and DEXA scan of the whole body, bone, and joint. Invitation letters were sent to people who live within a reasonable traveling distance to one of the imaging assessments centres and not on any other

information that has already been collected about the participants. Participants were excluded from the study if they have any metal or electrical implant (e.g., pacemaker) in their body, or if they have had recent surgery (less than six weeks), or if they had any medical problem that makes it difficult to take the scans (e.g., severe hearing or breathing problems, or tremors).

5.1.3 Measurement of inflammation

Activation of the immune system and inflammation associated with obesity has been implicated in the pathogenesis of T2DM. This activation can be detected by an increase in several markers, including cytokines and WBC. Most previous epidemiological research has focused on the role of CRP in predicting risk of type 2 diabetes (Wang *et al.*, 2013). Unfortunately, CRP measurement was not available at the time of analysis, so a decision was made to use only WBCs count as a marker of inflammation. Besides, total and differential WBCs (neutrophils, lymphocytes, monocytes, eosinophils, and basophils) are widely recognized as a non-specific marker for systematic inflammation (Dixon & Brien, 2006). They are an objective marker of acute infection, tissue damage, and other inflammatory conditions. Studies have shown that an increase in the WBC count is closely related with all-cause mortality (Sun *et al.*, 2005; Willems *et al.*, 2010; Wang *et al.*, 2018). In addition, evidence from large epidemiological studies suggests an association between total and peripheral WBCs count and diabetes risk (Schmidt *et al.*, 1999; Vozarova *et al.*, 2002; Twig *et al.*, 2013). Furthermore, Chen *et al.* showed that both CRP and WBC were elevated in obesity, and weight loss results in reductions of both CRP and WBC to similar extents (Chen *et al.*, 2009).

Unlike cytokines or even CRP, WBC is measured routinely and cheaply, which makes the biomarker interesting to study academically for potentially wider indications than infection. Therefore, study of WBC as a biomarker of metabolic disease is at the opposite end of the spectrum of pragmatism compared to our previous novel work on miRNA.

Figure 5-1: Locations of UK Biobank assessment centres throughout the UK

Taken from: https://biobank.ctsu.ox.ac.uk/crystal/exinfo.cgi?src=UKB_centres_map

5.2 Hypothesis

I hypothesised that:

1. Fat accumulation and distribution and WBC count differ between participants with and without T2DM.

2. Abdominal fat accumulation, particularly VAT, is independently associated with T2DM.

3. WBC count is more associated with abdominal fat accumulation.

4. WBC count is the link between the relationship of VAT accumulation and T2DM.

5.3 Aims

Using the UK Biobank cohort, I will investigate in a cross-sectional analysis:

1. Different patterns of body fat distribution (anthropometrics measurements, bio-impedance measurements, DEXA measurements, and MRI measurements) in both sexes and their association with T2DM.

2. The association between the body fat distribution measurements and WBC count.

3. The association of WBC count with T2DM.

4. Investigate whether WBC count is a link between adiposity and the development of T2DM.

5.4 Methods

5.4.1 Study population

To conduct this cross-sectional study, I included all body fat measurements available in the UK Biobank cohort at the time of analysis (data download Sept 2018). To do this, I used two datasets, the baseline assessment dataset, which includes the anthropometric measurements and the bio-impedance analysis variables, and the imaging study dataset, which included the DEXA and MRI body fat variables. The total number of participants included were ≈ 500,462 participants from the baseline study and ≈ 5995 participants from the imaging study. Due to these cohorts being substantially different in size and having different baseline dates, I treated them as separate cohorts in analysis and used all available data for each model.

The present study was superficially ethically approved by UK Biobank in July 2017: application ID 20152.

https://www.ukbiobank.ac.uk/2017/07/ms-tahani-al-ramah-the-relationship-between-adiposity-and-low-grade-inflammation-as-mediators-of-metabolic-dysfunction/

5.4.2 Variables included

This section provides brief information on the variables used to conduct the current study. More details are available at the UK Biobank website:

http://www.ukbiobank.ac.uk/

5.4.2.1 Demographic variables

Age

Age of attending assessment centre variable was used. It is a derived variable from date of birth and date of attending assessment centre. Age was recorded in years. More details are available at:

https://biobank.ctsu.ox.ac.uk/showcase/field.cgi?id=21022

Ethnicity

In this study, I used the ethnic background variable to categorise the ethnicity of the participants. This variable was created from the combination of consecutive branching questions asked during the baseline assessment using the touchscreen. In this variable, ethnicity was categorised into eight groups (white, black, Asian, mixed, Chinese, other ethnic groups, do not know, and prefer not to answer). I recorded the ethnicity group as white, south Asian, black, Chinese, and others. Participants with "Don't know" and "Prefer not to answer" responses were excluded from our analysis (n = 1961). More details are available at:

https://biobank.ctsu.ox.ac.uk/showcase/field.cgi?id=21000

Townsend Deprivation index

The Townsend deprivation index is a measure of material deprivation within a population that was first introduced by sociologist Peter Townsend in 1987. It is calculated by using four variables of the population of each area, and a score is given accordingly. These variables are unemployment, non-car ownership, non-home ownership, and household overcrowding. The higher the Townsend index score, the greater the degree of deprivation in this area. Townsend deprivation index was measured for each participant joining UK Biobank, and I used continuous scores derived for and published by the study. The score was calculated using the preceding national census output areas and their postcode. More details are available at:

https://biobank.ctsu.ox.ac.uk/showcase/field.cgi?id=189

Smoking and Alcohol status

These variables were taken during the initial assessment visit using the touchscreen. Both variables were categorised into never, former, current, or prefer not to answer. More details are available at:

https://biobank.ctsu.ox.ac.uk/showcase/field.cgi?id=20116

5.4.2.2 Type 2 Diabetes (T2DM) variable

I identified participant as having baseline T2DM if they reported yes in diabetes diagnosed by doctor variable, which was obtained by touchscreen input, or if they self-reported specific conditions related to diabetes during interview (specifically, diabetes associated neuropathy/ulcers, diabetes associated nephropathy, and diabetes associated eye disease).

http://biobank.ctsu.ox.ac.uk/crystal/coding.cgi?id=6

I excluded patients with type one diabetes (T1DM) if the patient reported starting insulin within the one-year diagnosis of diabetes. This means patients with T1DM are not in the comparator group.

5.4.2.3 Anthropometric measurements

Weight

Weight was measured by a variety of means during the assessment. I used the field that merges all these values into a single item. Weight was recorded in Kg. More details are available at:

https://biobank.ctsu.ox.ac.uk/showcase/field.cgi?id=21002

Height measurement

Standing height variable was used which was obtained using a Seca 240 cm height measure stand. Height was recorded in centimetres. More details are available at:

https://biobank.ctsu.ox.ac.uk/showcase/field.cgi?id=12144

Body Mass Index (BMI)

BMI was calculated using weight in kilograms divided by the square of the height in metres (kg/m^2). Height and weight used for the calculation were measured during the initial assessment centre visit. More details are available at:

https://biobank.ctsu.ox.ac.uk/showcase/field.cgi?id=21001

Hip and waist circumference measurements

Hip and waist circumference measurements were collected from participants using a Seca 200 cm tape measure. Waist circumference was measured at the level of the umbilicus. For hip circumference measurement, the tape was lowered to the broadest part of the hips. Readings were recorded in centimetres. More details are available at:

https://biobank.ctsu.ox.ac.uk/showcase/field.cgi?id=48 and 49

5.4.2.4 Bioimpedance measurement

Whole-body bio-impedance data were measured using a Tanita BC418MA body composition analyser. Measurement was done by passing an extremely low alternating current via the trunk, legs, and arms. All the data captured by the Tanita analyser were transmitted directly into the assessment centre software. Data included are: Whole-body fat mass, right and left legs fat mass, right and left arms fat mass, and trunk fat mass. More details are available at:

https://biobank.ctsu.ox.ac.uk/showcase/label.cgi?id=100009

5.4.2.5 Dual-Energy X-Ray Absorptiometry (DEXA)

Dual-energy X-ray absorptiometry (DEXA) measurements were taken using the GE-Lunar iDXA machine. A radiographer analysed scans and all measurements, including bone mass and body composition, were directly transferred to the UK Biobank servers. DEXA variables included are android fat mass, gynoid fat mass, arms fat mass, legs fat mass, total fat mass, trunk fat mass, and VAT mass. More details are available at:

https://biobank.ctsu.ox.ac.uk/showcase/label.cgi?id=124

5.4.2.6 MRI

All measurements were performed using a Siemens Aera 1.5 T scanner (Syngo MR D13) (Siemens, Erlangen, Germany) with the dual-echo Dixon Vibe protocol, covering neck to knees. All fat measurements were measured in Volume (ml). Visceral adipose tissue (VAT) was defined as the fat within the abdominal cavity

between the top of the femoral head and the top of the thoracic vertebrae T9, excluding fat outside the abdominal skeletal muscles and fat and lipids within and posterior of the spine and posterior of the back muscles. Abdominal subcutaneous adipose tissue (ASAT) was defined as subcutaneous fat in the abdomen from the top of the femoral head to the top of the thoracic vertebrae T9. Total trunk fat was a derived variable calculated as a sum of VAT and ASAT volumes. More details are available at:

https://biobank.ctsu.ox.ac.uk/showcase/refer.cgi?id=163332

5.4.2.7 White Blood Cells (WBCs) Analysis

The Complete Blood Count (CBC) was measured on fresh samples collected in a 4 ml EDTA during baseline recruitments. Samples were analysed within 24 hours of blood draw at the UK Biobank central laboratory using Beckman Coulter LH750 instruments. Total and differential WBCs (lymphocytes, monocytes, neutrophils, eosinophils, and basophils) results were measured as an absolute number per unit volume and proportions of the overall WBCs. Instrument quality control and calibration were performed according to the manufacturer's recommendations. More details are available at:

https://biobank.ctsu.ox.ac.uk/showcase/label.cgi?id=100002

5.4.3 Statistical Analysis

All analyses were pre-specified with an analysis plan, and a specific application to UK biobank to undertake this work. All body fat measurements were treated as continuous variables. Variables were tested for normality by visual inspection of their histograms and normal Q-Q plots. Descriptive statistics were expressed as mean ± SD. Comparison between mean values of a continuous variable in the two groups, no baseline diabetes (ND) and type 2 diabetes (T2DM) were evaluated using Student's two-tailed *t*-test. Separate tables were done for each measurement method (anthropometric, bio-impedance, DEXA, and MRI).

Pearson correlation (r) was used to investigate the association of WBC count with body fat measurements. Separate tables were done for each measurement method (anthropometric, bio-impedance, DEXA, and MRI). Additionally, a simple linear

regression analysis was performed to determine whether WBC count is an independent predictor for the presence of T2DM. Multiple linear regression analysis was also performed to adjust for potential confounders: age, ethnic background, smoking status, and socioeconomic status, as they are proven to be associated with both body fat and inflammation. I included BMI in the adjustment models although it is not a confounder for body fat, as I wanted to test if the associations of a specific body fat measurements with T2DM remained significant even after correcting for body mass using BMI. Standardised beta coefficient (SBC) was used to compare the effect each fat measurement has on WBC count. Specific assumptions of linearity were checked for linear regression models, constant variance, the linear association, and normality of residuals. Both sexes were analysed separately, regardless of evidence for interaction as a pre-specified.

The association between body fat measurements and T2DM risk was determined by calculating the odds ratio (OR) and 95% confidence interval (CI) using binary logistic regression analysis, assumption for logistic regression analysis was checked (dependent variable were binary, little or no multicollinearity among the independent variables, linearity of independent variables and log odds, and large sample size). Similarly, different models were run to adjust for potential confounders, the same confounders as for linear regression. Both genders were analysed separately as pre-specified.

All the statistical tests were performed using IBM SPSS (Statistical Package for Social Sciences) windows version 24 (SPSS Inc. Chicago, USA) and Microsoft Excel 2016. The criterions for statistical significance was $p < 0.05$. As this is a hypothesis-generating exploratory study, I did not employ any formal correction for multiple comparisons. However, this should be borne in mind when interpreting *p*-values from comparisons.

5.5 Results

5.5.1 Description of adiposity in the UK Biobank Cohort grouped by sex and diabetes

The baseline assessment data were used for both anthropometric and bio-impedance analysis (N ≈ 500,462 participants). The imaging study data were used for DEXA and MRI (N ≈ 5,170 and 5,995 participants, respectively). Both data set were classified according to sex and the presence or absence of baseline Type 2 diabetes (T2DM). Each group was analysed separately. Results were summarized in Table 5-1 for anthropometric characteristics, Table 5-2 for bio-impedance measurements, Table 5-3 for DEXA measurements, and Table 5-4 for MRI measurements.

Overall, data from this study confirmed well-documented differences in body fat composition and distribution between males and females. Males were 14.3 kg heavier than females. Meanwhile, females had higher total body fat measured by both bio-impedance (4.8 kg higher) and DEXA (1.9 kg higher). Regarding fat location, females accumulate more fat in the peripheral area, which was demonstrated mainly by DEXA measurements (Table 5-3). Females had more fat in the gynoid area (1.1 kg), legs (2.2 kg) and arms (0.5 kg). In contrast, men accumulate more fat in the central area shown by the waist (12.1 cm), trunk fat (1.9 kg), android fat (0.5 kg), and VAT (1.1 kg).

Table 5-4 shows the details of the truncal fat measured by MRI. Both sexes had almost similar truncal fat in both groups ND and T2DM. After separating each compartment of the truncal region into ASAT and VAT, men had more VAT fat (ND = 4.9 ± 2.3 and T2DM = 6.9 ± 2.9) while women had more ASAT fat (ND = 8.0 ± 3.4, T2DM = 11.2 ± 4.4).

The prevalence of diabetes in this study population was higher in males (6%) compared to females (3%). All body fat measurements were higher in patients with T2DM compared to patients without diabetes (ND) in both sexes ($p < 0.001$). In comparison with males, the mean difference between the ND and T2DM groups for most body fat measurements were higher in females compared to males except for VAT (measured by both DEXA and MRI), which was higher in males. In fact, VAT

measurements in females with diabetes (1.7 ± 0.9 for DEXA and 4.7 ± 2.0 for MRI) was similar to VAT measurements in males without diabetes (1.7 ± 0.9 for DEXA and 4.9 ± 2.3 for MRI). The mean amounts of visceral fat were 1.7 kg for DEXA and 4.7L for MRI in females with diabetes and 2.4 kg for DEXA and 6.9L for MRI in males with diabetes.

Table 5-1: Baseline anthropometric assessment characteristics from UK Biobank by sex and T2DM status

Variable	Total Cohort N ≈ 500462	Females N ≈ 272372				Males N ≈ 228090			
		ND N ≈ 264436 Mean (SD)	T2DM N ≈ 7936 Mean (SD)	Mean Difference (95% CI)	*P*-value	ND N ≈ 214175 Mean (SD)	T2DM N ≈ 13915 Mean (SD)	Mean Difference (95% CI)	*P*-value
Weight (kg)	78.1 (15.9)	71.1 (13.8)	84.9 (18.3)	13.9 (13.6 - 14.2)	< 0.001	85.4 (13.9)	94.6 (17.7)	9.2 (9.0 – 9.4)	< 0.001
BMI (kg/m^2)	27.4 (4.8)	26.9 (5.1)	32.9 (6.6)	6.0 (5.8 – 6.1)	< 0.001	27.6 (4.1)	31.1 (5.2)	3.5 (3.4 – 3.6)	< 0.001
Waist (cm)	90.3 (13.5)	84.3 (12.2)	100.3 (14.1)	16.0 (15.8 – 16.3)	< 0.001	96.4 (11.0)	106.1 (13.2)	9.7 (9.5 – 10.0)	< 0.001
Hips (cm)	103.4 (9.2)	103.1 (10.1)	112.2 (14.0)	9.1 (8.8 – 9.3)	< 0.001	103.2 (7.4)	107.5 (10.0)	4.3 (4.2 – 4.5)	< 0.001

ND: non-diabetes, **T2DM:** type two diabetes mellitus, **SD:** standard deviation, **CI:** confident interval.

Table 5-2: Baseline body fat analysis measured by bio-impedance from UK Biobank by sex and T2DM status

Variable	Total Cohort N ≈ 492444	Females N ≈ 268309				Males N ≈ 224135			
		ND N ≈ 260581	T2DM N ≈ 7728	Mean Difference	*P*-value	ND N ≈ 210687	T2DM N ≈ 13448	Mean Difference	*P*-value
Total body fat (kg)	24.9 (9.6)	26.7 (9.8)	36.7 (12.5)	10.1 (9.9 – 10.3)	< 0.001	21.9 (7.9)	28.4 (10.3)	6.5 (6.3 – 6.7)	< 0.001
Trunk fat (kg)	13.8 (5.2)	13.5 (5.2)	18.0 (6.0)	4.5 (4.3 – 4.6)	< 0.001	13.6 (4.9)	17.6 (6.0)	4.0 (3.9 – 4.0)	< 0.001
Arms fat (kg)	1.2 (0.6)	2.1 (1.1)	3.3 (1.7)	1.2 (1.2 – 1.3)	< 0.001	1.6 (0.7)	2.2 (1.0)	0.6 (0.55 -0.58)	< 0.001
Legs fat (kg)	4.1 (1.7)	7.8 (2.6)	10.7 (3.7)	2.9 (2.9 – 3.0)	< 0.001	4.6 (1.8)	6.0 (2.5)	1.4 (1.3 – 1.4)	< 0.001

ND: non-diabetes, **T2DM:** type two diabetes mellitus, **SD:** standard deviation, **CI:** confident interval

Table 5-3: Baseline body fat analysis measured by DEXA from UK Biobank by sex and T2DM status

Variables	Total Cohort N ≈ 5170	Females N ≈ 2713				Males N ≈ 2457			
		ND N ≈ 2665	T2DM N ≈ 48	Mean Difference	*P-value*	ND N ≈ 2373	T2DM N ≈ 84	Mean Difference	*P-value*
Total body fat (kg)	25.8 (9.0)	26.7 (9.5)	35.6 (11.5)	8.9 (6.1 – 11.2)	< 0.001	24.8 (8.9)	30.4 (10.8)	5.6 (3.6 – 7.5)	< 0.001
Trunk fat (kg)	14.6 (5.9)	13.9 (5.9)	20.9 (7.3)	7.0 (5.2 – 8.7)	< 0.001	15.2 (6.1)	19.6 (7.6)	4.3 (3.0 – 5.7)	< 0.001
Arms fat (kg)	2.7 (1.0)	2.9 (1.0)	4.0 (1.6)	1.1 (0.8 – 1.4)	< 0.001	2.4 (0.8)	2.8 (0.9)	0.4 (0.3 -0.6)	< 0.001
Legs fat (kg)	7.8 (3.1)	9.1 (3.1)	9.8 (4.0)	0.7 (0.2 – 1.6)	0.118	6.3 (2.3)	7.1 (2.8)	0.8 (0.3 -1.3)	0.002
Android fat (kg)	2.4 (1.2)	2.2 (1.1)	3.6 (1.4)	1.4 (1.0 – 1.7)	< 0.001	2.7 (1.2)	3.6 (1.5)	0.8 (0.2 – 1.4)	< 0.001
Gynoid fat (kg)	4.1 (1.5)	4.7 (1.5)	5.5 (2.1)	0.8 (0.3 – 1.2)	0.012	3.6 (1.3)	4.2 (1.7)	0.7 (0.4 – 0.9)	< 0.001
VAT mass (kg)	1.2 (0.9)	0.8 (0.6)	1.7 (0.9)	0.9 (0.7 – 1.1)	< 0.001	1.7 (0.9)	2.4 (1.1)	0.7 (0.5 – 1.0)	< 0.001

ND: non-diabetes, **T2DM:** type two diabetes mellitus, **SD:** standard deviation, **CI:** confident interval, **VAT:** visceral adipose tissue

Table 5-4: Baseline body fat analysis measured by MRI from UK Biobank by sex and T2DM status

Variable	Total Cohort N ≈ 5995	Females N ≈ 3145				Males N ≈ 2850			
		ND N ≈ 3095	T2DM N ≈ 50	Mean Difference	*P*-value	ND N ≈ 2754	T2DM N ≈ 96	Mean Difference	*P*-value
Total trunk (L)	10.7 (4.5)	10.6 (4.5)	15.8 (5.5)	5.3 (4.0 – 6.6)	< 0.001	10.8 (4.4)	14.0 (4.8)	3.2 (2.3 – 4.1)	< 0.001
ASAT (L)	7.0 (3.2)	8.0 (3.4)	11.2 (4.4)	3.2 (2.3 – 4.2)	< 0.001	5.9 (2.5)	7.1 (2.9)	1.2 (0.7 – 1.7)	< 0.001
VAT (L)	3.7 (2.2)	2.6 (1.5)	4.7 (2.0)	2.1 (1.6 – 2.6)	< 0.001	4.9 (2.3)	6.9 (2.9)	2.1 (1.5 – 2.7)	< 0.001

ND: non-diabetes, **T2DM:** type two diabetes mellitus, **SD:** standard deviation, **CI:** confident interval, **ASAT:** abdominal subcutaneous adipose tissue, **VAT:** visceral adipose tissue.

5.5.2 The association between adiposity and type 2 diabetes

Binary logistic regression models were used to examine which body fat measurements were associated with diabetes. First, I used univariate binary logistic regression to examine the crude association between each adiposity measurement and T2DM. After that, all the models were re-run adjusting for age, BMI, ethnic background, smoking status, and socioeconomic status. The binary logistic results were summarised in Table 5-5 for anthropometric characteristics, Table 5-6 for bio-impedance measurements, Table 5-7 for DEXA measurements, and Table 5-8 for MRI measurements.

The univariate logistic regression analyses showed a significant association between all adiposity measurements and T2DM in both sexes (p < 0.001). Specifically, VAT measured by both DEXA and MRI showed the strongest association with T2DM and was greater in females compared to males; in females OR (95% CI) = 4.90 (3.34 - 7.07) for DEXA and 1.858 (1.61 – 1.94) for MRI and in males OR (95% CI) = 1.94 (1.60 - 2.35) for DEXA and 1.39 (1.28 – 1.50) for MRI. Android fat was also strongly associated with diabetes.

After adjusting, the direction of the effect of some of the body fat measurements on T2DM changed from positive to inverse, making it appear as if this body fat measurement protects against T2DM. For example, for hip circumference the crude effect was, OR (95% CI) = 1.06 (1.06 - 1.06) in females and 1.062 (1.06 - 1.06) in males p < 0.001. After adjustment including BMI, the direction of the effect changed to 0.95 (0.95 – 0.96) in females and 0.97 (0.96 – 0.97). This pattern was also true for arms fat, legs fat, and gynoid fat. Moreover, all the bio-impedance body fat measurements (total body fat, trunk fat, arms fat, and legs fat) change the direction of the association with diabetes from positive to negative (p < 0.001) after adjustment (Table 5-6). This includes truncal fat, which was positively associated with type 2 diabetes using both DEXA and MRI measurement.

In females, after adjusting for age, BMI, ethnic background, smoking status, and socioeconomic status, the relationship between diabetes and most measurements of adiposity disappeared except for waist, trunk fat measured by both DEXA and MRI, android fat, and VAT measured by both DEXA and MRI. The highest association was with VAT measured by DEXA, OR (95% CI) = 3.97 (2.32 - 6.79), android fat, OR

(95% CI) = 1.63 (1.14 – 2.23), VAT measured by MRI, OR (95% CI) = 1.58 (1.29 – 1.94), and trunk fat measured by MRI, OR (95% CI) = 1.11 (1.02 – 1.22). In males, after adjusting for age, BMI, ethnic background, smoking status, and socioeconomic status the association disappeared between adiposity and diabetes except for waist, OR (95% CI) = 1.04 (1.04 – 1.04), VAT measured by DEXA, OR (95% CI) = 1.59 (1.20 – 2.12), and VAT measured by MRI, OR (95% CI) = 1.27 (1.14 – 1.41). All the associations continued to be higher in females.

