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**Circulating miRNA Biomarkers in
Metabolic Syndrome and Clinical Obesity
and their Link with Triple Negative Breast Cancer**

A thesis submitted to the University of Dublin, Trinity College
towards a degree of Doctor of Philosophy

By

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Based on research carried out under the supervision of

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Summary

Metabolic syndrome (MetS), which is a spectrum of conditions including abdominal obesity, dyslipidemia, hypertension, and insulin resistance are increasing in prevalence globally and lead to an increased risk of cardiovascular disease, Type 2 diabetes mellitus and cancer. The focus of this project was to seek blood-based biomarkers for MetS, in the form of miRNAs, extracellular vesicles and proteins (*i.e.* neuromedin U and interleukin 6). Additionally, triple negative breast cancer (TNBC) risk has been epidemiologically linked to MetS and obesity. This association was addressed by investigating the metabolic phenotype of two TNBC cell line variants, by assessing their energy synthesising pathways.

Profiling of the miRNA content of plasma from obese and MetS participants in an intervention study identified miR-758-3p as a potential diagnostic biomarker for MetS independent of obesity. This miRNA was also shown to have potential as a biomarker for response to dietary intervention with whey protein, as there was a positive clinical outcome for participants randomised to the whey dietary group following a high fat meal. Studies of extracellular vesicles (EV) secreted from healthy control, obese and MetS participants revealed that EV quantities may be used to diagnose MetS, independent of obesity, as EV quantities from MetS participants were found to be significantly higher than the quantities of healthy controls and obese participants. Global miRNA profiling of the EV miRNA content identified miR-100-5p as a potential diagnostic biomarker for MetS; interestingly, miR-758-3p was not found in the EVs from the same cohort of participants. Finally, we investigated the metabolic phenotype of two TNBC cell line variants and found there to be a significant decrease in the oxygen consumption and a significant increase in the glycolysis of the aggressive TNBC cell line variant compared to the less aggressive parent TNBC cell line variant. The increased migration and invasion of these cells may be mediated, at least in part, by altered oxygen consumption and glycolysis.

In conclusion, the research presented here has identified a potential diagnostic biomarker for MetS (*i.e.* miR-758-3p) that distinguished MetS from obesity, a central feature of MetS. This biomarker may also indicate response of an individual to dietary intervention. EV quantities isolated from MetS participants

may be useful as a diagnostic tool to identify individuals with MetS; that may also distinguish MetS from obesity. Not only do the EVs themselves have potential as a diagnostic biomarker, their miRNA content also has potential, as we identified one miRNA to be detected in the MetS cohort and undetected in the healthy controls (*i.e.* miR-100-5p). The association between MetS, obesity and TNBC may be metabolism. We have shown that the altered metabolic phenotype of the more aggressive TNBC cell line variant may explain the increased aggressiveness of these cells.

Abbreviations

AACE	American Association for Clinical Endocrinology
ABCA1	ATP-binding cassette transporter
ABCG2	ATP-binding cassette sub-family G member 2
<i>AdipQ</i>	Adiponectin
AHA	American Heart Association
ANOVA	Analysis of Variance
ApoB	Apolipoprotein B
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
BAT	Brown Adipose Tissue
BCRP	Breast Cancer Resistance Protein
BMI	Body Mass Index
BP	Blood Pressure
BSA	Bovine Serum Albumin
CAD	Coronary Artery Disease
cDNA	Complementary DNA
CERP	Cholesterol efflux regulatory protein
CHD	Coronary Heart Disease
CM	Conditioned Medium
CRP	C-reactive protein
C _T	Cycle Threshold
CVD	Cardiovascular Disease
DCA	Dichloroacetate
dFBS	Depleted Fetal Bovine Serum
DNA	Deoxyribonucleic Acid
ECAR	Extracellular Acidification Rate
EGIR	European Group for the Study of Insulin Resistance
ELISA	Enzyme-Linked Immunosorbent Assay
ER+	Estrogen Receptor +
EV	Extracellular Vesicle
FA	Fatty Acid
FBS	Fetal Bovine Serum
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazine
FdG	Fluorodeoxyglucose
g	Measurement of Relative Centrifugal Force
gDNA	Genomic Deoxyribonucleic Acid
GLP-1	Glucagon-like Peptide-1
GLUT	Glucose Transport Receptor
HCC	Hepatocellular carcinoma
HCL	Hypercholesterolemia
HDL	High Density Lipoprotein
HER2+	Human Epidermal Growth Factor Receptor Positive
HPT	Hypertension
IDF	International Diabetes Federation
IGF-1	Insulin-like Growth Factor Receptor-1
IL-6	Interleukin 6
IL-6R	Interleukin 6 Receptor
IPC	Interplate Calibrator
IR	Insulin Resistance