Table 5-5: Binary logistic regression analysis of the association between anthropometric measurements and T2DM from the UK Biobank by sex

Variable	Females			Males		
	N	OR (95% CI)	*P*-Value	N	OR (95% CI)	*P*-Value
Weight^ (kg)	272095	1.05 (1.05 - 1.05)	< 0.001	227753	1.04 (1.04 - 1.04)	< 0.001
Weight#	270690	1.05 (1.05 - 1.06)	< 0.001	226178	1.04 (1.04 - 1.05)	< 0.001
Waist^ (cm)	272372	1.08 (1.08 - 1.08)	< 0.001	228090	1.07 (1.06 - 1.07)	< 0.001
Waist*	270531	1.08 (1.08 - 1.09)	< 0.001	225912	1.04 (1.04 - 1.04)	< 0.001
Hip^ (cm)	272353	1.06 (1.06 - 1.06)	< 0.001	228050	1.06 (1.06 - 1.06)	< 0.001
Hip*	270537	0.95 (0.95 - 0.96)	< 0.001	225914	0.97 (0.96 - 0.97)	< 0.001

^: unadjusted, **#:** adjusted for age, ethnicity and deprivation, ***:** adjusted for age, BMI, ethnicity, and deprivation.
T2DM: type two diabetes mellitus, **OR:** odds ratio, **CI:** confident interval

Table 5-6: Binary logistic regression analysis of the association between bio-impedance measurements and T2DM from the UK Biobank by sex

Bio-impedance measurements	Females			Males		
	N	OR (95% CI)	*P*-Value	N	OR (95% CI)	*P*-Value
Total body fat^ (kg)	268251	1.07 (1.07 - 1.07)	< 0.001	223395	1.08 (1.07 - 1.08)	< 0.001
Total body fat*	266813	0.99 (0.98 - 0.99)	< 0.001	221686	1.00 (0.99 - 1.00)	0.137
Trunk fat^ (kg)	268163	1.14 (1.13 - 1.14)	< 0.001	224000	1.14 (1.14 - 1.14)	< 0.001
Trunk fat*	266727	0.97 (0.97 - 0.98)	< 0.001	222280	1.01 (1.00 - 1.02)	0.006
Arms fat^ (kg)	268194	1.73 (1.70 – 1.75)	< 0.001	224097	2.00 (1.96 - 2.03)	< 0.001
Arms fat*	266758	0.68 (0.65 - 0.73)	< 0.001	222377	0.90 (0.86 - 0.94)	< 0.001
Legs fat^ (kg)	268296	1.29 (1.28 - 1.95)	< 0.001	224135	1.31 (1.30 - 1.32)	< 0.001
Legs fat*	266859	0.869 (0.85 - 0.89)	< 0.001	222415	0.95 (0.94 - 0.97)	< 0.001

^: unadjusted, ***:** adjusted for age, BMI, ethnicity, and deprivation.
T2DM: type two diabetes mellitus, **OR:** odds ratio, **CI:** confident interval

Table 5-7: Binary logistic regression analysis of the association between DEXA measurements and T2DM from the UK Biobank by sex

DEXA measurements	Females			Males		
	N	**OR (95% CI)**	***P*-Value**	**N**	**OR (95% CI)**	***P*-Value**
Total fat^ (kg)	2713	1.07 (1.05 - 1.10)	< 0.001	2457	1.05 (1.03 - 1.07)	< 0.001
Total fat*	2700	0.99 (0.94 - 1.04)	0.597	2447	1.01 (0.97 - 1.05)	0.633
Trunk fat^ (kg)	2713	1.14 (1.10 - 1.19)	< 0.001	2457	1.09 (1.06 - 1.12)	< 0.001
Trunk fat*	2700	1.09 (1.01 - 1.17)	0.029	2447	1.05 (0.99 - 1.10)	0.107
Arms total fat^ (kg)	2713	1.89 (1.57 - 2.26)	< 0.001	2457	1.66 (1.34 - 2.05)	< 0.001
Arms total fat*	2700	1.14 (0.82 - 1.58)	0.444	2447	0.96 (0.67 - 1.38)	0.822
Legs total fat^ (kg)	2713	1.07 (0.98 - 1.16)	0.118	2457	1.11 (1.04 - 1.19)	0.002
Legs total fat*	2700	0.71 (0.62 - 0.82)	< 0.001	2447	0.92 (0.82 - 1.03)	0.125
Android fat^ (kg)	2713	2.03 (1.69 - 2.45)	< 0.001	2457	1.54 (1.34 - 1.77)	< 0.001
Android fat*	2700	1.63 (1.14 - 2.23)	0 .008	2447	1.23 (0.95 - 1.59)	0.110
Gynoid fat^ (kg)	2713	1.30 (1.12 - 1.50)	< 0.001	2457	1.30 (1.15 - 1.46)	< 0.001
Gynoid fat*	2700	0.63 (0.48 - 0.82)	0.001	2447	0.92 (0.74 - 1.15)	0.474
VAT^ (kg)	2678	4.90 (3.34 - 7.07)	< 0.001	2457	1.94 (1.59 - 2.35)	< 0.001
VAT*	2607	3.97 (2.32 - 6.79)	< 0.001	2421	1.59 (1.20 - 2.12)	0.001

^: unadjusted, ***:** adjusted for age, BMI, ethnicity, and deprivation.
T2DM: type two diabetes mellitus, **OR:** odds ratio, **CI:** confident interval, **VAT:** visceral adipose tissue.

Table 5-8: Binary logistic regression analysis of the association between MRI measurements and T2DM grouped by sex from the UK Biobank

Variable	Females			Males		
	N	OR (95% CI)	*P*-Value	N	OR (95% CI)	*P*-Value
Trunk fat^ (L)	3145	1.20 (1.15 - 1.26)	< 0.001	2850	1.15 (1.10 – 1.19)	< 0.001
Trunk fat*	3131	1.11 (1.02 – 1.22)	0.023	2837	1.08 (1.00 – 1.15)	0.045
ASAT^ (L)	3145	1.23 (1.15 - 1.31)	< 0.001	2850	1.16 (1.09 – 1.23)	< 0.001
ASAT*	3131	1.02 (0.90 - 1.61)	0.713	2837	0.92 (0.82 – 1.04)	0.167
VAT^ (L)	3145	1.86 (1.61 - 2.14)	< 0.001	2850	1.38 (1.28 – 1.5)	< 0.001
VAT*	3131	1.58 (1.29 – 1.94)	< 0.001	2837	1.27 (1.14 – 1.41)	< 0.001

^: unadjusted, ***:** adjusted for age, BMI, ethnicity, and deprivation.
T2DM: type two diabetes mellitus, **OR:** odds ratio, **CI:** confident interval, **ASAT:** abdominal subcutaneous adipose tissue, **VAT:** visceral adipose tissue.

5.5.3 The association between adiposity and WBCs

Person correlation analysis was used to examine the relationship between total WBC and differential WBC (neutrophils, lymphocytes, and monocytes) with adiposity measurements. Results are shown in Table 5-9 for anthropometric characteristics, Table 5-10 for bio-impedance measurements, Table 5-11 for DEXA measurements, and Table 5-12 for MRI measurements.

All the adiposity measurements were positively correlated with total and differential WBC ($p < 0.01$). Total WBC showed positive correlation with android fat ($r = 0.203$) and trunk fat ($r = 0.202$ for DEXA and $r = 0.182$ for MRI). Monocytes showed the strongest correlation with VAT ($r = 0.187$ for DEXA and 0.183 for MRI) compared to the other WBCs.

Table 5-9: Person correlation of WBC count and Anthropometric measurements from the UK Biobank

	WBC	Neutrophil	Lymphocyte	Monocytes	Weight	BMI	WC	HC
WBC	1							
Neutrophil	0.794**	1						
Lymphocyte	0.706**	0.154**	1					
Monocytes	0.452**	0.216**	0.301**	1				
Weight	0.101**	0.091**	0.034**	0.114**	1			
BMI	0.155**	0.138**	0.08**	0.089**	0.834**	1		
WC	0.158**	0.15**	0.055**	0.144**	0.887**	0.814**	1	
HC	0.103**	0.093**	0.053**	0.054**	0.79**	0.863**	0.735**	1

** Correlation is significant at the 0.01 level (2-tailed).
WBC: white blood cell, **BMI:** body mass index, **WC:** waist circumference, **HC:** hip circumference

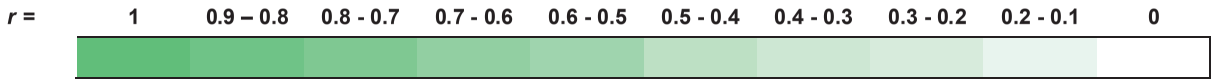


Table 5-10: Person correlation of WBC count and Bio-impedance measurements from the UK Biobank

	WBC	Neutrophil	Lymphocyte	Monocyte	Total body fat	Trunk fat	Arms fat	Legs fat
WBC	1							
Neutrophil	0.794**	1						
Lymphocyte	0.706**	0.154**	1					
Monocyte	0.452**	0.216**	0.301**	1				
Total body fat	0.138**	0.122**	0.085**	0.04**	1			
Trunk fat	0.138**	0.122**	0.072**	0.081**	0.943**	1		
Arms fat	0.136**	0.123**	0.082**	0.033**	0.955**	0.854**	1	
Legs fat	0.114**	0.097**	0.089**	- 0.019**	0.91**	0.727**	0.903**	1

** Correlation is significant at the 0.01 level (2-tailed).
WBC: white blood cell.

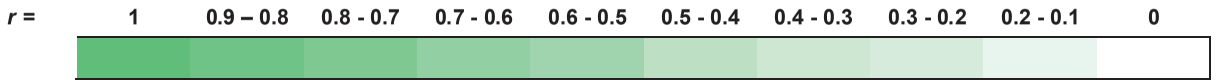


Table 5-11: Person correlation of WBC count and Body composition measurements done by DEXA from the UK Biobank

	WBC	Neutrophil	Lymphocyte	Monocyte	Total body fat	Trunk fat	Arms fat	Legs fat	Android fat	Gynoid fat	VAT
WBC	1										
Neutrophil	0.794**	1									
Lymphocyte	0.706**	0.154**	1								
Monocyte	0.452**	0.216**	0.301**	1							
Total body fat	0.183**	0.134**	0.17**	0.074**	1						
Trunk fat	0.202**	0.154**	0.163**	0.121**	0.954**	1					
Arms fat	0.163**	0.117**	0.171**	0.031*	0.895**	0.783**	1				
Legs fat	0.098**	0.062**	0.137**	0.032*	0.832**	0.631	0.826**	1			
Android fat	0.203**	0.157**	0.153**	0.138**	0.919**	0.986	0.735	0.566**	1		
Gynoid fat	0.134**	0.097**	0.149**	0.002	0.909**	0.763	0.857	0.956	0.703**	1	
VAT	0.176**	0.138**	0.106**	0.187**	0.647**	0.816	0.411	0.169	0.863	0.326**	1

** Correlation is significant at the 0.01 level (2-tailed), * Correlation is significant at the 0.05 level (2-tailed), **WBC:** white blood cell, **VAT:** visceral adipose tissue

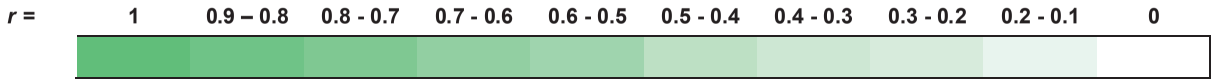
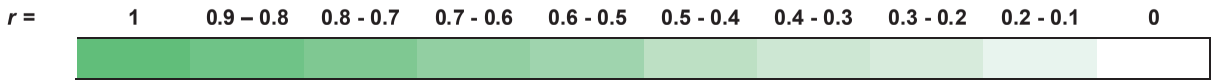


Table 5-12: Person correlation of WBC count and Abdominal MRI measurements from the UK Biobank

	WBC	Neutrophil	Lymphocyte	Monocytes	Trunk fat	ASAT	VAT
WBC	1						
Neutrophil	0.794**	1					
Lymphocyte	0.706**	0.154**	1				
Monocytes	0.452**	0.216**	0.301**	1			
Trunk fat	0.182**	0.162**	0.102**	0.121**	1		
ASAT	0.154**	0.131**	0.11**	0.043**	0.889**	1	
VAT	0.15**	0.142**	0.051**	0.183**	0.761**	0.381**	1

** Correlation is significant at the 0.01 level (2-tailed), * Correlation is significant at the 0.05 level (2-tailed).
WBC: white blood cell, **ASAT:** abdominal adipose tissue, **VAT:** visceral adipose tissue.



To measure the association of each measurement of adiposity with total WBC count, I performed simple linear regression analysis. Multiple linear regression analysis was also performed to adjust for: age, BMI, ethnic background, smoking status, and socioeconomic status. Each sex was analysed separately, Table 5-13 for females and Table 5-14 for males. In females, WC, android fat, and VAT (both DEXA and MRI) showed the strongest effect on WBC count (SBC = 0.189, 0.232, 0.258, and 0.190 respectively). These results were modestly attenuated after the adjustment. In males, the same fat measurements had a similar but slightly lesser effect on WBC count with the addition of trunk fat measured by both DEXA and MRI (SBC = 0.185 and 0.198 respectively).

Table 5-13: Linear regression analysis of the association of WBC count with adiposity (females) from UK biobank

Exposure	N	Model R square	Beta coefficient (per 1 unit increase in exposure) (95% CI)	Standardized beta coefficient (per 1 SD)	*P*-value
Anthropometric measurements					
Weight**^** (kg)	258135	0.018	0.020 (0.019 - 0.020)	0.134	< 0.001
Weight[#]	257826	0.061	0.021 (0.020 - 0.021)	0.141	< 0.001
WC**^** (cm)	258390	0.035	0.031 (0.03 - 0.031)	0.186	< 0.001
WC*	257677	0.077	0.020 (0.018 - 0.021)	0.119	< 0.001
HC**^** (cm)	258374	0.017	0.026 (0.025 - 0.026)	0.129	< 0.001
HC*	257682	0.077	- 0.023 [- 0.025 – (- 0.021)]	- 0.115	< 0.001
Bio-impedance measurements					
Total body fat**^** (kg)	254562	0.024	0.032 (0.031 - 0.033)	0.155	< 0.001
Total body fat*	254194	0.075	- 0.013 [- 0.016 - (- 0.011)]	- 0.065	< 0.001
Trunk fat**^** (kg)	254471	0.020	0.055 (0.054 - 0.057)	0.141	< 0.001
Trunk fat *	254105	0.054	- 0.011 [- 0.014 - (- 0.008)]	- 0.027	< 0.001
Arms fat**^** (kg)	254555	0.027	0.471 (0.460 - 0.482)	0.165	< 0.001
Arms fat*	254188	0.075	- 0.276 [- 0.321 - (- 0.231)]	- 0.097	< 0.001
Legs fat**^** (kg)	254614	0.026	0.187 (0.182 - 0.191)	0.160	< 0.001
Legs fat*	254247	0.076	- 0.177 [- 0.195 - (- 0.159)	- 0.152	< 0.001
DEXA measurements					
Total body fat**^** (kg)	2619	0.038	0.033 (0.027 - 0.039)	0.196	< 0.001
Total body fat*	2612	0.094	- 0.002 (- 0.013 - 0.01)	- 0.011	0.750
Trunk fat**^** (kg)	2619	0.052	0.061 (0.051 - 0.071)	0.227	< 0.001
Trunk fat*	2612	0.097	0.025 (0.007 - 0.042)	0.092	0.006
Arms fat**^** (kg)	2619	0.033	0.283 (0.225 - 0.341)	0.183	< 0.001
Arms fat*	2612	0.094	- 0.001 (- 0.092 - 0.093)	0.00	0.988
Legs fat**^** (kg)	2619	0.010	0.052 (0.032 - 0.071)	0.099	< 0.001
Legs fat*	2612	0.103	- 0.073 [- 0.101 - (- 0.046)]	- 0.142	< 0.001
Android fat**^** (kg)	2619	0.054	0.326 (0.274 - 0.378)	0.232	< 0.001
Android fat*	2612	0.098	0.147 (0.059 - 0.235)	0.105	0.001
Gynoid fat**^** (kg)	2619	0.02	0.150 (0.110 - 0.190)	0.142	< 0.001
Gynoid fat*	2612	0.097	- 0.092 [- 0.152 - (- 0.032)]	- 0.088	0.003
VAT**^** (kg)	2586	0.066	0.713 (0.609 - 0.816)	0.258	< 0.001
VAT*	2579	0.109	0.510 (0.366 - 0.654)	0.184	< 0.001
MRI measurements					
Trunk fat**^** (L)	3018	0.030	0.072 (0.057 - 0.087)	0.172	< 0.001
Trunk fat*	3012	0.054	0.026 (0.000 - 0.051)	0.061	0.051
ASAT**^** (L)	3018	0.023	0.089 (0.066 - 0.106)	0.151	< 0.001
ASAT*	3012	0.053	- 0.002 (- 0.038 - 0.033)	- 0.004	0.899
VAT**^** (L)	3018	0.036	0.247 (0.201 - 0.292)	0.190	< 0.001
VAT*	3012	0.061	0.161 (0.098 - 0.224)	0.124	< 0.001

^: unadjusted, **[#]:** adjusted for age, ethnicity, and deprivation, ***:** adjusted for age, BMI, ethnicity, and deprivation.
IDV: independent variable, **USBC:** unstandardized beta coefficient, **SBC:** standardised beta coefficient, **CI:** confident interval, **WC:** waist circumference, **HC:** hip circumference, **ASAT:** abdominal adipose tissue**, VAT:** visceral adipose tissue.

Table 5-14: Linear regression analysis of the association of WBC count with adiposity (Males) from the UK Biobank

Exposure	N	Model R square	Beta coefficient (per 1 unit increase in exposure) (95% CI)	Standardized beta coefficient (per 1 SD)	*P*-value
Anthropometric measurements					
Weight^ (kg)	218539	0.006	0.011 (0.011 - 0.012)	0.074	< 0.001
Weight#	218256	0.067	0.014 (0.013 - 0.014)	0.090	< 0.001
WC^ (cm)	218847	0.023	0.029 (0.028 - 0.030)	0.151	< 0.001
WC*	217999	0.079	0.020 (0.018 - 0.021)	0.102	< 0.001
HC^ (cm)	218813	0.005	0.02 (0.019 - 0.021)	0.069	< 0.001
HC*	218001	0.079	- 0.024 [- 0.026 – (- 0.021)]	- 0.084	< 0.001
Bio-impedance measurements					
Total body fat^ (kg)	214403	0.018	0.036 (0.035 - 0.037)	0.135	< 0.001
Total body fat*	213949	0.078	0.018 (0.015 - 0.021)	0.068	< 0.001
Trunk fat^ (kg)	214971	0.018	0.058 (0.056 - 0.060)	0.133	< 0.001
Trunk fat*	214512	0.078	0.024 (0.020 - 0.028)	0.056	< 0.001
Arms fat^ (kg)	215065	0.014	0.553 (0.534 - 0.573)	0.119	< 0.001
Arms fat*	214606	0.077	0.065 (0.051 - 0.106)	0.013	0.009
Legs fat^ (kg)	215102	0.018	0.224 (0.217 - 0.231)	0.133	< 0.001
Legs fat*	214643	0.078	0.094 (0.079 - 0.108)	0.056	< 0.001
DEXA measurements					
Total body fat^ (kg)	2388	0.028	0.030 (0.028 - 0.038)	0.168	< 0.001
Total body fat*	2385	0.065	0.017 (0.005 - 0.029)	0.095	0.006
Trunk fat^ (kg)	2388	0.032	0.047 (0.037 - 0.058)	0.180	< 0.001
Trunk fat*	2385	0.067	0.033 (0.015 - 0.050)	0.123	< 0.001
Arms fat^ (kg)	2388	0.023	0.307 (0.226 - 0.387)	0.151	< 0.001
Arms fat*	2385	0.063	0.108 (- 0.013 - 0.228)	0.053	0.079
Legs fat^ (kg)	2388	0.014	0.084 (0.056 - 0.112)	0.120	< 0.001
Legs fat*	2385	0.062	0.010 (- 0.029 - 0.049)	0.014	0.631
Android fat^ (kg)	2388	0.034	0.243 (0.191 - 0.295)	0.185	< 0.001
Android fat*	2385	0.068	0.173 (0.088 - 0.258)	0.131	< 0.001
Gynoid fat^ (kg)	2388	0.020	0.173 (0.124 - 0.222)	0.140	< 0.001
Gynoid fat*	2385	0.062	0.044 (- 0.031 - 0.119)	0.035	0.253
VAT^ (kg)	2363	0.034	0.319 (0.250 - 0.387)	0.185	< 0.001
VAT*	2360	0.066	0.188 (0.090 - 0.287)	0.109	< 0.001
MRI measurements					
Trunk fat^ (L)	2761	0.039	0.074 (0.061 - 0.088)	0.198	< 0.001
Trunk fat*	2757	0.075	0.050 (0.028 - 0.072)	0.133	< 0.001
ASAT^ (L)	2761	0.030	0.114 (0.09 - 0.139)	0.173	< 0.001
ASAT*	2757	0.072	0.059 (0.021 - 0.097)	0.090	0.009
VAT^ (L)	2761	0.036	0.137 (0.111 - 0.163)	0.191	< 0.001
VAT*	3012	0.075	0.074 (0.039 - 0.110)	0.104	< 0.001

^: unadjusted, **#:** adjusted for age, ethnicity, and deprivation, ***:** adjusted for age, BMI, ethnicity, and deprivation.
IDV: independent variable, **USBC:** unstandardised beta coefficient, **SBC:** standardised beta coefficient, **CI:** confident interval, **WC:** waist circumference, **HC:** hip circumference, **ASAT:** abdominal adipose tissue**, VAT:** visceral adipose tissue.

5.5.4 The association of WBC count with type 2 diabetes by sex

Binary logistic regression analysis was used to investigate the association between WBC count and diabetes. Results are shown in Table 5-15. Total WBC count was associated with diabetes in both sexes, OR = 1.21 (1.20 - 1.23), $p > 0.001$ for females and 1.12 (1.12 - 1.13), $p < 0.001$ for males. After controlling for BMI, age, smoking, and deprivation, the association remained strong in females while it deteriorated in males but remained significant. Furthermore, when analysing each type of WBC separately, they were associated with diabetes, but these associations change after adjusting. Out of the three types of WBC types, monocytes showed the strongest relationship with diabetes in males, OR (95% CI) = 1.73 (1.63 - 1.83) which decreased after adjustment for age, BMI, smoking status and deprivation, OR (95% CI) = 1.20 (1,12-1.28). In females, monocytes were highly associated with diabetes, OR (95% CI) = 1.43 (1.32 - 1.54) but adjustment led to a massive reduction in the association, OR = 1.10 (1.01 - 1.19). Moreover, neutrophils showed association with diabetes, OR (95% CI) = 1.31 (1.29 - 1.33) for females and 1.21 (1.20 - 1.23) for males, which didn't change much after adjusting for age, BMI, smoking status and deprivation. The association between T2DM and neutrophils were considerably higher in females compared to males.

Table 5-15: Binary logistic regression analysis of the association between WBC count and T2DM from the UK Biobank by sex

Variable	Females			Males		
	N	OR (95% CI)	*P*-Value	N	OR (95% CI)	*P*-Value
WBC^ (10^9 cells/Litre)	258960	1.21 (1.20 - 1.23)	< 0.001	219313	1.12 (1.12 - 1.13)	< 0.001
WBC*	256743	1.13 (1.11 - 1.14)	< 0.001	216843	1.06 (1.05 - 1.06)	< 0.001
Lymphocytes^ (10^9 cells/Litre)	258490	1.12 (1.10 - 1.14)	< 0.001	218899	1.04 (1.03 - 1.05)	< 0.001
Lymphocytes*	256280	1.06 (1.05 - 1.08)	< 0.001	216438	1.02 (1.01 - 1.03)	< 0.001
Monocytes^ (10^9 cells/Litre)	258490	1.43 (1.32 - 1.54)	< 0.001	218899	1.73 (1.63 - 1.83)	< 0.001
Monocytes*	256280	1.1 (1.01 - 1.19)	0.017	216438	1.20 (1.12 - 1.28)	< 0.001
Neutrophil^ (10^9 cells/Litre)	258490	1.31 (1.29 - 1.33)	< 0.001	218899	1.21 (1.20 - 1.23)	< 0.001
Neutrophil*	256280	1.24 (1.22 - 1.26)	< 0.001	216438	1.14 (1.13 - 1.15)	< 0.001

^: unadjusted, *****: adjusted for age, BMI, smoking status, and deprivation.
T2DM: type two diabetes mellitus, **WBC:** white blood cell, **OR:** odds ratio, **CI:** confident interval.

Next, I repeated Table 5-5, Table 5-6, Table 5-7, and Table 5-8 (Binary logistic regression analysis of the association between adiposity and diabetes) showing only central adiposity measurements since they are more associated with T2DM. I adjusted for age, BMI, smoking status, and deprivation and adjusted for the total WBC count. The reason for adjusting for total WBC count was to investigate whether the relationship between adiposity and diabetes was mediated by WBC count. Results are demonstrated in Table 5-16. In females, the association between diabetes and WC, trunk fat, android fat, and VAT remained significant even after the adjustment for age, BMI, smoking status and deprivation and total WBC count.

The strongest association with T2DM was by VAT measured by DEXA, OR (95% CI) = 3.67 (2.10 – 6.43), android fat measured by DEXA, OR (95% CI) = 1.67 (1.15 – 2.42), and VAT measured by MRI, OR (95% CI) = 1.64 (1.33 – 2.02). In males, the association between diabetes and both WC and VAT remained significant after the adjustment for age, BMI, smoking status and deprivation and total WBC count with VAT showing the strongest association, OR (95% CI) = 1.53 (1.14 – 2.05) measured by DEXA and OR (95% CI) = 1.27 (1.14 – 1.42) measured by MRI.

Table 5-16: Binary logistic regression analysis of the association between central adiposity and T2DM from the UK Biobank by sex

Variable	Females			Males		
	N	OR (95% CI)	*P*-Value	N	OR (95% CI)	*P*-Value
Anthropometric measurements						
Waist^ (cm)	272372	1.08 (1.08 - 1.08)	< 0.001	228090	1.07 (1.064 - 1.067)	< 0.001
Waist*	270531	1.08 (1.08 - 1.09)	< 0.001	225912	1.04 (1.04 - 1.04)	< 0.001
Waist♦	256686	1.08 (1.08 - 1.09)	< 0.001	216806	1.04 (1.03 - 1.04)	< 0.001
Bio-impedance measurements						
Trunk fat^ (kg)	268163	1.14 (1.13 - 1.14)	< 0.001	224000	1.14 (1.14 - 1.14)	< 0.001
Trunk fat*	266727	0.97 (0.97 - 0.98)	< 0.001	222280	1.01 (1.00 - 1.02)	0.006
Trunk fat♦	253139	0.98 (0.98 - 0.99)	< 0.001	213346	1.01 (1.00 - 1.02)	0.004
DEXA measurements						
Trunk fat^ (kg)	2713	1.14 (1.10 - 1.19)	< 0.001	2457	1.09 (1.06 - 1.12)	< 0.001
Trunk fat*	2700	1.09 (1.01 - 1.17)	0.029	2447	1.05 (0.99 - 1.10)	0.107
Trunk fat♦	2607	1.09 (1.01 - 1.17)	0.025	2378	1.04 (0.98 - 1.10)	0.172
Android fat^ (kg)	2713	2.03 (1.69 - 2.45)	< 0.001	2457	1.54 (1.34 - 1.77)	< 0.001
Android fat*	2700	1.63 (1.14 - 2.23)	0 .008	2447	1.23 (0.95 - 1.59)	0.110
Android fat♦	2607	1.67 (1.15 - 2.42)	0.007	2378	1.20 (0.93 - 1.56)	0.169
VAT^ (kg)	2678	4.90 (3.34 - 7.07)	< 0.001	2457	1.94 (1.59 - 2.35)	< 0.001
VAT*	2607	3.97 (2.32 - 6.79)	< 0.001	2421	1.59 (1.20 - 2.12)	0.001
VAT♦	2575	3.67 (2.10 - 6.43)	< 0.001	2353	1.53 (1.14 - 2.05)	0.004
MRI measurements						
Trunk fat^ (L)	3145	1.20 (1.15 - 1.26)	< 0.001	2850	1.15 (1.10 – 1.19)	< 0.001
Trunk fat*	3131	1.11 (1.02 – 1.22)	0.023	2837	1.08 (1.00 – 1.15)	0.045
Trunk fat♦	3005	1.13 (1.03 - 1.24)	0.014	2748	1.07 (1.0 - 1.15)	0.066
ASAT^ (L)	3145	1.23 (1.153 - 1.311)	< 0.001	2850	1.16 (1.09 – 1.23)	< 0.001
ASAT*	3131	1.02 (0.90 - 1.61)	0.713	2837	0.92 (0.82 – 1.04)	0.167
ASAT♦	3005	1.00 (0.91 - 1.18)	0.626	2748	0.91 (0.81 - 1.03)	0.133
VAT^ (L)	3145	1.86 (1.61 - 2.14)	< 0.001	2850	1.38 (1.28 – 1.5)	< 0.001
VAT*	3131	1.58 (1.29 – 1.94)	< 0.001	2837	1.27 (1.14 – 1.41)	< 0.001
VAT♦	3005	1.64 (1.33 - 2.02)	< 0.001	2131	1.27 (1.14 - 1.42)	< 0.001

^: unadjusted, ***:** adjusted for age, BMI, ethnicity, and deprivation, **♦:** adjusted for age, BMI, ethnicity, deprivation, and WBC
T2DM: type two diabetes mellitus, **OR:** odds ratio, **CI:** confident interval, **ASAT:** abdominal adipose tissue**, VAT:** visceral adipose tissue.

Finally, I repeated Table 5-15, but this time, I investigated whether the association of WBC with diabetes was mediated by body fat distribution. Here, I wanted to test whether the association between diabetes and WBCs was linked to these specific adiposity measurements (Table 5-17, for total WBC count), (Table 5-18, for Lymphocytes count), (Table 5-19, for monocytes count), and (Table 5-20, for neutrophil count).

In females, the association between T2DM and WBC count remained significant even after adjusting for the different body fat measurements. Out of the three sub-types of WBC count, neutrophils showed the strongest association with T2DM after adjusting for all the chosen body fat measurements (Table 5-20). The strongest association between T2DM and neutrophils was shown after the adjustment of trunk fat and android fat measured by DEXA, OR (95% CI) = 1.48 (1.22 – 1.81), and 1.46 (1.19 – 1.78) respectively.

In males, the adjustment of BMI and WC didn't change the association between T2DM and WBC count. The strongest associations were with monocytes, OR (95% CI) = 1.20 (1.12 – 1.28), and 1.18 (1.11 – 1.26) respectively. Adjusting for the other body fat measurements (android fat, trunk fat, and VAT) remove the association between WBC count and T2DM.

Table 5-17: Binary logistic regression analysis of the association between WBC count and T2DM from the UK Biobank by gender (Adjusted for BMI or WC)

Variable	Females			Males		
	N	OR (95% CI)	*P*-Value	N	OR (95% CI)	*P*-Value
WBC (unadjusted)	258960	1.21 (1.20 - 1.23)	< 0.001	219313	1.12 (1.12 - 1.13)	< 0.001
WBC (+BMI)	256743	1.13 (1.11 - 1.14)	< 0.001	216843	1.06 (1.05 - 1.06)	< 0.001
WBC (+WC)	257080	1.11 (1.10 - 1.12)	< 0.001	217363	1.05 (1.05 - 1.06)	< 0.001
WBC (+Android, DEXA)	2612	1.35 (1.15 - 1.57)	< 0.001	2380	1.03 (0.90 - 1.18)	0.663
WBC (+Trunk fat, DEXA)	2612	1.37 (1.17 - 1.59)	< 0.001	2380	1.03 (0.90 - 1.18)	0.664
WBC (+VAT, DEXA)	2580	1.23 (1.04 - 1.46)	0.016	2355	1.02 (0.89 - 1.18)	0.740
WBC (+Trunk fat, MRI)	3009	1.09 (1.02 - 1.16)	0.007	2750	1.02 (0.89 - 1.15)	0.823
WBC (+VAT, MRI)	3009	1.08 (1.01 - 1.16)	0.018	2750	1.02 (0.89 - 1.15)	0.814

The above are adjusted for age, ethnicity, smoking status, and deprivation + (body fat measurement).
T2DM: type two diabetes mellitus, **OR:** odds ratio, **CI:** confident interval**, WC:** waist circumference, **VAT:** visceral adipose tissue.

Table 5-18: Binary logistic regression analysis of the association between WBC count and T2DM from the UK Biobank by gender (Adjusted for body fat measured by bio-impedance)

Variable	Females			Males		
	N	OR (95% CI)	*P*-Value	N	OR (95% CI)	*P*-Value
Lymphocytes (unadjusted)	258490	1.12 (1.10 - 1.14)	< 0.001	218899	1.04 (1.03 - 1.05)	< 0.001
Lymphocytes (+BMI)	256280	1.06 (1.05 - 1.08)	< 0.001	216438	1.02 (1.01 - 1.03)	< 0.001
Lymphocytes (+WC)	256615	1.06 (1.04 - 1.07)	< 0.001	216957	1.02 (1.02 - 1.03)	< 0.001
Lymphocytes (+Android, DEXA)	2612	1.44 (1.03 - 2.02)	0.033	2378	1.00 (0.71 - 1.38)	0.943
Lymphocytes (+Trunk fat, DEXA)	2612	1.47 (1.05- 2.04)	0.024	2378	0.98 (0.70 - 1.38)	0.919
Lymphocytes (+VAT, DEXA)	2580	1.29 (0.85 - 1.95)	0.233	2353	0.97 (0.69 - 1.38)	0.876
Lymphocytes (+Trunk fat, MRI)	3008	1.07 (0.97 - 1.19)	0.196	2748	1.03 (0.76 - 1.39)	0.860
Lymphocytes (+VAT, MRI)	3008	1.07 (0.95 - 1.21)	0.271	2748	1.06 (0.80 - 1.39)	0.700
The above are adjusted for age, ethnicity, smoking status, and deprivation + (body fat measurement). **T2DM:** type two diabetes mellitus, **OR:** odds ratio, **CI:** confident interval, **WC:** waist circumference, **VAT:** visceral adipose tissue.						

Table 5-19: Binary logistic regression analysis of the association between WBC count and T2DM from the UK Biobank by gender (Adjusted for DEXA body fat measurements)

Variable	Females			Males		
	N	OR (95% CI)	*P*-Value	N	OR (95% CI)	*P*-Value
Monocytes (unadjusted)	258490	1.43 (1.32 - 1.54)	< 0.001	218899	1.73 (1.63 - 1.83)	< 0.001
Monocytes (+BMI)	256280	1.10 (1.01 - 1.19)	0.033	216438	1.20 (1.12 - 1.28)	< 0.001
Monocytes (+WC)	256615	1.06 (1.02 - 1.11)	0.006	216957	1.18 (1.11 - 1.26)	< 0.001
Monocytes (+Android, DEXA)	2612	1.44 (0.58 - 3.53)	0.432	2378	0.64 (0.16 - 2.54)	0.525
Monocytes (+Trunk fat, DEXA)	2612	1.52 (0.64 - 3.61)	0.347	2378	0.64 (0.16 - 2.54)	0.523
Monocytes (+VAT, DEXA)	2580	1.06 (0.26 - 4.26)	0.938	2353	0.65 (0.16 - 2.57)	0.537
Monocytes (+Trunk fat, MRI)	3008	1.26 (0.45 - 3.52)	0.655	2748	1.22 (0.36 - 4.18)	0.754
Monocytes (+VAT, MRI)	3008	1.06 (0.30 - 3.80)	0.927	2748	1.27 (0.37 - 4.34)	0.706
The above are adjusted for age, ethnicity, smoking status, and deprivation + (body fat measurement). **T2DM:** type two diabetes mellitus, **OR:** odds ratio, **CI:** confident interval, **WC:** waist circumference, **VAT:** visceral adipose tissue.						

Table 5-20: Binary logistic regression analysis of the association between WBC count and T2DM from the UK Biobank by gender (Adjusted for MRI fat measurements)

Variable	Females			Males		
	N	OR (95% CI)	*P*-Value	N	OR (95% CI)	*P*-Value
Neutrophil (unadjusted)	258490	1.31 (1.29 - 1.33)	< 0.001	218899	1.21 (1.20 - 1.23)	< 0.001
Neutrophil (+BMI)	256280	1.24 (1.22 - 1.26)	< 0.001	216438	1.14 (1.13 - 1.15)	< 0.001
Neutrophil (+WC)	256615	1.21 (1.19 - 1.22)	< 0.001	216957	1.13 (1.12 - 1.15)	< 0.001
Neutrophil (+Android, DEXA)	2612	1.46 (1.19 - 1.78)	< 0.001	2378	1.07 (0.90 - 1.27)	0.473
Neutrophil (+Trunk fat, DEXA)	2612	1.48 (1.22 - 1.81)	< 0.001	2378	1.07 (0.90 - 1.27)	0.463
Neutrophil (+VAT, DEXA)	2580	1.30 (1.04 - 1.61)	0.019	2353	1.06 (0.89 - 1.26)	0.531
Neutrophil (+Trunk fat, MRI)	3008	1.35 (1.13 - 1.61)	0.001	2748	1.01 (0.86 - 1.19)	0.904
Neutrophil (+VAT, MRI)	3008	1.27 (1.05 - 1.53)	0.012	2748	1.00 (0.85 - 1.18)	0.999
The above are adjusted for age, ethnicity, smoking status, and deprivation + (body fat measurement). **T2DM:** type two diabetes mellitus, **OR:** odds ratio, **CI:** confident interval, **WC:** waist circumference, **VAT:** visceral adipose tissue.						

5.6 Discussion

The present study was conducted to explore the links between body fat distribution, WBC count, and T2DM using the UK Biobank cohort. To accomplish that, all the adiposity measurements available in the UK Biobank study were used: anthropometric measurements, bio-impedance measurements, DEXA scans, and MRI scans to conduct this study. Each of these methods employs different ways to measure body fat, either direct or indirect measurement.

5.6.1 Adiposity and T2DM

In the current study, the prevalence of diabetes was higher in males (6%) compared to females (3%). This was similar to previous studies done in several large cohort studies (Yang, Dou & Song, 2010; Anjana *et al.*, 2011; Logue *et al.*, 2011; Soriguer *et al.*, 2012).

This study showed that central obesity was independently associated with T2DM in both sexes and was a strong predictor for the development of T2DM than total body fat. Out of all the central obesity measurements used in this study, VAT was a strong predictor for T2DM. The association of central adiposity with diabetes is well established. Several longitudinal studies have confirmed that visceral fat is more strongly associated with T2DM than total body fat (Neeland *et al.*, 2012; Wander *et al.*, 2013; Nordström *et al.*, 2016). Moreover, the association between VAT and T2DM in this study was stronger in females compared to males, although females with T2DM had less VAT than males with T2DM. In general, sex plays an important role in fat distribution. Adult females have more subcutaneous fat and can accommodate excess energy better than males by expanding the SAT. Therefore, females must put on more weight to reach the point where SAT become dysfunctional, leading to ectopic fat accumulation. Meanwhile, males ability to store excess fat in SAT is low leading to more ectopic fat accumulation with weight gain (Sattar, 2013). Also, studies have shown that differences in body fat distribution between males and females tend to diminish after menopausal age. Female body fat distribution after the menopause shows an increase of abdominal fat shifting to android or visceral adiposity, leading to a similar risk for diabetes to males. This provides an explanation of why men develop diabetes at a lower

BMI leading to a higher prevalence of diabetes among men. In general, younger females need to gain more weight than males to reach a threshold of ectopic fat accumulation that leads to insulin resistance and dysglycaemia.

5.6.2 Total and peripheral WBC count

Total and peripheral WBC count has been suggested to be associated with diabetes risk (Vozarova *et al.*, 2002; Veronelli *et al.*, 2004; Twig *et al.*, 2013; Vatcheva *et al.*, 2015). A recent meta-analysis of 20 studies including ;90,000 participants demonstrated a positive correlation between increased WBC level and diabetes risk (Gkrania-Klotsas *et al.*, 2010). The aim of this study was to assess whether WBC count is associated with both body fat distribution and T2DM and can be a link between the two.

5.6.2.1 Adiposity and WBC count

In the current study, the association of adiposity with WBC count was investigated. All adiposity measurements were positively correlated with total and differential WBCs with VAT showing the strongest correlation with monocyte concentration. Generally, in this study VAT showed the strongest association with WBC count in both sexes. These findings were in agreement with previous studies showing that obesity, and more particularly central obesity, are associated with elevated peripheral and total WBC count (Panagiotakos *et al*. 2005; Dixon & Brien 2006; Farhangi *et al*. 2013), but extend these by comparing a range of different measurements in far larger samples size. All previous reports used anthropometric measurements or bioimpedance to measure body fat, which does not adequately represent the distribution and the actual amount of body fat, and they are less useful as an independent marker for metabolic risk.

Borga *et al*. have examined different modes of adiposity measurement in UK Bio bank, using data from 4753 subjects (Borga *et al.*, 2018). They report that whole-body measurements of adipose tissue (AT) by DEXA and quantitative MRIs show excellent agreement with linear correlation of 0.99, and coefficient of variation (CV) of 4.5% for fat (computed from AT), but the agreement was found significantly lower for visceral adipose tissue, with a CV of >20%. Our study adds to this information by relating adiposity measurements to real clinical outcomes.

In UK Biobank, our use of DEXA, and MRI scans which are proven to be a more reliable and precise for body fat measurements. A recent study by Sung *et al.* using a non-contrast computed tomography (CT) to measure visceral fat showed that visceral adiposity was associated with higher circulating WBC count (Sung *et al.*, 2018). The study included 3291 Taiwanese with a mean age of 49.8 ± 9.8 years. Together with MRI scans, CT is considered the gold standard for analysing body fat composition and distribution (Borga *et al.*, 2018). Therefore, by including MRI for body fat measurements, the results of this study add robust evidence on the relationship between VAT and WBCs. In line with chapter 1, there are clear biological mechanisms to suggest why VAT is particularly proinflammatory (Figure 1-7). The evolutionary reasons that VAT may be proinflammatory are still being debated (West-Eberhard, 2019).

5.6.2.2 WBC count and T2DM

A growing body of evidence suggests that inflammation can activate various signal transduction cascades, including many of the pathways that are associated with insulin action and pancreatic ß-cell function which leads to insulin resistance and T2DM (Prattichizzo *et al.*, 2018). In the current study, this possibility was investigated using total and differential WBC count. The results showed that elevated total and differential WBC count were risk factors for developing T2DM in both sexes, although this appears to be mediated by visceral fat deposits. Previous prospective studies have investigated the association between WBC count and T2DM; a recent prospective study was done by Gu *et al.* examined the relationship between the incidence of diabetes and WBC count in 47,678 Chinese participants without diabetes (Gu *et al.*, 2018). In a period of 9 years, 1463 participants developed diabetes. They found that participants with elevated WBC count were at higher risk of developing T2DM in both groups' participants with obesity and participants without obesity compared with participants with low WBC count. In addition, a prospective cohort study by Volzorova *et al.* on Pima Indians with baseline normal glucose tolerance found that total WBC counts to be an independent risk factor for the development of T2DM, after controlling for age, gender and percent of body fat (Vozarova *et al.*, 2002). All these studies showed that the relationship between T2DM and WBC was independent of body weight. However, this study has specific strengths beyond existing relevant literature by using a large sample size (> 400,000 participants).

In contrast, other studies showed that the relation between WBC and T2DM was found only in patients with obesity. A cohort study was done by Twig *et al*. to examine if WBC count was independently associated with T2DM among 24,897 young Israeli men (Twig *et al.*, 2013). Participants were periodically screened for diabetes during a mean follow-up of 7.5 years. They found that high WBC count was associated with an increased risk of diabetes in men with overweight or obesity and not normal-weight men (BMI ≤ 25). Furthermore, a second cohort study was done on 3000 Mexican American adults with obesity and followed them for ten years (Vatcheva *et al.*, 2015). They showed that WBC count was related to the risk of T2DM. The relationship they found was only seen in participants with obesity, and not in overweight or normal-weight participants. Age and ethnicity can partially explain the cause for conflicting results as these studies included participants of varying age and ethnicity, which are independent risk factors for T2DM. Besides, these studies use BMI to try to link the association between T2DM and WBC count which cannot distinguish between lean and fat mass and provides no indication of body fat distribution which may be a key for the association with WBCs and not total obesity *per se*.

5.6.3 WBC count a putative link between obesity and T2DM

Further examination of the association between body fat distribution, WBC count, and T2DM was done. In the previous sections, it was shown that VAT was an independent risk factor for developing diabetes in both sexes and VAT was associated with an increase in total and differential WBC concentration. Also, T2DM was associated with a high WBC count. Thus, the possibility that WBCs could be the link between VAT and T2DM was examined since it has been shown that VAT is an independent risk factor for T2DM (Frikke-Schmidt *et al.*, 2016; Janochova, Haluzik & Buzga, 2019). In the current study, WBC count was associated with VAT but was not linked to T2DM. This was observed by the higher odds ratio in participants with T2DM that remained significant even after adjusting for WBC count (Table 5-16). The results of this study was in agreement with other study that showed that inflammation agreed with a recent far smaller study done by Rakotoraivelo *et al*. were they compare the expression of several pro- and anti-inflammatory cytokines in serum, SAT, and VAT from 89 obese patients undergoing bariatric surgery to 13 control patients (Rakotoarivelo *et al.*, 2018). They showed that in obese patients, the expression of cytokines is higher in VAT compared to

SAT and is independent of their diabetes status. Moreover, a study was done using the randomised double-blind CANTOS trial to test the effects of canakinumab (IL-1β inhibitor) on reduces incident diabetes (Everett *et al.*, 2018). The authors randomised 10,061 patients with prior myocardial infarction and C-reactive protein (CRP) ≥ 2 mg/l to placebo or canakinumab. Despite significant reductions in CRP and IL-6, IL-1β inhibition with canakinumab over five years did not reduce the risk of new-onset diabetes. There was only temporary improvement of HbA1c, nor did it have long-lasting effects on glycemia among those with diabetes.

The findings of the current study were consistent with the hypothesis that ectopic fat accumulation as a marker for subcutaneous adipose tissue dysfunction is the link between obesity and T2DM (Sattar & Gill, 2014) and that inflammation associated with T2DM could simply be a bystander related ectopic fat accumulation. That does not mean that inflammation derived from adipose tissue is necessarily unimportant to other chronic conditions such as CVD (West-Eberhard, 2019).

Another major finding of this study was gender differences in the immune response in T2DM. In this study, it was shown that central obesity was responsible for the association between WBCs and T2DM in males. While in females, the association between WBCs, particularly neutrophils, and T2DM is not entirely explained by central adiposity. This was evident by the remainder of the association even after adjusting for central adiposity measurements. The reason for this is not clear but may be due to differences in their responses to inflammatory stimuli. Gender differences in immune responses have been documented (Bhatia, Sekhon & Kaur, 2014). Females have better immune capabilities with higher immunoglobulin levels and stronger immune and inflammatory responses than males. This is evident in their lower incidence of infections but increased susceptibility to autoimmune diseases (Zandman-Goddard *et al.*, 2013). Nevertheless, this difference diminishes after menopause, which may indicate that it is most probably due to the effect of sex hormones (Yang *et al.*, 2016).

5.7 Summary

5.7.1 What was already known?

- Excess VAT is associated with insulin resistance (IR) and type 2 diabetes (T2DM).

- VAT and central fat mass generally are associated with inflammation measured by cytokines

- Inflammation may play a role in the development of T2DM.

5.7.2 What does this study add?

- VAT is an independent risk factor for developing T2DM: after adjustment each kg increase in VAT measured by DEXA was associated with 4-fold higher odds of T2DM in females and 1.5-fold in males.

- WBCs count was associated with VAT.

- The association between WBCs and T2DM is probably mainly explained by fat deposits in the visceral region, although the converse is not true.

5.7.3 What are the implications and future research needs?

- For metabolic risk assessment, body fat distribution measurements provide supporting information to crude total body fat.

- Understanding the role of body fat distribution in the pathogenesis of T2DM is important for the development of therapeutic strategies to treat the disease.

5.8 Conclusion

Assessing body fat distribution is likely to be more important to clinical metabolic risk than total body fat. Visceral fat despots measured by DEXA or MRI are best, but if not pragmatic, waist circumference may be acceptable. Bio-impedance is not a useful substitute for measuring regional fat deposits.

5.8.1 Strength and limitation

One of the major advantages of using UK Biobank to conduct our study, besides its large sample size, is that all the measurements have been performed in a unified and standardized way on all participants. Although the UK biobank cohort is not representative of the general population, its well-design, comprehensive phenotyping, and large size sample make it a reliable source to measure associations and risk factors between baseline characteristics and disease outcomes. There are potential limitations of this study. Lack of laboratory data to accurately diagnose T2DM. First, the cross-sectional approach used does not provide strong evidence in cause-effect relationships. Therefore, causality cannot be inferred from this work. Second, although I adjusted for many variables to minimize the potential for confounding, I cannot rule out the role of unmeasured confounders. Third, participants in the UK Biobank cohort are relatively healthy and may not be fully representative of the U.K. population (Manolio *et al.*, 2012). The work requires external validation and will have greater power as greater numbers of participants undergo MRI.

6 REDUCTION OF ADIPOSE LEVELS OF INFLAMMATION WHEN TREATING OBESITY: FEASIBILITY STUDY

6.1 Introduction

Given the fact that obesity is one of the strongest risk factors for developing T2DM (De Ferranti & Mozaffarian, 2008; Malnick, 2006; Bray, 2004), strong evidence suggests that tackling obesity early before the development of ß-cell dysfunction, or even after diagnosis, can improve or even reverse T2DM (American Diabetes Association, 2018). Obesity is a complex problem with no single solution to apply to everyone. Several strategies for weight loss have been introduced; among these methods, bariatric surgeries were considered the most effective treatment in obtaining substantial and durable weight loss in severe obesity.

Bariatric surgery (BS) can be defined as a surgical intervention that leads to large scale weight loss in obese patients. Since its development in the early 1950s, a lot of modification and improvements have been done to the original procedure (Nguyen & Varela, 2016). The development of new techniques led to the following two types of bariatric surgeries: 1. Gastric restriction procedures that decrease food intake and promote a feeling of satiety after meals, 2. Gastric restriction procedures with malabsorption that reduce the absorption of calories, proteins, and other nutrients. Recently, all procedures are done laparoscopically and include four types which are: laparoscopic adjustable gastric banding (LAGB), sleeve gastrectomy (SG), Roux-en-Y gastric bypass (RYGB), and biliopancreatic diversion with or without duodenal switch (BPD+DS), Figure 6-1 (Pareek *et al.*, 2018). The efficacy of bariatric surgery for sustained weight loss over time is well established. In a meta-analysis including 136 studies, 5 of them were randomised, with a total of 22,094 patients undergoing metabolic surgery showed a mean weight loss of 40 kg (Buchwald *et al.*, 2004). In another meta-analysis done by Gloy *et al.* including 11 randomised control trials with 796 obese patients (Gloy *et al.*, 2013b), bariatric surgery resulted in a 26 kg greater weight loss than nonsurgical treatment (95% confidence interval 21-26kg).

Recent evidence has shown that bariatric surgery is not just better for losing weight but is also superior in improving the metabolic dysfunction associated with obesity, particularly diabetes. The glycaemic improvement was thought to be only related to caloric restriction and malabsorption, but more recent studies have demonstrated that alterations of the gastrointestinal tract associated with bariatric surgery change the secretion of gut hormones and neurotransmitters

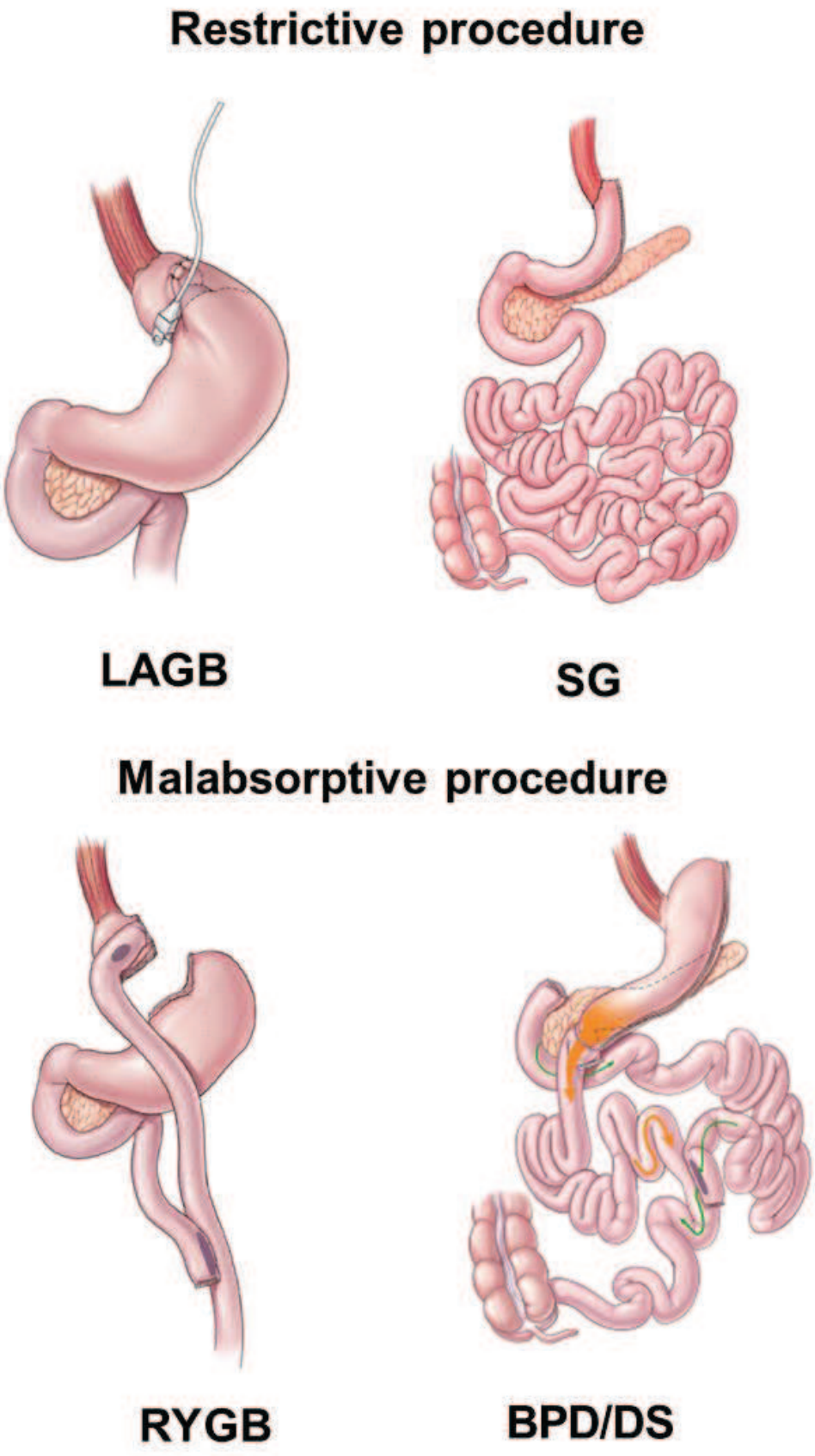


Figure 6-1: Types of bariatric surgeries.
LAGB: Laparoscopic adjustable gastric banding, **SG:** sleeve gastrectomy, **RYGB:** Roux-en-Y gastric bypass, **BPD/DS:** biliopancreatic diversion with or without duodenal switch. Reproduced from (Schauer *et al.*, 2016) with permission.

affecting appetite, satiety, energy expenditure, and glucose metabolism (Le Roux *et al.*, 2006; Miras & le Roux, 2013). Evidence continues to accumulate, showing that bariatric surgery is superior in achieving glycaemic control than conventional medical therapy. Recently, 11 randomised controlled clinical trials Table 6-1 were published comparing bariatric surgery with medical intervention in 794 obese patients with T2DM (Schauer *et al.*, 2016). The surgical procedures evaluated were RYGB (7 studies), LAGB (4 studies), BPD (1 study), and SG (1 study). The number of patients in each study ranged from 38 for the smallest study and 150 patients for the most extensive study. Postoperative follow up in each study ranged from 6 months to 5 years. Despite the variability in the 11 RCTs in both design and patients' characteristics, they were all, except one study (Ding *et al.*, 2015), supportive of the superiority of bariatric surgery compared to non-surgical treatments for glycaemic control. The study that didn't agree (Ding *et al.*, 2015) showed that LAGB and intensive medical diabetes and weight management program have similar 1-year benefits on diabetes control (remission for LAGB and medical treatment was 33% and 23%, respectively, P = 0.46). The reason for this result could be because the BS used was LAGB, which is less effective than the other BS (Himpens, 2008). Also, the studied population included patients with advanced T2DM (HbA1c 8.2% ± 1.2%, 40% on insulin) who might have less response to intervention.

The most apparent effect of BS is loss of up to half of total adipose tissue mass within the first year after surgery along with improvements in metabolic function (Galanakis *et al.*, 2015). The improvement in metabolic health observed after BS could be related to the restore function of SAT and most importantly the change in adipocyte size since the size of adipocytes dramatically influence the body metabolic function. Several studies investigating adipocyte size after BS found that adipocytes become smaller reaching diameters similar to lean controls (Cancello *et al.*, 2013; Aghamohammadzadeh *et al.*, 2013). In line with these observations, Anderson *et al.* reported that improvements in whole body insulin sensitivity 2 years after RYGB correlated strongly with a larger reduction in adipocyte size (Andersson *et al.*, 2014).

Also, it is worth considering the impact of bariatric surgery on the function of adipose tissue as an immunological organ capable of modulating not only immune populations in other tissues but also metabolic outcomes.

To advance our understanding of metabolic disturbance associated with obesity and why bariatric surgery is superior to other treatment options the **RE**duction of **A**dipose **L**evels of **I**nflammation when **T**reating obesit**Y** (REALITY) study was proposed. In this study BS will be used as a model of metabolic change (before and after weight loss) to study inflammatory markers, hormonal changes, adipocyte size, and miRNA expression. Conducting such a study is not easy, and Glasgow has specific practical challenges. Firstly, getting patients to participate in such studies and donating tissue samples is challenging. Secondly: there is low number of bariatric surgeries performed per year (~ 45) carried out at only two hospitals: Queen Elizabeth hospital or Glasgow Royal Infirmary, which means small sample number, and it is vital to be available for opportunities to enrol. Thirdly is the location of hospitals in Glasgow. Tissue sample collected must remain viable to be used for further analysis. The hospitals are located far away (~3.1 miles) from the university lab, so transportation of the samples must be organised. Finally, there is no standard published method for sample collection, handling, and processing, and analysing, to use directly therefore, the assessment of the reproducibility of the methods that will be used must be done before implementing them in a large-scale study. So, before starting this study, I will do a feasibility study to test the ability to overcome these challenges.

Table 6-1: Randomised control trials (RCTs) of bariatric surgeries vs. medical therapy for T2DM

	Study	Design	No. of patients	Follow up (Months)	T2DM remission rate		*P*-Value
					BS Group	MT Group	
1	(Dixon *et al.*, 2008)	LAGB vs control	60	24	73%	13%	<0.001
2	(Liang *et al.*, 2013)	RYGB vs control	101	12	90%	0%	<0.0001
3	(Halperin *et al.*, 2014)	RYGB vs control	38	12	58%	16%	0.03
4	(Wentworth *et al.*, 2014)	LAGB vs control	51	24	52%	8%	0.001
5	(Parikh *et al.*, 2014)	RYGB, LAGB, SG vs control	57	6	65%	0%	0.0001
6	(Mingrone *et al.*, 2015)	RYGB vs BPD vs control	60	60	42% and 68%	0%	0.003
7	(Ikramuddin *et al.*, 2015)	RYGB vs control	120	24	44%	9%	<0.001
8	(Courcoulas *et al.*, 2015)	RYGB vs LAGB vs control	69	36	40% and 29%	0%	0.004
9	(Ding *et al.*, 2015)	LAGB vs control	45	12	33%	23%	0.46
10	(Cummings *et al.*, 2016)	RYGB vs control	43	12	60%	5.9%	0.002
11	(Schauer *et al.*, 2017)	RYGB vs SG vs controls	150	60	29% and 23%	5%	<0.05

BS: bariatric surgery, **MT:** medical therapy, **LABG:** laparoscopic adjustable gastric band, **RYGB:** Roux-en-Y gastric bypass **SG:** Sleeve gastrectomy, **BPD:** biliopancreatic diversion. Reproduced from (Schauer *et al.*, 2016) with permission.

6.2 Aims

Before starting the REALITY (Reduction of adipose levels of inflammation when treating obesity) study, a pilot study was done to assess the feasibility of conducting such a study in Glasgow. The aim of the study was to:

1. Assess patient willingness to participate and to give adipose tissue samples at the time of surgery.

2. Determine the optimal collection, handling, and storage of blood and adipose samples for studying adipocyte morphology and miRNA expression.

3. Test the reproducibility of the chosen methods that will be used later in the large-scale study.

Research Questions:

Can I recruit patients to the study?

Can I transport samples to the University within a sufficient time window?

How best to transport samples to the university?

Do I have the resources and equipment needed to conduct the study?

Am I able to extract enough (> 100 ng) total RNA?

What is the best way to normalise miRNA expression in both adipose tissue and plasma samples?

Am I able to measure the expression of selected miRNAs in both plasma and adipose tissue?

Am I able to get good quality Haematoxylin and Eosin (H&E) slides to measure adipocyte diameter?

6.3 Methods

6.3.1 Generation of protocol

In order to determine the optimal collection, handling, and storage of blood and adipose tissue samples, I need first to decide what type of analytical methods I am going to use in our study. This was done by first, going through the literature to look for methods and protocols for studying adipocyte morphology and miRNA expression. Second, discuss the methods with other groups in the building who are expert in this field (Dr. Ian Salt) to make sure that I can conduct these protocols in our lab and to receive the proper training on these protocols under their supervision, and our own Standard Operating Procedure developed.

6.3.1.1 MiRNA expression

RNA is highly sensitive to degradation, and although this may not be as much of an issue for mature miRNA the issue requires study. Collection and handling samples can significantly affect the amount and quality of RNA extracted from samples. For circulating miRNAs, I decided to use the same protocol I standardised in the previous studies (Chapter 2.2). For adipose tissue, I will compare two methods for tissue preservation: 1. Snap freezing, on dry ice, is the most common method used for stabilising tissue samples intended for RNA analysis. 2. Submerging the tissue in RNA*Later*, which is a stabilising reagent that helps to stabilise the RNA and prevent degradation.

6.3.1.2 Adipocyte diameter

Two methods are commonly used for measuring adipocyte diameter: Collagenase digestion and histological analysis. The histological method is the commonly used method when the main aim is adipose tissue morphology. The disadvantages of this method are, it is more time consuming, require well-trained personnel, and the mean fat cell size is lower compared with collagenase digestion (Laforest *et al.*, 2017). In the collagenase digestion, adipose tissue is digested with collagenase to separate mature adipocytes from the stroma-vascular fraction by floatation. This method can be performed using basic laboratory equipment and is less time consuming. The main limitation of this method is it must be determined immediately after isolation, if the cell suspension is stored it causes changed in

the cell morphology such as distortion in larger isolated adipocytes (Gamage, Yashodara & Senanayake, 2018). Therefore, I decided to use the histological method because immediate analysis cannot be done in our arrangement. Besides, histological slide can be stored and used to assess adipocyte cell size in a retrospective manner.

6.3.2 Analytical plan

The next step was to organise with hospital staff involved in specimen collection to explain the study and what is exactly needed from them. I first met with the surgeon involved, Mr. Simon Gibson, and the Anaesthetist to develop a system that worked with the normal flow of their operating theatre. After that, instructions on how to collect and preserve the sample were explained to the surgeon and all the medical staff involved in the process of sample collection.

Finally, a Letter of access to conduct research through NHS Greater Glasgow and Clyde was approved (Appendix 4) and GCP (Good clinical practice) license was granted (Appendix 5) to start our study.

6.3.3 Patient Recruitment

This study was conducted in accordance with the protocol approved by the Research Ethics Committee based on the ethical standards in the Helsinki declaration (Appendix 6).

Invitation letters (Appendix 7) and information leaflet explaining the scientific background of the study were sent by post to 5 patients who were: participating in the SCOTS Study (Logue *et al.*, 2015), were due to have bariatric surgery in Greater Glasgow and Clyde and have consented to be contacted for future research studies. During their pre-operative clinic appointment, the researcher explained the study further and answered the patient's questions. Patients who agreed to participate signed informed consent (Appendix 8). Details demographic, anthropometric, and biochemical characteristics were taken from the preoperative assessment, around two weeks prior to the operation. Bariatric procedures were performed laparoscopically by a single surgeon.

6.3.4 Sample Collection and Processing

All surgeries were performed by Mr. Simon Gibson at the Queen Elizabeth University Hospital (QEUH). All preparations such as tubes, fixatives, equipment, labelling were done early in the morning at the day of the surgery in the University Lab then taken to QEUH by taxi before the surgery started. Upon arrival at the surgical theatre, tubes were hand into the surgical staff for samples collection.

For blood samples, they were taken by the Consultant anaesthetist before general anaesthesia was induced. All patients were fasting before the surgery. Samples were kept on ice until processed. After approximately 2 hours, at the end of the surgery, $2cm^3$ of subcutaneous adipose tissue was taken by the surgeon from the laparoscopic port site. The AT sample was divided by the surgeon in the operation theatre into three small pieces and placed in the labelled sterile tube. After that, non-sterile theatre staff delivered the sample outside the theatre. Immediately, one sample was snap frozen on dry ice, and the other two were fixed, one in RNA*Later*, and the other one in Zinc Formalin. The sample was then transferred by the researcher using a taxi back to the university lab. Upon arrival at the University Laboratory (approximately within 1 hour of the collection), samples were immediately processed and stored at − 80 ℃ for subsequent analysis (Figure 6-2).

6.3.4.1 Blood

Fasting blood samples were taken by the Consultant Anaesthetist from the patient having their bariatric surgery, having fasted for at least 12 hours and before general anaesthesia was induced (3 × 9 ml EDTA tube and 1 × 4 ml plain). Samples were kept on ice until processed within 4 hours of collection. Upon their arrival to University Laboratory, one plain tube and one EDTA tube were immediately centrifuged at 3000 rpm for 15 min. The harvested serum and plasma were stored frozen at $-80\,℃$ for subsequent analysis in 300 µl labelled aliquots to avoid frequent freezing and thawing. The second EDTA tube was used for measuring glycated haemoglobin (HbA1C). The last EDTA blood tube was aliquoted and stored at $-80\,℃$ as whole blood for total RNA extraction (which was carried out within one month of blood collection) and subsequent measuring miRNA expression. Serum samples were used for measuring glucose, insulin, and high sensitivity C

reactive protein (CRP). The plasma sample was used for measuring total adiponectin.

6.3.4.2 Adipose Tissue

Two cm^3 of subcutaneous adipose tissue was removed by the surgeon via the port site of the laparoscopic equipment at the end of the bariatric surgery procedure. Freshly excised adipose tissue was immediately dissected into three small pieces and kept in the sterile labelled tubes handed in before the surgery. Two of the three pieces were used for RNA extraction and miRNA quantification and expression. The first piece was fixed in RNA*Later* (Thermo Fisher Scientific, Loughborough, Cat: AM7020), which is an aqueous solution that rapidly penetrates fresh tissues to stabilize and protect RNA and eliminates the need to process or freeze samples immediately. The second piece was immediately snap frozen on dry ice. The last piece was fixed in zinc formalin for morphological studies. All samples were kept on ice until they were transported to the university lab (~ 1 hour).

6.3.5 Biochemical Analysis

Fasting blood Glucose (FBG), high sensitivity CRP, HbA1C, and insulin were analysed in the clinical biochemistry laboratory at the University of Glasgow. FBG, CRP, and HbA1C were analysed using an automated clinical analyser Cobas C311 (Roche Diagnostics; Burgess Hill, UK). Insulin was measured by a commercial ELISA (Mercodia, Uppsala, Sweden). The intra-assay coefficient of variation was 5·5%, and the inter-assay coefficient of variation was 9·7% for insulin. Insulin sensitivity was calculated using the HOMA-IR formula (Chapter **Error! Reference source not found.**). Plasma adiponectin was measured with ELISA Kit (Quantikine Human Adiponectin/Acrp30 Immunoassay, Cat. No. SRP300). Intra- and Inter- coefficient of variation is 4.1% and 4% respectively at adiponectin concentration = 7 µg/ml. Details on methods used are found in the general methods (Chapter 2.4.1).

6.3.6 miRNA Extraction and Expression

Total RNA was extracted from plasma and adipose tissue samples (RNA*Later* and snap frozen). The expressions of mir-221, mir-222, mir-144, mir-155, mir-192 and mir-193b (targeted based on previous literature, chapter 3.1.2) were measured using real-time quantitative polymerase chain reaction (RT-qPCR). Samples from each patient were run simultaneously. Details on the methodology are found in the general methods of chapter 2.2.

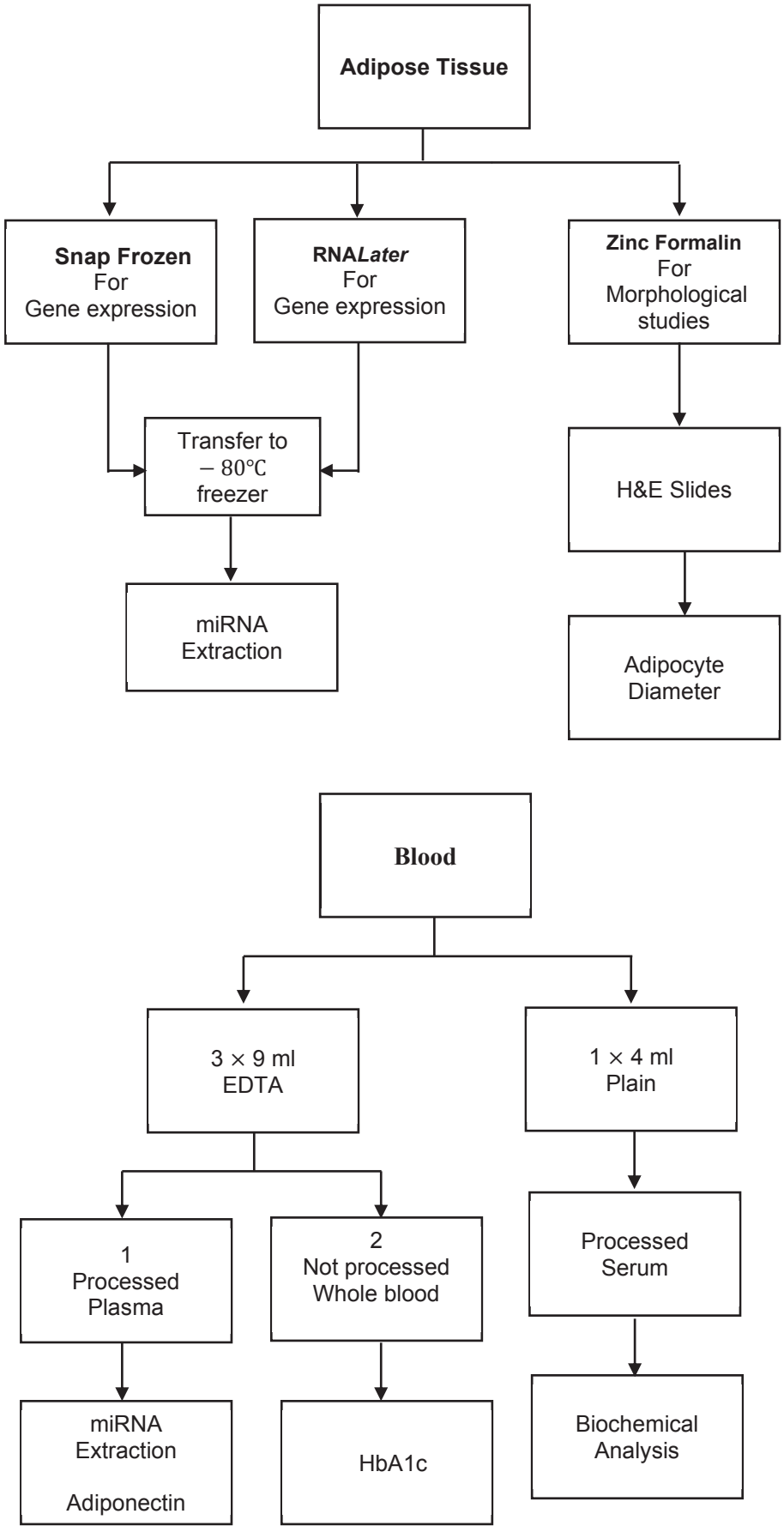


Figure 6-2: Sample processing in the Feasibility study

6.3.6.1 Normalisation and quality control for qRT-PCR results

Normalisation of qRT-PCR results in reference (housekeeping) gene was done for both samples plasma and adipose tissues.

Plasma

Mir-16 was tested as an endogenous control for plasma in this study in addition to using mir-39 as an exogenous control. Details are found in chapter 2.3.1.

Adipose Tissue

Three endogenous controls miRNAs were tested (mir-103, mir-331, and RNU44) using both snap frozen adipose tissue and RNA*later* fixed adipose tissue.

6.3.7 Measuring Adipocyte Diameter

Adipose tissue fixed in 10% zinc formalin was used for adipose tissue morphological analyse.

6.3.7.1 Slide Preparation

Adipose tissue $\sim 4\ mg$ pieces taken were fixed in 10 % Zinc Formalin Fixative (ZFF) over 24 hours at room temperature. After that, tissue was switch to 70% alcohol and kept at 4℃ until processing.

6.3.7.2 Processing and Embedding

Fixed tissues were placed into embedding cassettes in a tissue processor (Shandon Excelsior, Thermo Scientific), and dehydrated through a serial alcohol gradient and xylene before placing in paraffin wax ready for sectioning. Conditions for embedding were as follows:

1. 70 % alcohol 2 hours

2. 80 % alcohol 3 hours

3. 95 % alcohol 4 hours

4. 100 % alcohol (1) 4 hours

5. 100 % alcohol (2) 5 hours

6. 100 % alcohol (3) 5 hours

7. 100 % alcohol (4) 6 hours

8. 50 % alcohol / 50 % Xylene 4 hours

9. Xylene (1) 5 hours

10. Xylene (2) 5 hours

11. Wax (1) 5 hours

12. Wax (2) 5 hours

13. Wax (3) 6 hours

6.3.7.3 Sectioning of Tissue Block

Adipose tissue blocks were trimmed on a manual rotary microtome (RM2235, Leica) to a five µm thick ribbons. Paraffin ribbons were placed in a water bath at 40°C after they were collected onto silane treated slides (Clarity Microscope Slides). Eight slides were obtained from each sample with 3 - 4 sections on each slide. Sections were baked on to slides at 60°C for 4 hours followed by 40°C overnight. Once slides were prepared, they were stored at room temperature until staining.

6.3.7.4 Haematoxylin & Eosin staining

Paraffin-embedded adipose tissue sections were rehydrated by moving down an alcohol gradient. Paraffin was removed from sections by two consecutive washes in Histoclear for 7 minutes. This was followed by washing in 100% ethanol for 7 minutes, 95% ethanol for 7 minutes, and finally, 70% ethanol for 7 minutes. Sections were rehydrated by keeping them in water for 7 minutes. After that, sections were stained in Haematoxylin for 2 minutes, followed by 5 minutes wash

under running tap water. Slides were dipped into 70% ethanol for 30 seconds then stained with Eosin for 2 minutes. Sections were dehydrated again by running them up a concentration gradient of ethanol: Two 30 seconds washes of 95% ethanol, followed by two washes in 100% alcohol (one for 1 minute and the second for 7 minutes). Finally, slides underwent two washes in Histoclear for 5 minutes before being mounted using DPX. Slides were left overnight to dry.

6.3.7.5 Images

Slides were first viewed using a light microscope (Olympus BX41) on an X 10 objective lens. Images from each section were taken with a QImaging Go-3 camera attached to the microscope. Generated images were then opened using QCapture Pro 6.0 software. Serial static digital images were captured and stored in separate folders corresponding to specimen number. The total number of images per sample were 12. All images were stored in uncompressed 24-bit color TIFF format.

6.3.7.6 Image Analysis (Semiautomated method)

All images generated were analysed using *Adiposoft*, an open-source ImageJ plugin software for the analysis of adipose tissue cellularity in histological sections which was developed in MATLAB (MathWorks, Natick, MA). The software can be downloaded from:

http://imagej.net/Adiposoft#Installation

The software was validated and considered to be a fast and accurate quantitative analysis for adipocytes measurements (Galarraga *et al.*, 2012). Calibration of the software was carried out before image analysis. This was achieved by using a Micrometre Glass Slide, also known as a Stage Graticule Slide, for Microscope Calibration 1mm Horizontal Scale 100 divisions -10 µm intervals (Olympus, Tokyo) (Figure 6-3). The calibration was done using the same microscope and magnification lens (X 10 magnification). The minimum threshold for adipocyte diameter was set to 25 µm (Galarraga *et al.*, 2012). Adipocyte number, area, and diameter data were generated by the program after scanning 12 images for each patient. The histological images were also manually analysed and edited. Cells touching the screen edge, overlapping with adjacent cells or distorted by tissue cutting were excluded from the analysis. The final image represented whole

undistorted adipocytes of variable dimensions and shape (Figure 6-4). Data were saved as spreadsheet files for further statistical analysis.

6.3.8 Statistical Analysis

The selected miRNAs were normalized to cel-mir-39 and expressed as Ct. Variables were tested for normality by visual inspection of their histograms and normal Q-Q plots. Non-parametric variables were normalized by log transformation to use parametric tests. Descriptive statistics were expressed as mean ± SD for normally distributed variables, and median ± inter-quartile range for non-normally distributed variables. Comparison between mean values of the two methods of adipose tissue fixation, RNA*Later* (RL), and snap freezing on dry ice (SF) was evaluated using Student's two-tailed *t*-test. Adipocyte size was grouped according to diameter into four groups small < 50 µm, medium 50 – 69 µm, large 70 – 89 µm, and very large > 90 µm (Verboven *et al.*, 2018). Bar chart graphics were used to describe adipocyte diameter distribution

All the statistical tests were performed using IBM SPSS (Statistical Package for Social Sciences) windows version 22 (SPSS Inc. Chicago, USA) and Microsoft Excel 2016. The criterions for statistical significance was $p < 0.05$.

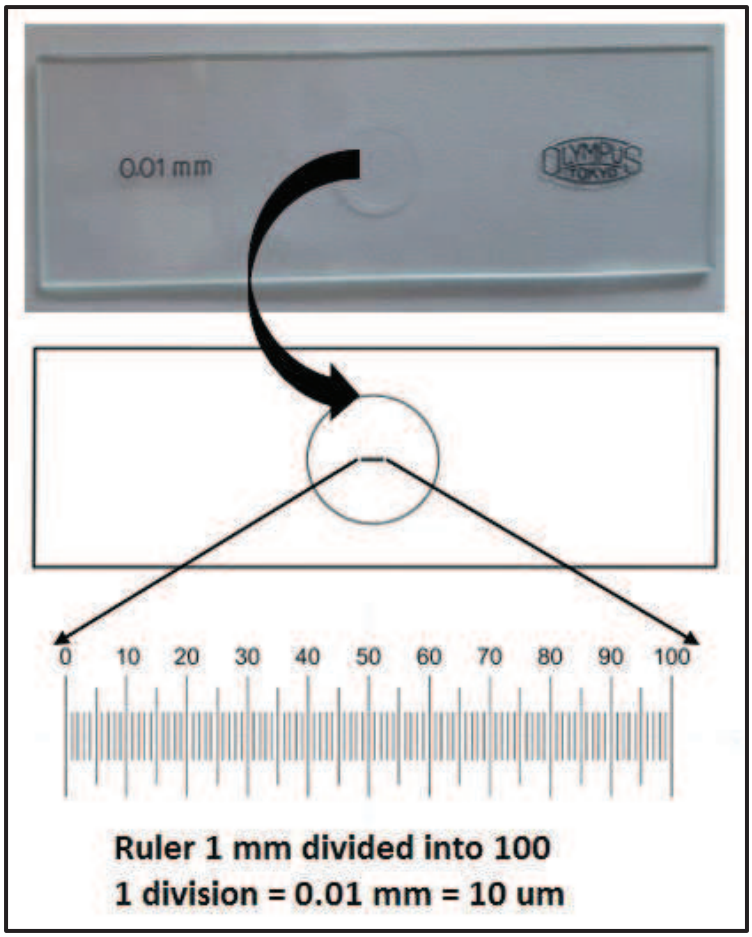


Figure 6-3: Micrometre glass slide used to calibrate adipocyte images

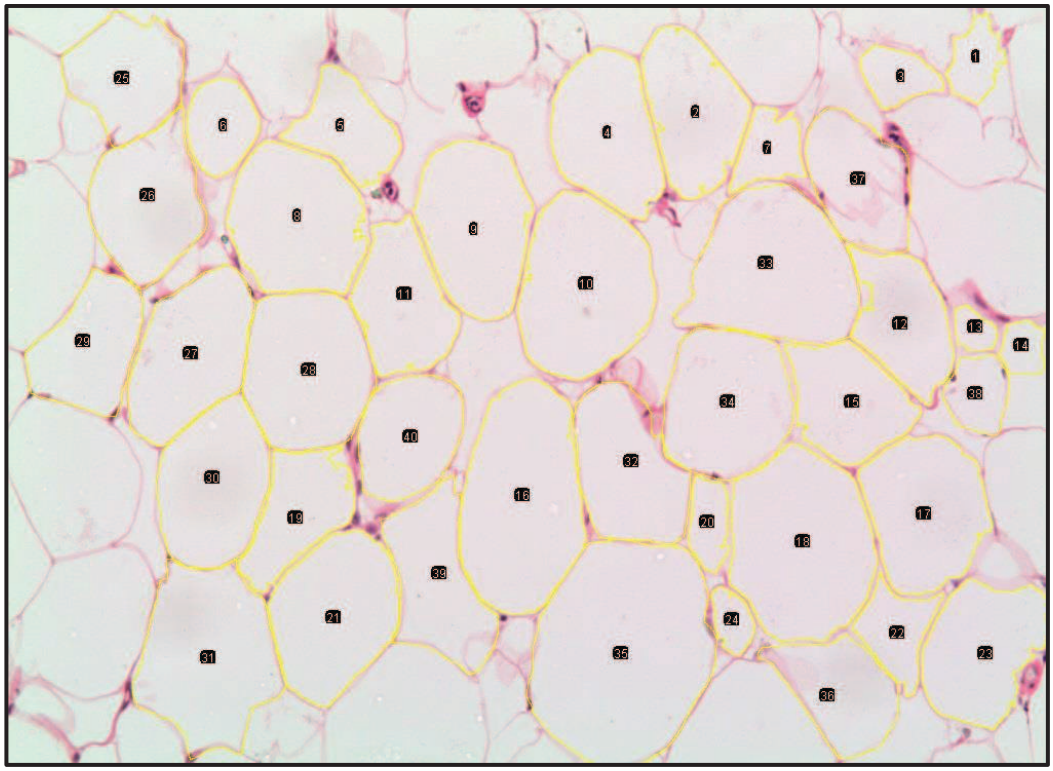


Figure 6-4: Adipose tissue image from a study patient, analysed by *Adiposoft* program

6.4 Results

6.4.1 General characteristics of patients included in the study

Table 6-2 summarises the general characteristics of the study population. A total of 5 patients undergoing bariatric surgery (4 Females and 1 Male) were included with a mean age of 37.4 ± 11.1 years. All patients were apparently healthy, with two patients showing slightly elevated blood pressure. Four patients were non-smokers, and one patient quit smoking prior to surgery. Mean BMI was 45 ± 3.7 kg/m^2, which is categorised as class III obesity according to WHO guidelines. Patients were normoglycemic, glucose = 5.3 ± 0.4 mmol/L and HbA1C = 38.6 (36-41) mmol/mol. In addition, patients were insulin sensitive, mean HOMA-IR = 1.1 and adiponectin = 3.2 µg/ml. High sensitive CRP was slightly elevated mean CRP = 5.3 mg/L.

6.4.2 Adipose tissue samples

I compared the two methods, snap freezing, on dry ice (SF) and submerging the tissue in RNA*Later* (RL), in terms of total RNA extracted, expression of housekeeping genes, and the expression of the six miRNA I am interested in.

6.4.2.1 Total RNA Extracted

The total RNA concentration was isolated from both snap frozen and RNA*Later* adipose tissue samples from all five patients (Table 6-3). The mean concentration of total RNA extracted was 36.4 ng/µl from snap frozen adipose tissue samples and 40.4 ng/µl from RNA*Later* adipose tissue sample. There was no difference in RNA concentration between the RNA*Later* adipose tissue samples and the snap frozen adipose tissue samples ($p > 0.05$) in terms of the amount of total RNA extracted.

Table 6-2: Patients Characteristics

Demographic Characteristics	**Mean (SD)**
Age (years)	37.4 (11.1)
Blood pressure (mm Hg)	119/81 (16/9)
Anthropometric Characteristics	
Body weight (kg)	127.6 (7.1)
Body mass index (kg/m^2) *	45 (3.7)
Biochemical Characteristics (Fasting)	
Plasma Glucose (mmol/L)	5.3 (0.4)
Insulin (pmol/L) *	5.2 (2.1-9.3)
HOMA-IR*	1.1 (0.25-2.3)
HbA1C (mmol/mol)	38.6 (36-41)
Adiponectin (µg/mL) *	3.2 (1.1-9.9)
High sensitivity C-reactive protein (mg/L) *	5.3 (2.6-21.2)

***:** Variables w not normally distributed were summarized as median (inter-quartile range).
HOMA-IR: Homeostatic model assessment for insulin resistance, **HbA1c:** Haemoglobin A1c, **SD:** standard deviation.

Table 6-3: Total RNA concentration from five patients using two preservative methods (snap frozen and RNA*Later*).

Patient	RNA*Later* Conc. (ng/µL)	Snap Frozen Conc. (ng/µL)
1	20.8	39.5
2	23.6	29.9
3	40.4	36.4
4	44.5	27.9
5	42.3	41.6
Median (IQR)	40.4 (22.2 – 43.4)	36.4 (28.9 – 40.3)
Conc : concentration, **IQR :** inter quarter range		

6.4.2.2 Normalisation of miRNA expression

Housekeeping genes or control genes were used to normalise the expression of genes between different samples. However, the expression level of these housekeeping genes may vary among tissues. In this study, I tested three housekeeping genes (mir-103, mir-331, and RNU44) which have been used for normalizing miRNA expression adipose tissue. Table 6-4 show the comparison between the expression of these housekeeping genes of the two adipose tissue samples (snap frozen vs. RNA*Later*). There is no difference in the expression of these selected housekeeping genes between the two adipose tissue samples ($p > 0.05$, for all three). When comparing the expression of the three housekeeping genes in adipose tissue RNU44 was highly expressed (Ct = 24) and more stable between samples (SD > 0.25) than the other two housekeeping genes (mir-103 and mir-331).

6.4.2.3 Expression of targeted miRNAs

Table 6-5 shows the expression of six miRNA in both adipose tissue samples. These miRNAs were chosen to be included in our study based on our previous literature search (Chapter 3.1.2), which showed evidence that their expression change by the influence of BMI and glycaemic control. There was no difference in their expression between the two adipose tissue methods (RNA*Later* and snap frozen). In general, mir-193b was highly expressed in adipose tissue compared to the other miRNAs.

Table 6-4: Comparing the expression of housekeeping genes between adipose tissue samples collected using RNA*Later* or snap frozen.

miRNA	AT (RL) Ct Mean (SD)	AT (SF) Ct Mean (SD)	*P*-Value
Mir-103	31.9 (1)	31.9 (0.7)	0.972
Mir-331	31.0 (1.5)	31.5 (1.7)	0.627
RNU44	24.0 (0.2)	24.0 (0.1)	0.803

miRNA: micro RNA, **Ct:** cycle threshold, **AT:** Adipose tissue, **SF:** snap frozen, **RL:** RNA*Later*, **SD:** standard deviation

Table 6-5: Comparing the expression of the selected six miRNAs in both snap frozen and RNA*Later* adipose tissue samples.

miRNA	AT (RL) Ct Mean (SD)	AT (SF) Ct Mean (SD)	*P*-Value
Mir-222	30.5 (1.6)	31.0 (1.4)	0.590
Mir-221	31.4 (0.9)	32.3 (1.2)	0.232
Mir-192	34.7 (0.7)	35.1 (0.4)	0.073
Mir-193b	26.6 (1.5)	27.2 (1.3)	0.971
Mir-144	34.0 (0.9)	35.0 (0.6)	0.422
Mir-155	34.1 (1.1)	34.1 (1.1)	0.501

miRNA: micro RNA, **Ct:** cycle threshold, **AT:** Adipose tissue, **SF:** snap frozen, **RL:** RNA*Later*, **SD:** standard deviation

6.4.3 Adipocyte Diameter

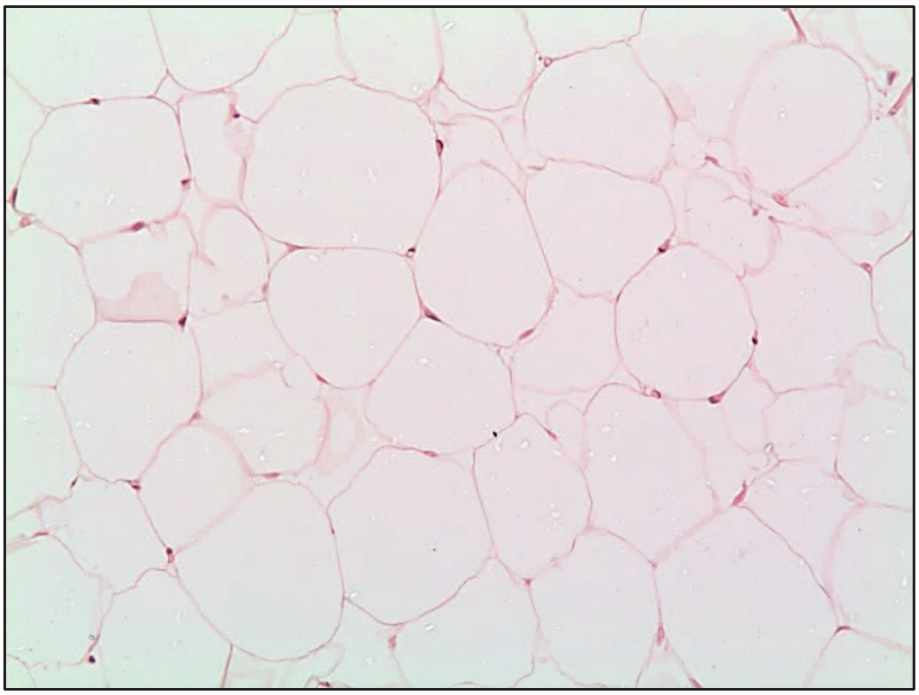
Adipocyte size was categorised into four groups small < 50 µm, medium 50 – 69 µm, large 70 – 89 µm, and very large > 90 µm. A bar chart was constructed showing the adipocyte size distribution for each participant as shown in section b of figuresFigure 6-5, Figure 6-6, Figure 6-7, Figure 6-8, and

Figure 6-9 while section a demonstrate one H&E section from each patient SAT sample. Table 6-6 describes the adipocytes measurements in SAT samples taken from all five patients. The mean adipocyte diameter was 58.2 ± 26 µm. In this study, participants had higher proportions of small adipocytes.

Table 6-6: SAT adipocyte measurement analysis in all five patients

Patients	Total Adipocytes counted	Adipocyte Diameter (µm) Mean (SD)	Small < 50 (%)	Medium 50 – 69 (%)	Large 70 – 89 (%)	V. Large > 90 (%)
1	634	59.8 (29)	47.3	18.6	15.0	19.1
2	839	58.2 (26)	46.8	21.8	16.4	14.9
3	927	59.4 (23)	40.3	28.2	21.3	10.2
4	721	57.7 (25)	46.6	23.9	16.8	12.8
5	752	56.1 (27)	52.4	20.7	12.5	14.4
SD: standard deviation						

a



b

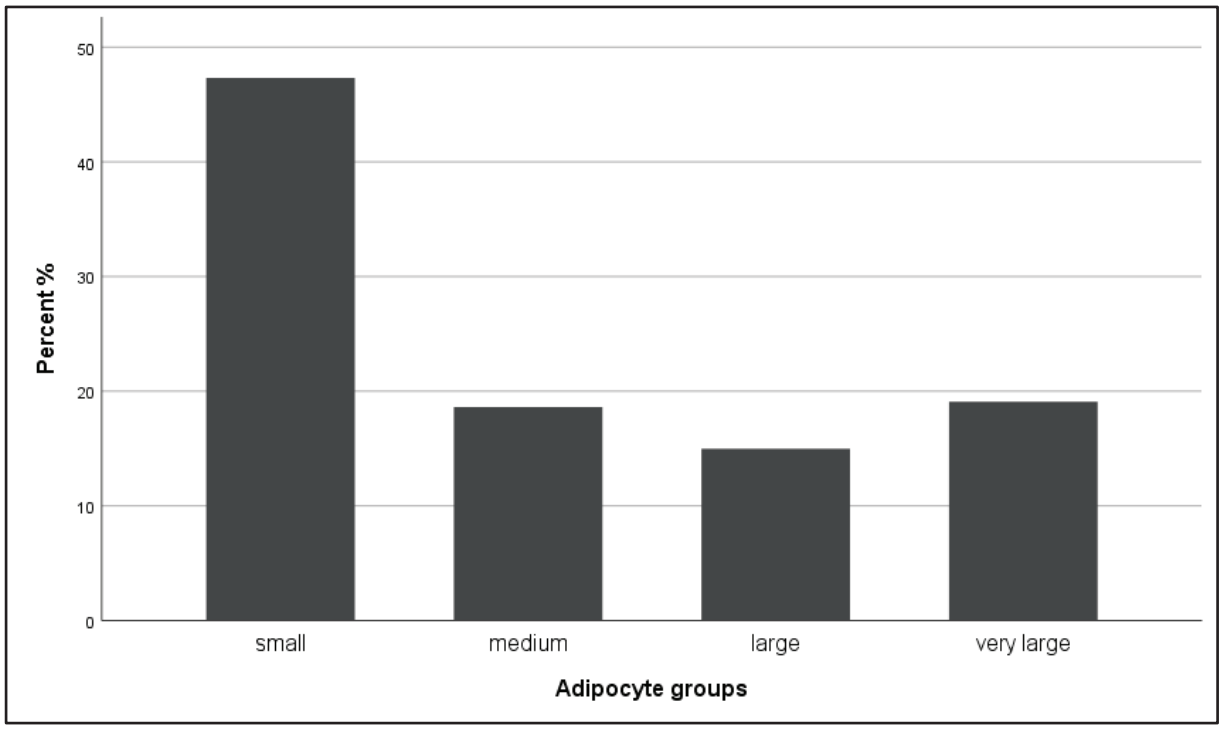
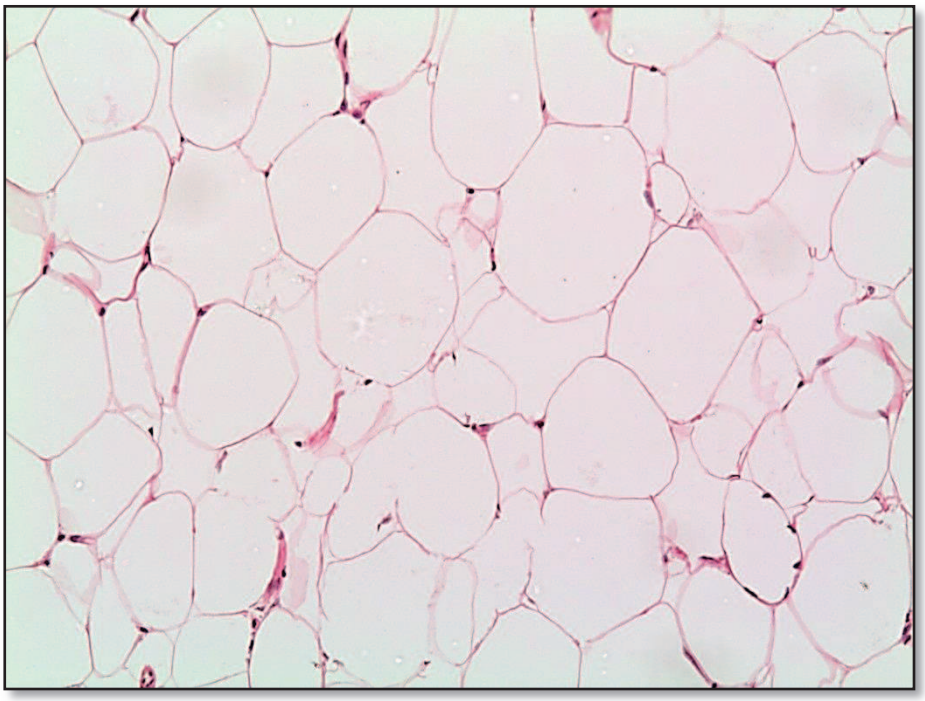


Figure 6-5: Patient 1 SAT
a) H&E slide showing adipocytes. b) bar chart represents adipocyte size distribution categorised into small < 50 µm, medium 50 – 69 µm, large 70 – 89 µm, and very large > 90 µm. The mean adipocyte diameter = 59.8 ± 29µm.

a



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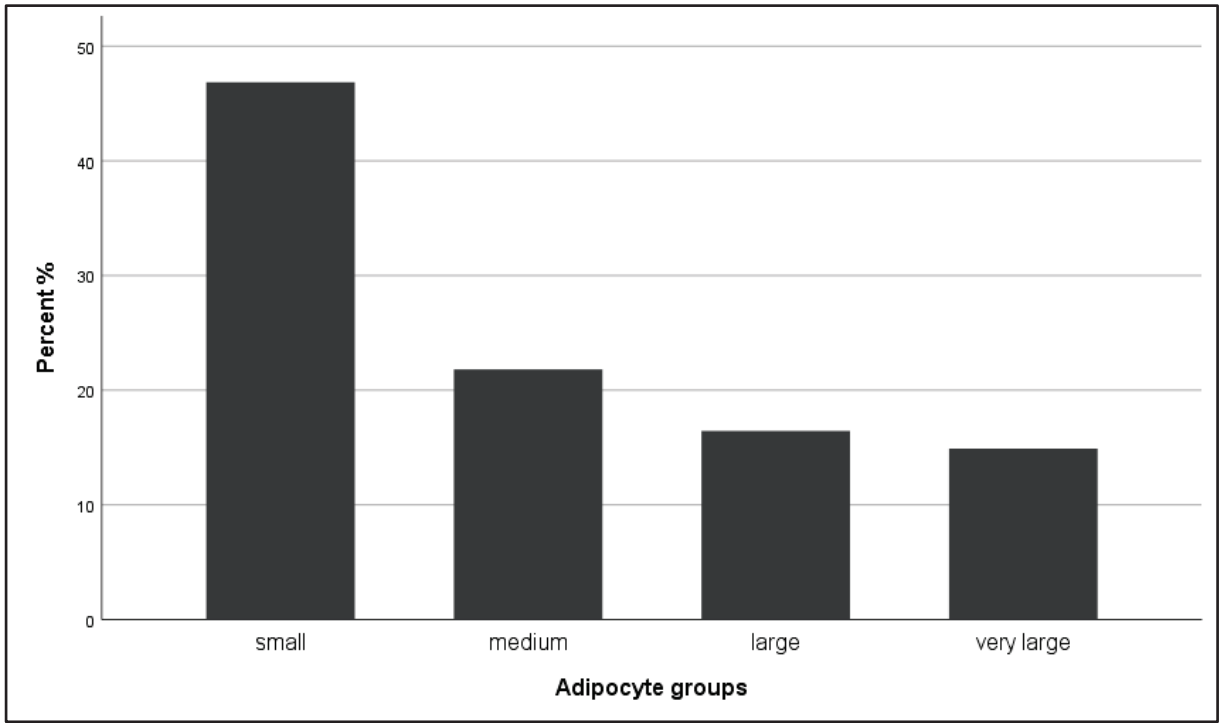
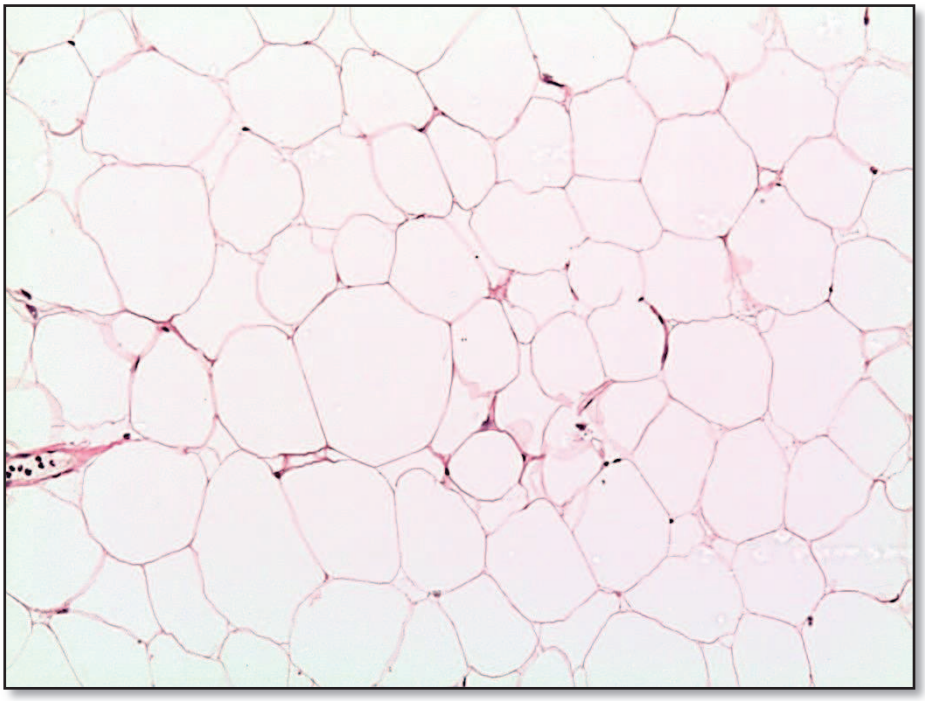


Figure 6-6: Patient 2 SAT
a) H&E slide showing adipocytes. b) bar chart represents adipocyte size distribution categorised into small < 50 µm, medium 50 – 69 µm, large 70 – 89 µm, and very large > 90 µm. The mean adipocyte diameter = 58.2 ± 26 µm.

a



b

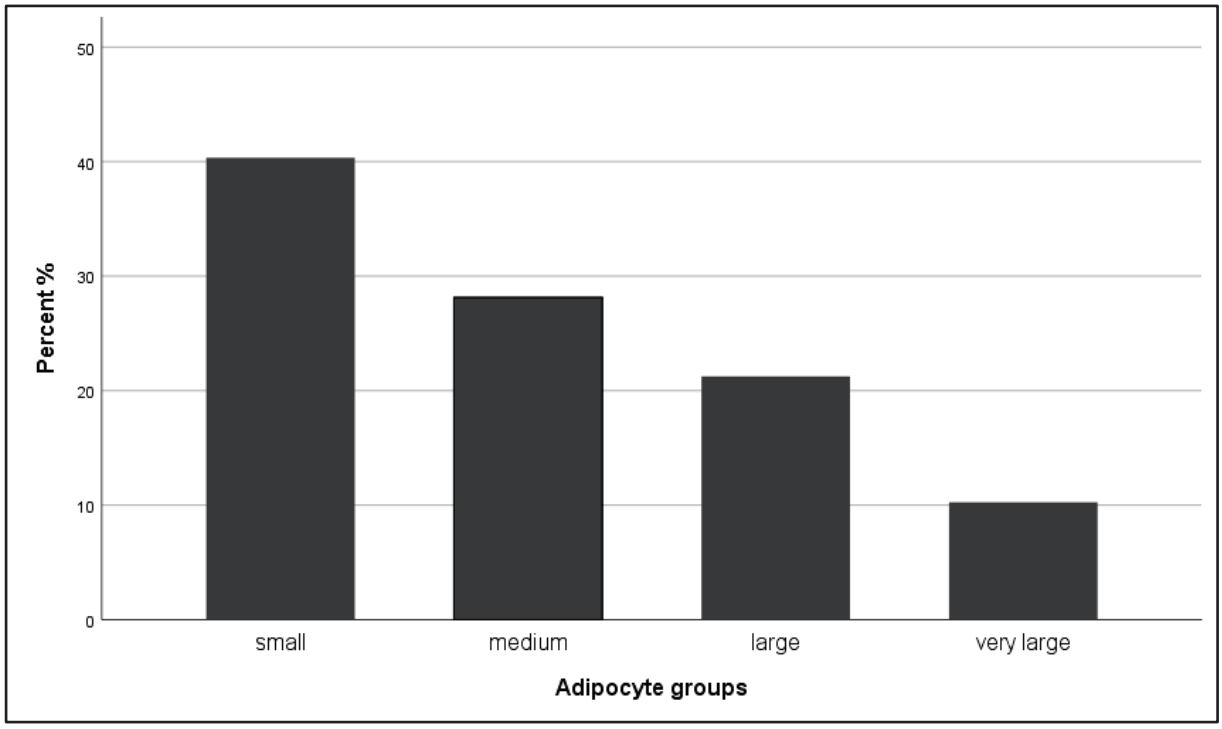


Figure 6-7: Patient 3 SAT
a) H&E slide showing adipocytes. b) bar chart represents adipocyte size distribution categorised into small < 50 µm, medium 50 – 69 µm, large 70 – 89 µm, and very large > 90 µm. The mean adipocyte diameter = 59.4 ± 23 µm.

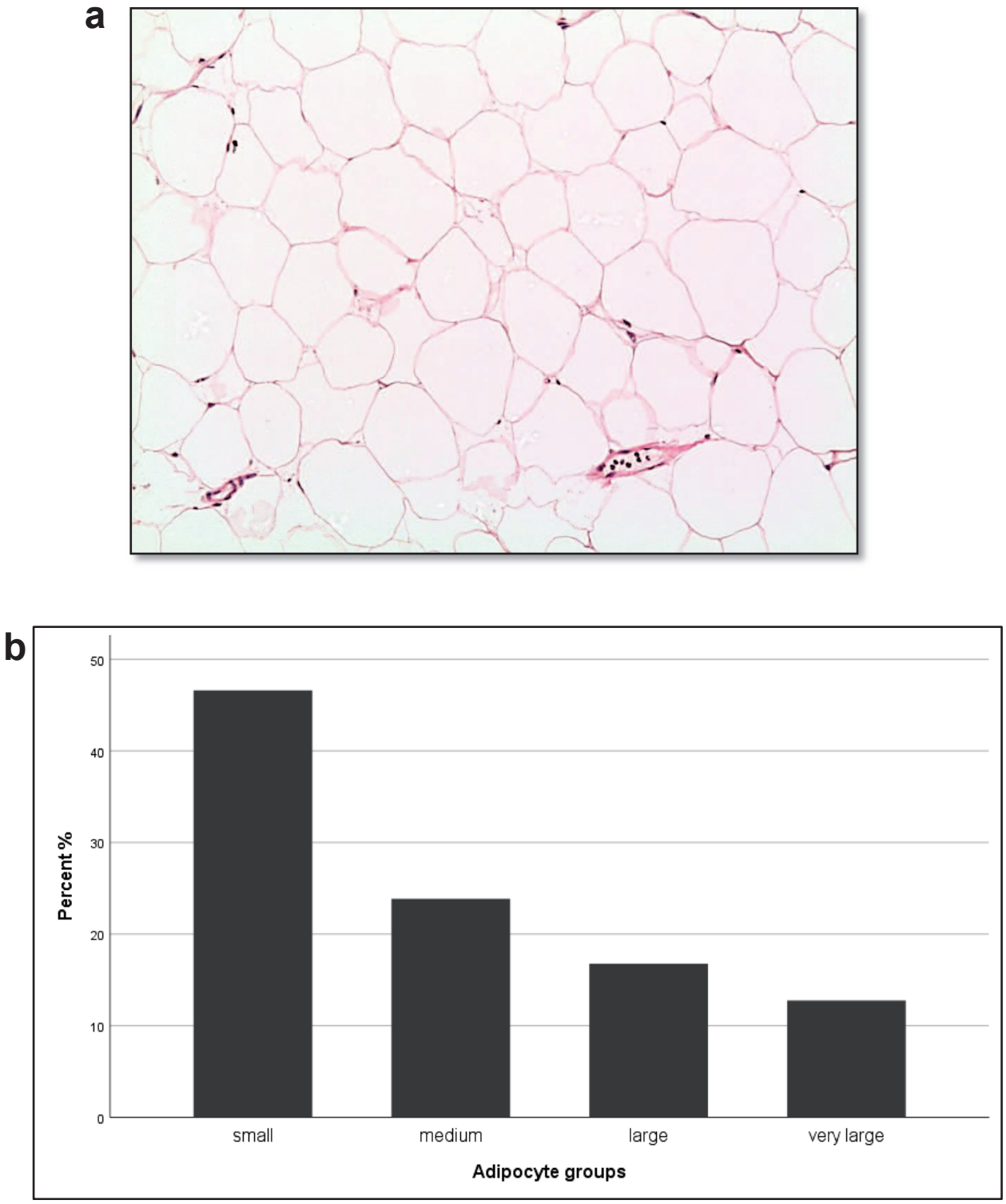


Figure 6-8: Patient 4 SAT
a) H&E slide showing adipocytes. b) bar chart represents adipocyte size distribution categorised into small < 50 µm, medium 50 – 69 µm, large 70 – 89 µm, and very large > 90 µm. The mean adipocyte diameter = 57.7 ± 25 µm.

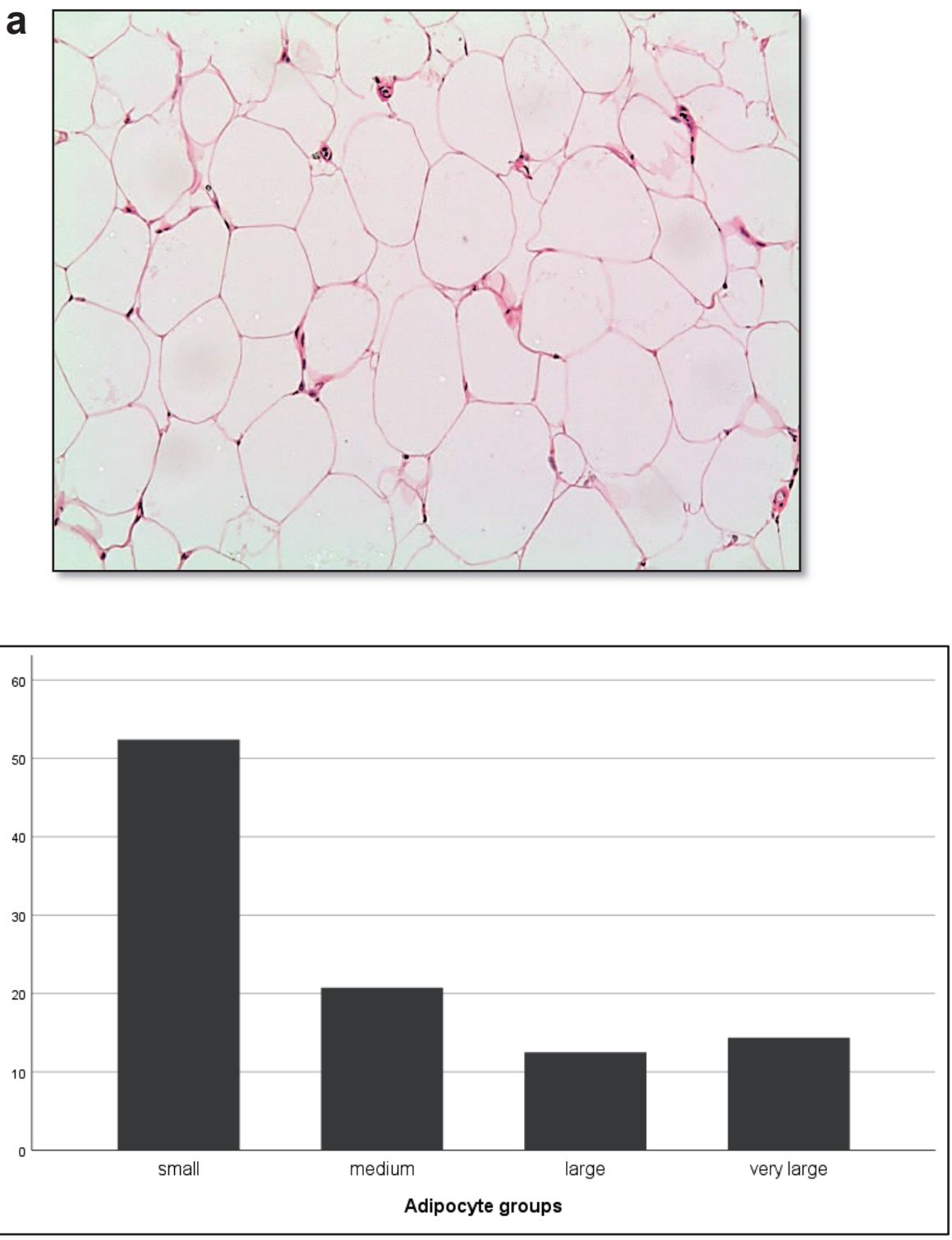


Figure 6-9: Patient 5 SAT
a) H&E slide showing adipocytes. b) bar chart represents adipocyte size distribution categorised into small < 50 µm, medium 50 – 69 µm, large 70 – 89 µm, and very large > 90 µm. The mean adipocyte diameter = 56.1 ± 27 µm.

6.4.4 Plasma samples

6.4.4.1 Normalisation of miRNA Expression

Two types of controls to normalise the expression of circulating miRNAs were tested in our study, endogenous control (mir-16) and exogenous control (mir-39) (Table 6-7). The endogenous control, mir-16, was highly expressed in our samples (Ct = 21.2) but showed more variation between the samples (SD > 0.25). Alternatively, the exogenous control (cel-miR-39) results, which is one of the widely accepted controls for circulating miRNA, was highly expressed in our samples and showed less variation between the samples (SD < 0.25).

6.4.4.2 Expression of targeted miRNAs

All six-circulating miRNA was detected in the plasma of our patients. Circulating mir-221 and mir-222 were highly expressed in patient's plasma samples (mean Ct = 24.3 and 25.1 respectively). Among the six circulating miRNAs, mir-193b showed the least expression in patient's samples (Ct = 33.8) (Table 6-8).

Table 6-7: Normalising expression for circulating miRNAs

Mir-16 Mean (SD)	Mir-39 Mean (SD)
21.2 (0.9)	23.6 (0.24)
SD: standard deviation	

Table 6-8: The mean expression of circulating miRNAs in plasma samples

miRNA	Ct Mean (SD)
Mir-222	25.1 (2.0)
Mir-221	24.3 (1.8)
Mir-192	29.0 (1.2)
Mir-193b	33.8 (1.3)
Mir-144	30.1 (1.0)
Mir-155	29.8 (2.2)
AT: Adipose tissue, **Ct:** cycle threshold, **SD:** standard deviation	

6.5 Discussion

The primary outcome of this study was to evaluate the feasibility of the proposed study, **RE**duction of **A**dipose **L**evels of **I**nflammation when treating obesi**TY** (REALITY) study.

6.5.1 Patient recruitment

Patients recruitment is an essential part of any clinical study. Insufficient recruitment results in failure of the study leading to a waste of time, funds, and other resources. To establish recruitment potential using this study design, invitation letters were sent to five patients who were already in the SCOTS study. Bariatric dieticians offered to give information sheets and letter to patients who agreed to participate in a future study. All five patients approached agreed to participate and signed a consent form. Since none of the five patients refused, this reassured me that the study was acceptable to patients, and recruiting enough patients in the main study would be possible.

6.5.2 Sample collection and processing

Since the timing of sample collection and sample processing is a critical factor affecting the quality of results, effort was made to made sure that samples arrive at the lab as quickly as possible. In this study, transportation of viable samples to the lab was quick (~ 1 hour) although the hospital was ~ 3.1 miles distant.

6.5.3 Adipose tissue samples

6.5.3.1 Total RNA Extracted

Isolating good quality RNA from adipose tissue was challenging due to its high content of triglyceride. Partially degraded or poor yield RNA can cause misleading results, and it is a big concern, especially when working with a limited amount of tissue from human biopsies. A variety of factors during sample collection, processing, and storage can negatively impact results and were considered in the current study.

To avoid RNA degradation, endogenous RNases must be inactivated immediately after adipose tissue harvesting. Two methods for adipose tissue preservation were

tested: snap-freezing AT samples on dry ice and placing AT samples in RNA*Later* solution (an aqueous, nontoxic collection solution that stabilizes and protects cellular RNA in freshly harvested tissue and cell samples). To decide which method was better for adipose tissue collection, comparison between the two methods was made in terms of total RNA yield. The total RNA concentration was isolated from both snap frozen and RNA*Later* adipose tissue samples. There was no difference between the total RNA extracted from both samples ($p > 0.05$), but The RNA*Later* method had a trend towards a slightly higher concentration of total RNA than snap-frozen method (mean of 40.4 ng/µl and 36.4 ng/µl, respectively). A disadvantage of the snap-frozen method was that samples must be preserved frozen until assay. Even brief thawing can result in RNA degradation and loss. During tissue processing (homogenization) samples will be at risk of thawing. Therefore, using RNA*Later* to preserve adipose tissue was easier for transportation and handling of adipose tissue samples.

6.5.3.2 Normalisation of miRNA expression

Normalisation of gene expression with a reference gene (housekeeping gene) is a significant step in gene quantification and analysis. An ideal housekeeping gene should show minimal variation in expression between samples and under the experimental conditions used and be adequately expressed in the tissue of interest. In the current study, three housekeeping genes (mir-103, mir-331, and RNU44) were tested in adipose tissue which have been used as an endogenous control in previous studies (Chapter 2.3.2). There was no difference in the expression of these selected housekeeping genes between the two adipose tissue samples ($p > 0.05$, for all three housekeeping genes). When comparing the expression of the three housekeeping genes (Table 6-4), RNU44 was found to be highly expressed (mean Ct = 24) and more stable between samples (SD < 0.25) than mir-103 and 331 (SD > 0.25). RNU44 was found to be the most suitable housekeeping gene in this study samples while the others will be avoided in our future study.

6.5.3.3 Expression of targeted miRNAs

In the present study, the ability of measuring the expression of miRNAs in adipose samples were tested using the same targeted six miRNAs used in chapter 3 (mir-

222, mir-221, mir-192, mir-193b, mir-155, and mir-144). The expression of these miRNAs was detected in SAT samples. After that, a comparison was made for the expression of the selected six miRNA between the two adipose tissue samples (RNA*Later* and snap frozen). There was no difference in their expression between the two methods ($p > 0.05$).

6.5.3.4 Adipocyte diameter

Large adipocyte size is associated with many of the adverse effects observed in metabolic diseases. Several methods have been described for measuring adipocyte size, but not all these methods are suitable for large-scale studies. Furthermore, some of these methods utilize equipment that is not available to all laboratories. Manual techniques are considered the most accurate way, but these are laborious and not suitable for large scale studies. More recently, a semi-automated protocol for quantitatively analysing size and number of adipocytes within adipose tissue which reduce, but do not eliminate human interaction. In the present study, a semi-automated method was tested using *Adiposoft* software, which is open-source software that provides fast and accurate quantitative measurements of adipose tissue cellularity in histological sections. The use of this technique to measure adipose tissue cellularity was feasible and can be easily implemented in a large-scale study. Total adipocytes were counted, and adipocyte diameter and percent were measured (Table 6-6). In this study, participants had higher proportions of small adipocytes than previously reported. In addition, the average SAT adipocyte diameter was 58.2 ± 26µm which is considered low compared to SAT adipocyte diameter in a study was done by (Verboven *et al.*, 2018). In their study, the average SAT adipocyte diameter for 17 men with obesity and without diabetes was 75.6 µm. There are several reasons for this difference. First, the average age for participants in this study were 37.4 ± 11 while in Verboven *et al.* was 48 (45-54) and age is an important factor that might affect adipocyte diameter. Secondly, participants in this study were insulin sensitive (HOMA-IR = 1.1) while in the other study, participants were insulin resistant (HOMA-IR = 4.7). Although the participants in this study were obese, they seem to maintain a normal metabolic function, metabolically healthy obesity (MHO) (Bell, Kivimaki & Hamer, 2014; Hinnouho *et al.*, 2015; Smith *et al.*, 2019). It is also possible that different tissue fixation techniques have some impact on adipocyte diameter. Finally, it is also possible that there are differences in the absolute calibration

between the digital photography and image analysis software used. One way to resolve the issue of calibration would be for me to repeat the study using the same equipment and SOP as Verboven *et al*. Any differences in size would then be attribuatble to incorrect calibration rather than systematic differences in technique and pre-anaytical variables. However, although absolute calibration may not be in agreement between studies, it is highly likely that the associations and internal correlations remain valid; methods with different calibrations tend to maintain good correlations.

6.5.4 Plasma samples

6.5.4.1 Normalisation of miRNA expression

For plasma samples, normalisation of the expression of the targeted six circulating miRNAs is a necessary step. Finding a suitable control gene to normalize the expression of the circulating miRNAs was challenging. For control genes, two types of controls were tested, mir-16, an endogenous control and cel-miR-39, an exogenous control (Table 6-7). The endogenous control (mir-16), was highly expressed in plasma samples (Ct = 21.2) but showed more variation between the samples (SD > 0.25). Alternatively, the exogenous control (cel-miR-39) results were highly expressed in the current samples and showed less variation (SD < 0.25). According to these results, a decision was made to use cel-miR-39 to normalize the expression of circulating miRNAs in the present study.

6.5.4.2 Expression of targeted miRNAs

The expression of the targeted six miRNAs were tested. All the six-circulating miRNA were detected in the plasma of the present study (Table 6-8).

6.6 Conclusion

In summary, the feasibility of the Reality study was tested and concluded that the protocol designed used was accomplishable and ready to be tested in a full-scale study. Overall, the validation of sample collection, processing, and the methods selected was done and were ready to apply. Most importantly, the study was acceptable to patients, and this will probably lead to satisfactory recruitment number for the main study. One of the significant strengths of this study was the establishment of teamwork among the bariatric surgery department in the QEUH and clinical research team at the University of Glasgow. The alliance created by this collaboration demonstrates the feasibility of conducting a future large-scale study.

6.6.1 Study strengths and limitations

This is the first study to test feasibility I was aware of using a formal protocol for adipose tissue and blood samples collection, handling, and transporting. A robust standard operating procedure for blood and adipose tissue was established for miRNAs extraction and expression as well as measuring adipocyte diameter that can be implemented in a large-scale study. Moreover, such study helped in dealing with critical issues raised and the appropriate respond to it. Although a feasibility study is a useful tool for large studies design, it has its limitations. First, there is no fixed pattern telling which components to include in the study and how-to assess and evaluate the outcomes; due to low power, it mostly relies on subjective judgment. Second, the costs of reliable and detailed analysis are high however, this was justified by the higher cost in case the large-scale study failure. Finally, the small sample size; but this is a facet of the pilot design. The study is sufficient to demonstrate more quantitively meaningful research is feasible.

7 General Discussion

7.1 Summary

The work presented in this thesis was intended to explore the connection between obesity and T2DM. As well as to assess the possibility of using circulating miRNA as potential biomarkers for both conditions. One of the advantages of the work in this thesis is use of multiple studies with different designs, size, and population phenotypes. This thesis included a small size feasibility study (5 participants from SCOT study) to, a medium cross-sectional and RCT study (154 participants from CAMERA trial), and finally, a very large-scale cross-sectional study (> 500,000 participants).

7.2 Major Findings

7.2.1 Suitability of the miRNA methodology for a large-scale analysis in a laboratory

This thesis presents some of the first large-scale epidemiological data investigating circulating miRNA biomarkers, and their associations with risk factors. In the initial stages of development of the thesis considerable laboratory time was spent developing a moderately high-throughput practical method to achieve this.

Circulating miRNAs exhibited promising potential to serve as effective non-invasive diagnostic biomarkers. To allow clinical use, larger scale studies are required to further test their sensitivity, specificity, and applicability as diagnostic biomarkers for clinical application and to reduce or prevent false positive or negative results. Unfortunately, all the methods currently utilised for the identification of miRNAs (such as PCR and microarrays) are expensive, time consuming, and require expertise which are not easy to apply in a large study. The development of a robust simple to use and sensitive methods for circulating miRNAs is a necessity before utilising them as diagnostic biomarkers.

In the last few years, progress has been made in the development of novel assay methodologies for the detection of circulating miRNA. One of these new methods is the use of aptamers, which are single stranded oligonucleotides that have high affinity and specificity in identifying target molecules. Ding *et al*. develop highly sensitive new method that uses aptamers that could identify and capture the

miRNAs in plasma and then analyse them on a full-featured micro-well detector. This method enables direct analysis of miRNAs in plasma without the need of miRNA extraction, reverse transcription, amplification, and normalisation (Ding *et al.*, 2019). This technology will make detection of circulating miRNAs in a large-scale study easy such as Generation Scotland cohort: 19,500 blood samples (Smith *et al.*, 2013) possible.

7.2.2 Circulating miRNAs as biomarkers

The emergence of circulating miRNAs as novel minimally invasive biomarkers is relatively new promising area of research. In addition to their exceptional stability, circulating miRNAs hold other unique characteristics including their ease of detection, detectability even in a small sample amount, and their association with established clinicopathological parameters. All these features make circulating miRNAs promising biomarker candidate for future clinical applications. Recently, accumulating body of evidence has been introduced to support circulating miRNAs as non-invasive diagnostic and prognostic biomarkers in a wide variety of diseases such as cancers (Sueta *et al.*, 2017; Al-qatati *et al.*, 2017), diabetes (Zhu & Leung, 2015; Sebastiani *et al.*, 2017), autoimmunity (Heegaard *et al.*, 2015) and cardiovascular diseases (Sayed *et al.*, 2015). However, most of the studies have been limited by small numbers and inconsistencies in results. So far and due to these issues, none of the suggested circulating miRNAs, including those in this thesis, succeeded as a biomarker. This is because it is not possible to compare results from studies that uses different samples, methods, and controls for normalisation.

In the first study (chapter 3), the selected circulating miRNAs (mir-221, mir-222, mir-192, mir-193b, mir-155, and mir-144) showed significant associations with markers of metabolic risk in a population without diabetes and with large waist and coronary heart disease. The associations were supported by an expanding literature showing their potential biological role for the biomarker shown in section3.6.2).

Among the six targeted circulating miRNAs, mir-144 displayed a specific association with insulin, HOMA-IR thus highlighting the potential role of mir-144 as a key player in insulin resistance, not mediated by body weight. This miRNA

appears to contribute to the regulation of insulin signalling which was supported by the findings of Karolina *et al.* were they showed that elevated circulating mir-144 correlate with the downregulation of insulin receptor substrate 1 (IRS1) at both mRNA and protein levels (Karolina *et al.*, 2011).

The results of this thesis suggest that the selected circulating miRNAs are possible and reproducible biomarkers for the following reasons. First, the use of large samples to investigate circulating miRNA which most of the studies lack. Second, in the initial stages a considerable laboratory time was spent developing a moderately high-throughput practical method to standardise the analytical part. Third, the associations with metabolic risk factors were specific and not providing overlapping information particularly for mir-144 which was specific with insulin and HOMA-IR. Finally, the associations with metabolic health markers were underpinned by an expanding literature around a potential biological role for the biomarker.

The present study supports the putative utilities of circulating miRNAs as reproducible biomarkers however, further standardisation of both preanalytical and analytical procedures, as well as automated solutions, are required before large scale epidemiological investigation and their use as a clinical tool can be considered.

7.2.3 Body fat distribution

It is well established that obesity is associated with increased risk for developing T2DM. People with obesity have a seven times greater risk of T2DM than people with normal weight (Abdullah *et al.*, 2010). Nevertheless, the relation between these two conditions is complicated and is not explained by total fat alone. A number of factors have been suggested as possible links between the two conditions. One connection suggested was body fat distribution. Previous studies have shown that body fat location particularly in the central area is more associated with T2DM then total body fat (Sattar and Gill, 2014; Nordström *et al.*, 2016; Janochova, Haluzik and Buzga, 2019). Therefore, the third study (chapter 5) was designed to look at the association between body fat distribution and T2DM. Here, the differences in body fat measurements between participants with and without T2DM in both genders using the UK biobank cohort was explored. In this

study, the hypothesis that fat mass and distribution vary by sex was conformed, a fact that has been previously well documented (Davis *et al.*, 2013; Chang, Varghese & Singer, 2018). A striking data demonstrating how different fat depots were associated with T2DM were presented. Specifically, visceral fat was an independent risk factor for T2DM. The results of this study corroborated with findings by several longitudinal studies which confirmed that visceral fat is more strongly associated with T2DM than total body fat (Neeland *et al.*, 2012; Wander *et al.*, 2013; Nordström *et al.*, 2016). If these associations were causal, it suggests interventions to mitigate visceral fat accumulation are most likely to be clinically beneficial in terms of reducing T2DM risk.

7.2.4 WBC count

Low-grade inflammation is a common phenomenon observed in both obesity and T2DM. Because of that, it has been speculated that inflammation plays a major role in the pathogeneses of T2DM. This was supported by several epidemiological studies demonstrating that markers of inflammation were correlated with or predicted incidents of T2DM (Duncan and Schmidt, 2006; Herder, Carstensen and Ouwens, 2013). In addition, a growing body of evidence suggested that inflammation can alter several pathways that are associated with insulin action and pancreatic ß-cell function leading to insulin resistance and T2DM (Prattichizzo *et al.*, 2018). Nonetheless, interventions with anti- inflammatory compounds have yielded limited or no benefit (Everett *et al.*, 2018). For those reasons, this study sought to investigate the use of WBC count as a marker for this low-grade inflammation is the link between adiposity and T2DM. This was supported by large epidemiological studies showing an association between total and peripheral WBCs count and diabetes risk (Schmidt *et al.*, 1999; Vozarova *et al.*, 2002; Twig *et al.*, 2013).

In this study it was shown that WBC count cannot be considered the sole or primary connection of T2DM rather than an important mechanism that is linked to the primary cause, VAT deposition. Our findings further added to the indecisive evidence surrounding the role of WBC count plays in T2DM.

7.2.5 Feasibility Study

To move the work forward it is important to plan for the next steps. To further explore and expand the association found in the previous studies in adipose tissue, since adipose tissue expandability and inflammation appears to be the drive of the metabolic dysfunction. To understand the role of SAT adipocyte in metabolic homeostasis, the REALITY study was considered where bariatric surgeries are going to be used to compare, adipocyte size, inflammation, and miRNA expression before and after weight loss. In the fourth study (chapter 6), a pilot study was conducted to assess the feasibility of the REALITY study and to work out all the logistics and requirements before conducting a large-scale study. The final conclusion of this pilot study was that the REALITY study can be implemented to success as it was carefully planned.

7.3 Future Perspectives

From the work I presented in this thesis I am planning to expand my analysis to further confirm my findings. This will be accomplished by:

1. Continue with the UK Biobank study but with further development of the ideas. The UK Biobank imaging study is still accruing participants, and more data are released every 6 - 12 months. The imaging study will continue until it reaches 100,000 participants which is predicted to be in 2022. This will allow us to include more participants in our study. Recently, further data become available from a range of biochemical assays of baseline samples from all participants. I will use HbA1c to categorise our study population to three different groups (Normal: ≤ 42 mmol/mol, Pre-diabetes: 42 - 47 mmol/mol, and Diabetes: ≥ 48 mmol/mol). This will allow us to monitor body fat distribution through the course of the disease, from pre to post disease establishment. Moreover, I will include CRP as an inflammatory biomarker. CRP is more widely used in epidemiological studies as a measurement of inflammation than WBC, and this will allow us to compare our findings with these studies. Finally, forthcoming availability of primary care data will allow us to investigate prospective links with incident type 2 diabetes, a stronger study design than cross-sectional data.

2. I will proceed with the full REALITY study with the implementation of the protocol I designed. The primary hypothesis to test is that subcutaneous adipocyte diameter is reduced after significant weight loss leading to metabolic improvement. The study will use bariatric surgery as a model of metabolic change by comparing before and after weight loss (after 1 year). I will measure SAT adipocyte size, inflammatory markers, hormonal changes, and miRNA expression. The first samples (baseline samples) of both blood and SAT samples will be collected during bariatric surgery. The other samples will be collected from the same patients after one year of surgery. To accomplish that I will be using a novel biopsy method that allows sampling from deep SAT compartment by a percutaneous bedside approach (Alderete *et al.*, 2015). The primary outcome will be reduction in SAT adipocyte diameter and its effect on inflammation and miRNA expression. For the sample size three scenarios were proposed:

a. To detect a clinically significant difference in one weight loss group of adipocyte size 63 μm and 76 μm with SD 21 μm at alpha = 0.05 and 80% power, assuming a correlation of r = 0.8 at the two timepoints, 11 participants will be needed.

b. To detect a clinically significant difference in one weight loss group of adipocyte size 63 μm and 70 μm with SD 21 μm at alpha = 0.05 and 80% power, assuming a correlation of r = 0.8 at the two timepoints, 31 participants will be needed.

c. To detect a clinically significant difference in one weight loss group of adipocyte size 63 μm and 68 μm with SD 21 μm at alpha = 0.05 and 80% power, assuming a correlation of r = 0.8 at the two timepoints, 58 participants will be needed.

Out of the three power calculations number c make more sense and will be use in future study.

7.4 Concluding remarks

This work has demonstrated the potential utility of circulating miRNA as biomarkers of metabolic disease, that also deserve further consideration as potential causal mediators. It also showed that ectopic fat deposition in the visceral area plays an important role in the development of metabolic diseases. Deeper understanding of how body fat distribution is regulated may lead to novel therapeutic treatments of metabolic complications associated with obesity.

APPENDICES

Appendix 1: Reverse Transcription

miRNA Dilution for RT

RNA must be diluted to 2 ng / µL. For each miRNA, a total of 9 ng of RNA is needed. Therefore, 9 ng x 8 miRNA=72. I rounded this up to 80 ng to account for potential loss of material while pipetting. To calculate volume of RNA needed, I used the following formula:

$$\frac{80}{Total\ Conc.of\ RNA} = \mathrm{X}\ \mu l\ \text{(amount of RNA)}$$

For each miRNA, a total volume of 5 µL of RNA is needed. Thus, for 8 miRNAs 40 µL in total was needed. 40 µL – X = amount of RNAse free water to make up the 40 µL.

Master Mix Preparation

One master mix for each miRNA was prepared according to the following:

	For 1 sample	For 8 ~ 10 Samples
dNTP (100 mM)	0.075 µL	0.75 µL
Multiscribe	0.5 µL	5 µL
10X RT Buffer	0.75 µL	7.5 µL
RNase Inhibitor	0.095 µL	0.95 µL
RNAse free water	0.08 µL	0.8 µL
Primer (5X; RT)	1.5 µL	15 µL

Appendix 2: Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Master Mix Preparation

Number to prepare depends on number of samples + 1 negative in triplicate.

e.g. for 8 samples:

$8 + 1 = 9 \times 3 = 27 \sim 30$

	n = 1	n = 30
2X TaqMan mastermix	5 µL	150 µL
Primers (20X)	0.5 µL	15 µL
RNase free water	µL	93 µL

Appendix 3: External Spike-in control (cel-miR-39) preparation

Stock Solution

Was prepared by adding 300 µL nuclease free water vortexed, spun. The final concentration was 2 x 1010 copies/µL stock. The stock control was aliquoted into 30 µL /tube and stored at $-80℃$.

Working Control

4 µL of the stock control solution was mixed with 16 µL nuclease free water to produce the First Step Solution (4 x 109 copies/µL). First Step Solution (2 µL) was further diluted with nuclease free water (48 µL) to produce a 1.6 x 108 copies/µL.

Appendix 4: Letter of access for research

Coordinator/Administrator: Ms Joanne McGarry/Mrs Elaine O'Neill
Telephone Number:0141 232 1815
E-Mail: elaine.o'neill2@ggc.scot.nhs.uk Website:
www.nhsggc.org.uk/r&d

Research & Development
West Glasgow ACH
Dalnair Street
Glasgow G3 8SW

01 June 2016

Tahani ALRamah
BHF Cardiovascular Research Centre
University of Glasgow
126 University Place
Glasgow G12 8TA

Dear Ms T ALRamah,

Letter of Access for Research

This letter confirms your right of access to conduct research through **NHS Greater Glasgow and Clyde** for the purpose and on the terms and conditions set out below. This right of access commences on **01/06/16** and ends on **31/06/17** unless terminated earlier in accordance with the clauses below.

You have a right of access to conduct such research as confirmed in writing in the letter of permission for research from this NHS organisation. Please note that you cannot start the research until the Principal Investigator for the research project has received a letter from us giving permission to conduct the project.

The information supplied about your role in research at **NHS Greater Glasgow and Clyde** has been reviewed and you do not require an honorary research contract with this NHS organisation. We are satisfied that such pre-engagement checks as we consider necessary have been carried out.

You are considered to be a legal visitor to **NHS Greater Glasgow and Clyde** premises. You are not entitled to any form of payment or access to other benefits provided by this NHS organisation to employees and this letter does not give rise to any other relationship between you and this NHS organisation, in particular that of an employee.

While undertaking research through **NHS Greater Glasgow and Clyde**, you will remain accountable to your employer the **University of Glasgow** but you are required to follow the reasonable instructions of **Lorna Forde** in this NHS organisation or those given on her/his behalf in relation to the terms of this right of access.

Where any third party claim is made, whether or not legal proceedings are issued, arising out of or in connection with your right of access, you are required to co-operate fully with any investigation by this NHS organisation in connection with any such claim and to give all such assistance as may reasonably be required regarding the conduct of any legal proceedings.

You must act in accordance with **NHS Greater Glasgow and Clyde** policies and procedures, which are available to you upon request, and the Research Governance Framework.

You are required to co-operate with **NHS Greater Glasgow and Clyde** in discharging its duties under the Health and Safety at Work etc Act 1974 and other health and safety legislation and to take reasonable care for the health and safety of yourself and others while on **NHS Greater Glasgow and Clyde** premises. You must observe the same standards of care and propriety in dealing with patients, staff, visitors, equipment and premises as is expected of any other contract holder and you must act appropriately, responsibly and professionally at all times.

If you have a physical or mental health condition or disability which may affect your research role and which might require special adjustments to your role, if you have not already done so, you must notify your employer and the health board's HR department prior to commencing your research role at the Health board.

You are required to ensure that all information regarding patients or staff remains secure and *strictly confidential* at all times. You must ensure that you understand and comply with the requirements of the NHS Confidentiality Code of Practice (http://www.dh.gov.uk/assetRoot/04/06/92/54/04069254.pdf) and the Data Protection Act 1998. Furthermore, you should be aware that under the Act, unauthorised disclosure of information is an offence and such disclosures may lead to prosecution.

You should ensure that, where you are issued with an identity or security card, a bleep number, email or library account, keys or protective clothing, these are returned upon termination of this arrangement. Please also ensure that while on the premises you wear your ID badge at all times or are able to prove your identity if challenged. Please note that this NHS organisation accepts no responsibility for damage to or loss of personal property.

We may terminate your right to attend at any time either by giving seven days' written notice to you or immediately without any notice if you are in breach of any of the terms or conditions described in this letter or if you commit any act that we reasonably consider to amount to serious misconduct or to be disruptive and/or prejudicial to the interests and/or business of this NHS organisation or if you are convicted of any criminal offence. You must not undertake regulated activity if you are barred from such work. If you are barred from working with adults or children, this letter of access is immediately terminated. Your employer will immediately withdraw you from undertaking this or any other regulated activity and you MUST stop undertaking any regulated activity immediately.

Your substantive employer is responsible for your conduct during this research project and may in the circumstances described above instigate disciplinary action against you.

NHS Greater Glasgow and Clyde will not indemnify you against any liability incurred as a result of any breach of confidentiality or breach of the Data Protection Act 1998. Any breach of the Data Protection Act 1998 may result in legal action against you and/or your substantive employer.

If your current role or involvement in research changes, or any of the information provided in your Research Passport changes, you must inform your employer through their normal procedures. You must also inform your nominated manager in this NHS organisation.

Yours sincerely,

Ms Joanne McGarry
Research Co-ordinator

Appendix 5: Good clinical practice certificate

NHS
SCOTLAND
NHS RESEARCH SCOTLAND

This is to certify that

TAHANI Y.M. ALRAMATH

Attended

NRS Introduction to Good Clinical Practice

Tuesday 10th November 2015

Course Content:

- History of Clinical Trials and the development of GCP
- Principles of GCP
- UK legislation – Statutory Instrument
- Informed consent process
- Investigator responsibilities
- Sponsor responsibilities
- Study conduct
- Safety reporting
- Trial Master Files, Investigator Site Files and data management
- QA/QC – Monitoring

This ICH E6 GCP Investigator Site Training meets the Minimum Criteria for ICH GCP Investigator Site Personnel Training identified by TransCelerate BioPharma as necessary to enable mutual recognition of GCP training among trial sponsors.

RCPS Code: [93898]

Signed: Shona McDermott

Shona McDermott

Assistant Director of Glasgow CRF: Education & Training

Appendix 6: R&D Management Approval GN15MD002

Administrator: Lorn Mackenzie R&D
Telephone Number: 0141 211 1743
E-Mail: Lorn.Mackenzie@ggc.scot.nhs.uk
Website: www.nhsggc.org.uk/r&d

Management Office
Western Infirmary
Tennent Institute
1st Floor 38 Church Street
Glasgow, G11 6NT,

20/01/2015

Dr Jennifer Logue
BHF Cardiovascular Research Centre
126 University Place
Glasgow, G12 8TA

NHS GG&C Board Approval

Dear Dr Logue,

Study Title:	REALITY REduction of Adipose Levels of Inflammation when Treating obesitY -Feasibility Study
Principal Investigator:	Dr Jennifer Logue
GG&C HB site	GG&C Weight Management Service, Mansion house Unit
Sponsor	NHS GG&C
R&D reference:	GN15MD002
REC reference:	15/EM/0018
Protocol no: (including version and date)	V1.0; 10.12.14

I am pleased to confirm that Greater Glasgow & Clyde Health Board is now able to grant **Approval** for the above study.

Conditions of Approval

1. **For Clinical Trials** as defined by the Medicines for Human Use Clinical Trial Regulations, 2004
    a. During the life span of the study GGHB requires the following information relating to this site
        i. Notification of any potential serious breaches.
        ii. Notification of any regulatory inspections.

It is your responsibility to ensure that all staff involved in the study at this site have the appropriate GCP training according to the GGHB GCP policy (www.nhsggc.org.uk/content/default.asp?page=s1411), evidence of such training to be filed in the site file.

2. **For all studies** the following information is required during their lifespan.
   a. Recruitment Numbers on a quarterly basis
   b. Any change of staff named on the original SSI form
   c. Any amendments – Substantial or Non-Substantial
   d. Notification of Trial/study end including final recruitment figures
   e. Final Report & Copies of Publications/Abstracts

Please add this approval to your study file as this letter may be subject to audit and monitoring.
Your personal information will be held on a secure national web-based NHS database.
I wish you every success with this research study

Yours sincerely,

Lorn Mackenzie
Senior Research Administrator

Appendix 7: Patient invitation Letter

Institute of Cardiovascular & Medical Sciences
BHF Glasgow Cardiovascular Research Centre
University Of Glasgow
126 University Place
Glasgow
G12 8TA

Dear [Insert name]

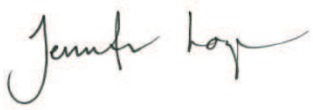
Thank you for already agreeing to take part in the Surgical Obesity Treatment Study (SCOTS) based at the University of Glasgow. You previously gave us permission to contact you to let you know about any other studies that we are doing. We are doing a further small study and we are inviting participants who are due to have their surgery in Glasgow to take part in it.

The extra study would involve donating a blood sample and a small piece of fat tissue which would be removed during your surgery. I am writing to invite you to consider participating in this extra study.

The study is funded by the NHS Greater Glasgow and Clyde Research Endowments and aims to recruit 5 people in the first instance. The study is supported by your bariatric surgery team in Glasgow.

Please take some time to read the enclosed patient information sheet and consider if you would like to take part in our study. The research team will be available at one of your appointments prior to your surgery so you can ask any questions and let us know if you would like to take part.

Yours sincerely



Dr Jennifer Logue
CSO Clinician Scientist
Senior Clinical Lecturer and Honorary Consultant in Metabolic Medicine
University of Glasgow

BHF Glasgow Cardiovascular Research Centre
Tel: +44(0)141 330 2569
Fax: + 44(0)141 330 6955
Email: jennifer.logue@glasgow.ac.uk
Website: scotsurgerystudy.org.uk
The University of Glasgow, Charity number SC004401

Appendix 8: Consent Form for The Reality Study

SCOTS Participant Identification Number:

CONSENT FORM

Title of Project: REALITY - REduction of Adipose Levels of Inflammation when Treating obesitY - Feasibility Study

Name of Researcher: Dr Jennifer Logue

Please initial box

1. I confirm I have read and understand the information sheet dated 10/12/2014 (Version 2.0) for the above study. I have had the opportunity to consider the information, ask questions, and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my medical notes may be looked at by individuals from regulatory authorities or the NHS board, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐
4. I agree to my blood and fat (adipose tissue) samples being stored for use in future studies ☐
5. I agree to take part in the above study. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature

When completed 1 for patient, 1 for researcher site file, 1 for upload to electronic case notes

2014_12_10 REALITY consent form v1.

Appendix 9: Poster for the 53rd EASD annual meeting, Lisbon, Portugal, 2017

Putative miRNA biomarkers of insulin resistance and the effect of metformin: data from the CAMERA trial

Tahani Alramah[1], Karine Pinel[1], David Preiss[2], Naveed Sattar[1], Jennifer Logue[1], Paul Welsh[1]

[1]Institute of Cardiovascular and Medical Sciences, University of Glasgow, Glasgow, UK. [2] Clinical Trial Service Unit and Epidemiological Studies Unit, University of Oxford, UK

Background & Aim

- Several circulating miRNAs have been reported to be associated with insulin resistance (IR) and are considered as potential diagnostic biomarkers, although studies have generally been limited by small size. It is also not clear whether these associations are causal.
- We aimed to:
  1) validate cross sectional associations of targeted miRNAs with IR within a large study of people without diabetes
  2) to explore the effect of metformin on these miRNAs.

Methods

- CAMERA study – randomised, placebo-controlled double-blinded trial performed in Glasgow, UK (2009-2012).
- 173 patients CHD patients (without diabetes)
- Metformin or placebo (1:1) for 18 months.
- miRNAs extracted from available baseline and 18 month visit plasma samples.
- The expressions of mir-221, 222, 144, 155, 192 and 193b (targeted based on previous literature) were measured using RT-qPCR.
- Spike-in of cel-miR-39 was used to normalise to a quality control. Repeat samples run as additional quality control.
- Stats – linear regression of ΔCT values for cross sectional analyses, and the
- Stats – $2^{-\Delta\Delta CT}$ method to investigate the randomised effect of metformin.

Figure 1: Trail Profile

Table 1: Cel-mir-39 intra and inter-Assay CV%

Intra-Assay		Inter-Assay	
Mean	24.93	Mean	24.93
SD	0.11	SD	0.86
CV	0.45	CV	3.54

CV: Coefficient of Variation, **SD:** Standard Deviation

Results

In cross sectional analysis at baseline:

- mir-144 showed the strongest association with markers of insulin sensitivity (Table 4 & 5).
- Other miRNAs showed variable associations with markers of IR and glycaemia (Table 4)
- No miRNA was associated with BMI.
- Mir-192 and 193b showed strong associations with liver enzymes and adjustment for body fat did not attenuate these associations (Table 3 and 4).

The Effect of Randomisation to Metformin on Circulating miRNAs:

- Baseline characteristics were well balanced between randomised groups, including for miRNA (Table 1).
- Over 18 months, metformin showed no effect on the expression of mir-221, 222, 144, 155, 192 and 193b(figure 2)

Table 2: Association of baseline miRNAs with baseline metabolic risk factors

	Placebo group (n=81)	Metformin group (n=73)
Demographic Characteristics		
Age (years)	64 (8)	63 (8)
Gender		
Male	61 (74%)	58 (80%)
Female	20 (25%)	15 (20%)
Blood pressure (mm Hg)	140/79 (20/11)	140/79 (16/11)
Anthropometric Characteristics		
Body mass index (kg/m²) *	30.7 (27.1-33.1)	29.4 (27.5-32.3)
Biochemical Characteristics (Fasting)		
Plasma Glucose (mmol/L)	5.2 (0.5)	5.3 (0.3)
Insulin (pmol/L) *	9.7 (7.5-14.4)	9.8 (7.2-13)
HOMA-IR*	2.3 (1.6-3.4)	2.3 (1.6-3.1)
HbA1c (mmol/mol) *	38.2 (36-40)	38.7 (37-41)
Triglyceride (mmol/L) *	1.5 (1.1-1.9)	1.4 (1.1-2.0)
Total Cholesterol (mmol?L) *	4.3 (3.6-4.7)	4.2 (3.6-4.7)
HDL (mmol/L) *	1.1 (1.0-1.4)	1.1 (1.0-1.3)
Alanine aminotransferase (U/L) *	26.0 (18.5-33.5)	23.0 (19.5-35)
γ-glutamyltransferase (U/L) *	35.0 (21.5-54)	34.0 (22.5-64)
High sensitivity C-reactive protein (mg/L) *	1.6 (0.9-3.2)	1.8 (0.8-3.9)
WBC (x10⁹/L) *	6.7 (5.6-8.1)	6.5 (5.8-7.5)
miRNA Expression		
mir-222 (CT)	28.8 (1.6)	28.8 (1.8)
mir-221 (CT)	26.6 (1.6)	26.6 (1.7)
mir-144 (CT)	35.0 (1.3)	35.0 (1.2)
mir-155 (CT)	32.0 (1.1)	32.7 (1.3)
mir-192 (CT)	33.8 (1.3)	33.7 (1.7)
mir-193b (CT)	34.1 (1.3)	34.0 (1.8)

* Not normally distributed variables, median (inter-quartile range) were used.

Table 3: Pearson correlation of liver enzymes vs miRNAs expression at baseline

		dCt mir-221	dCt mir-222	dCt mir-192	dCt mir-193b	dCt mir-155	dCt mir-144
GGT (U/L)	r	-0.236**	-0.103	-0.278***	-0.402***	-0.225**	-0.159*
ALT (U/L)	r	-0.227**	-0.143	-0.399***	-0.636***	-0.138	-0.156

dCt: delta Ct, **GGT:** Gamma-Glutamyl Transferase, **ALT:** Alanine Aminotransferase, *r:* Pearson Correlation Coefficient. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 4: Pearson correlation of insulin sensitivity markers vs miRNAs expression at baseline

		dCt mir-221	dCt mir-222	dCt mir-192	dCt mir-193b	dCt mir-155	dCt mir-144
HOMA-IR	r	-0.072	-0.086	-0.136	-0.152	-0.121	-0.207*
INS (pmol/L)	r	-0.052	-0.073	-0.115	-0.124	-0.096	-0.184*
Glucose (mmol/mol)	r	-0.135	-0.033	-0.187*	-0.175*	-0.165*	-0.251**
HbA1C (mmol/mol)	r	-0.202*	-0.245**	-0.147	-0.170*	-0.175*	-0.141

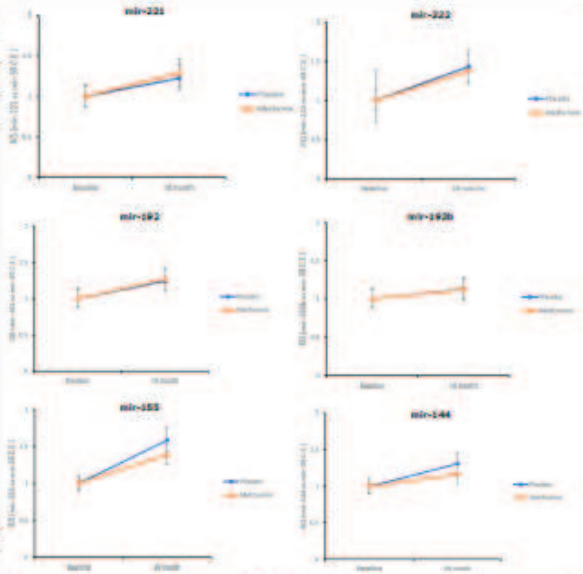
dCt: delta Ct, **INS:** Insulin, **HOMA-IR:** homeostatic model assessment for insulin resistance, *r:* Pearson Correlation Coefficient. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 5: Baseline associations of biomarkers with selected miRNA

MiRNA	Predictor	Model 1 B (95% CI) p-value	Model 2 B (95% CI) p-value	Model 3 B (95% CI) p-value
Mir-144	Glucose (mmol/L)	34% (15.1-50)	31.9% (10.4-48.1)	31.7% (8.9-48.8)
	Insulin (pmol/L)	2.5% (0.3-4.5)	2.2% (0.1-4.3)	2.3% (0.1 - 2.3)
	HOMA-IR	10.2% (2.5-17.2)	9.3% (1.5-16.4)	9.7% (1.5-17.1)
Mir-193b	GGT (U/L) (10 units increase)	8% (6 - 10.1)	8% (5-10.1)	8% (5-10.1)
	ALT (U/L) (10 units increase)	40.3% (30.3 - 50)	40.3% (30.4 - 50.1)	40.5% (30.6 - 50.1)
Mir-192	GGT (U/L) (10 units increase)	6% (3 - 9)	6% (2 - 9)	6% (2 - 9)
	ALT (U/L) (10 units increase)	28% (16 - 38)	27% (30.4 - 50.1)	29% (19 - 40)

Models demonstrate the association of one unit (ten units for LFTs) increase in risk factors with miRNA. Model 1: unadjusted Model 2: adjustment for age and sex Model 3: adjustment for age, sex and body fat GGT: Gamma-Glutamyl Transferase, ALT: Alanine Aminotransferase, HOMA-IR: homeostatic model assessment for insulin resistance

Figure 2: Line Graph Comparing the RQ change for each miRNA between Placebo and Metformin Groups.



Conclusions

- In this, one of the first studies to investigate the effect of a randomised intervention on miRNA expression, we observed.
  - i. cross-sectional associations of targeted miRNAs with biomarkers of metabolic risk – broadly consistent with literature.
  - ii. no effect of metformin randomised treatment on miRNA (vs placebo).
- These data support the putative utilities of miRNAs as reproducible biomarkers, but also highlight challenges in their use as surrogates for RCTs.

Appendix 10: Poster for Diabetes UK, Liverpool, 2019

University of Glasgow

Associations of targeted circulating microRNAs with insulin resistance in the CAMERA trial

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Introduction

- MicroRNAs (miRNAs) are a class of small, single stranded, non-coding RNAs molecules(20-25 nucleotides long) that regulate gene expression. They were thought to be exclusively intracellular until recently they were discovered in body fluids.
- Circulating miRNAs are remarkably stable in body fluids which made them a potential diagnostic biomarkers.
- Several circulating miRNAs have been reported to be associated with insulin resistance (IR).
- It is also not clear whether these associations are causal.
- Studies have generally been limited by small size.

Aim

- Investigate the association of the targeted circulating miRNAs with insulin sensitivity.
- Explore the potential usage of these circulating miRNAs as biomarkers for insulin resistance.
- Explore the effect of metformin on these miRNAs.

Table 4: Pearson correlation of insulin sensitivity markers vs miRNAs expression at baseline

	mir-221	mir-222	mir-192	mir-193b	mir-155	mir-144	INS	FBG
mir-221								
mir-222	0.619**							
mir-192	0.725**	0.487**						
mir-193b	0.602**	0.495**	0.801**					
mir-155	0.734**	0.596**	0.698**	0.611**				
mir-144	0.494**	0.405**	0.501**	0.482**	0.562**			
INS	- 0.052	- 0.073	- 0.115	- 0.124	- 0.096	- 0.184*		
FBG	- 0.135	- 0.033	- 0.187*	- 0.175*	- 0.165*	- 0.251**	0.286*	
HOMA-IR	- 0.072	- 0.086	- 0.138	- 0.152	- 0.121	- 0.287*	0.981**	0.981**

Methods

- CAMERA study - randomised, placebo-controlled double-blinded trial performed in Glasgow, UK (2009-2012).
- 173 patients CHD patients (without diabetes)
- Metformin or placebo (1:1) for 18 months (Figure 1).
- miRNAs extracted from available baseline and 18 month visit plasma samples.
- The expressions of mir-221, 222, 144, 155, 192 and 193b (targeted based on previous literature) were measured using RT-qPCR.
- Spike-in of cel-miR-39 was used to normalise. Repeat samples run as additional quality control.
- Stats:
  - linear regression of ΔCT values
  - $2^{-\Delta\Delta CT}$ method to investigate the randomised effect of metformin.

Figure 1: Trial Profile

Table 5: Baseline associations of biomarkers with selected miRNA

MiRNA	Predictor	Model 1 β (95% CI) p-value	Model 2 β (95% CI) p-value	Model 3 β (95% CI) p-value
Mir-144	Glucose (mmol/L)	34% (15.1-50)	31.8% (10.4-46.1)	31.7% (8.9-48.8)
	Insulin (pmol/L)	2.5% (0.3-4.5)	2.2% (0.1-4.3)	2.3% (0.1 - 2.3)
	HOMA-IR	10.2% (2.5-17.2)	9.3% (1.5-16.4)	9.7% (1.5-17.3)

Results

In cross sectional analysis at baseline:

- mir-144 showed the strongest association with markers of insulin sensitivity (Table 4&5).
- Adjustment for age, gender, and body fat did not attenuate these associations (Table 5).
- Other miRNAs showed variable associations with markers of IR and glycaemia (Table 4).
- No miRNA was associated with anthropometric measurements.

- **Prospectively:**
- Metformin randomisation did not change any miRNA (figure 2)

Table 2: Association of baseline miRNAs with baseline metabolic risk factors

	Placebo group (n=81)	Metformin group (n=73)
Demographic Characteristics		
Age (years)	64 (8)	63 (8)
Gender		
Male	61 (74%)	58 (80%)
Female	20 (25%)	15 (20%)
Blood pressure (mm Hg)	140/79 (20/11)	140/79 (18/11)
Anthropometric Characteristics		
Body mass index (kg/m^2) *	30.7 (27.1-33.1)	29.4 (27.5-32.3)
Biochemical Characteristics (Fasting)		
Plasma Glucose (mmol/L)	5.2 (0.5)	5.3 (0.3)
Insulin (pmol/L) *	8.7 (7.5-14.4)	9.8 (7.2-13)
HOMA-IR*	2.3 (1.6-3.6)	2.3 (1.6-3.1)
HbA1c (mmol/mol) *	38.2 (36-40)	38.7 (37-41)
Triglyceride (mmol/L) *	1.5 (1.1-1.9)	1.4 (1.1-2.0)
Total Cholesterol (mmol/L) *	4.3 (3.6-4.7)	4.2 (3.6-4.7)
HDL (mmol/L) *	1.1 (1.0-1.4)	1.1 (1.0-1.3)
Alanine aminotransferase (U/L) *	25.0 (18.5-33.5)	23.0 (19.5-35)
γ-glutamyltransferase (U/L) *	35.5 (21.5-54)	34.0 (22.5-64)
High sensitivity C-reactive protein (mg/L) *	1.6 (0.9-3.2)	1.8 (0.8-3.9)
WBC (x10^9/L) *	6.7 (5.6-8.1)	6.5 (5.8-7.5)
miRNA Expression		
mir-222 (dCt)	3.4 (1.7)	3.6 (1.7)
mir-221 (dCt)	1.4 (1.7)	1.4 (1.6)
mir-144 (dCt)	9.7 (1.4)	10.0 (1.5)
mir-155 (dCt)	7.4 (1.3)	7.4 (1.2)
mir-192 (dCt)	8.6 (1.6)	8.6 (1.5)
mir-193b (dCt)	9.1 (1.5)	9.0 (1.6)

* Not normally distributed variables, median (inter-quartile range) were used.
dCt: delta Ct, **HOMA-IR:** homeostatic model assessment for insulin resistance

Figure 2: Line Graph Comparing the RQ change for each miRNA between Placebo and Metformin Groups.

Conclusions

- Among the six targeted miRNAs, mir-144 displayed a strong positive association with glucose, HOMA-IR, and insulin thus highlighting the potential role of mir-144 as a key player in insulin resistance, not mediated by body weight.
- These data support the putative utilities of miRNAs as reproducible biomarkers, but also highlight challenges in their use as surrogates for RCTs.
- Further studies are required to investigate causality

Appendix 11: Conference support funding award

University of Glasgow | College of Medical, Veterinary & Life Sciences

J:admin/gradschool/committee/taac/2018-19/conferencesupportaward

28 February 2019

Ms Tahani Alramah
PhD student
ICAMS
(by email)

Dear Tahani

Application for Conference Support Funding 2018/2019

Following careful consideration by the Training and Awards Committee of your recent application for a Conference Support Award, I am pleased to inform you that you have been awarded **£400** towards the cost of your attendance at the Diabetes UK Professional Conference from 6-8 March 2019 in Liverpool.

It is a condition of this award that you submit a short report (no more than 250 words) to the MVLS Graduate School within one month of undertaking the skills training, describing: the event, who attended and summarising the skills gained.

Please be advised that you should meet the initial cost of the activity yourself or through your supervisor (they should advise us of the appropriate budget code in which to reimburse the costs through a journal entry form). Upon completion of the activity, **(if paying yourself)** you will be required to complete an **expenses form** and send this along with your receipts to the Graduate School. Payment will then be made to you by the Finance Office. If you have any further questions please contact the Graduate School at the following telephone number: (0141) 330 6498 or Audrey.Hillis@glasgow.ac.uk

Kind regards

Audrey Hillis

Audrey Hillis
Graduate School

Enc.

cc Dr Delyth Graham, PG Convener
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