LDL	Low Density Lipoprotein
MetS	Metabolic Syndrome
Mins	Minutes
miRNA	Micro Ribonucleic Acid
NAO	10-N-Nonyl Acridine Orange
NCEP:ATPIII	National Cholesterol Education Program-Third Adult Treatment Panel
NEP	Neprilysin
NHANES	National Health and Nutrition Examination Survey III
NHLBI	National Heart, Lung, and Blood Institute
NmU R1	Neuromedin U Receptor 1
NmU R2	Neuromedin U Receptor 2
NmU	Neuromedin U
NTA	Nanoparticle Tracking Analysis
OCR	Oxygen Consumption Rate
OGTT	Oral Glucose Tolerance Test
PBS	Phosphate Buffered Saline
PD	Pyruvate Dehydrogenase
PDK	Pyruvate Dehydrogenase Kinase
PET	Positron Electron Tomography
PR+	Progesterone Receptor Positive
Pre-miRNA	Precursor miRNA
PPAR- γ	peroxisome proliferator-activator receptor gamma
Pri-miRNA	Primary miRNA
PVDF	Polyvinylidene difluoride
qPCR	Quantitative Polymerase Chain Reaction
RAAS	Rennin-angiotensin-aldosterone System
RNA	Ribonucleic Acid
ROC	Receiver Operating Characteristics
ROS	Reactive Oxygen Species
rRNA	Ribosomal RNA
RT	Reverse Transcription
SDS	Sodium Dodecyl Sulfate
Secs	Seconds
MC-SFA	Medium Chain-Saturated Fatty Acids
SNP	Single Nucleotide Polymorphism
T2DM	Type 2 Diabetes
TEM	Transmission Electron Microscopy
TNBC	Triple Negative Breast Cancer
tRNA	Transfer RNA
VDAC1	Voltage Dependent Anion Channel 1
vLDL	Very Low Density Lipoprotein
WC	Waist Circumference
WHO	World Health Organisation
WP	Work Package
Yrs	Years
mRNA	Messenger RNA
DMEM	Dulbecco's Modified Eagles Medium

Chapter One: Introduction

Parts of this chapter are published in the following review articles:

Metabolic Syndrome: a closer look at the growing epidemic and its associated pathologies.

Sadhbh O'Neill and Lorraine O'Driscoll *Obesity Reviews* (2015) Jan; 16(1):1-12.

Circulating miRNA Biomarkers for Metabolic Syndrome.

Sadhbh O'Neill, Mette Bohl Larsen, Soren Gregersen, Kjeld Hermansen and Lorraine O'Driscoll *Trends in Endocrinology and Metabolism* (2016) 27(6): 363-374.

1. Introduction

1.1. Metabolic Syndrome

The term Metabolic Syndrome (MetS), first coined by Haller and Hanefeld [1] in 1975 is characterised as a combination of underlying risk factors that when - occurring together - culminate in adverse outcomes, including type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD) [2] and cancer [3] and thus an approximately 1.6-fold increase in mortality [4]. The major risk factors for developing MetS are physical inactivity and a diet high in both fats and carbohydrates; contributing to the two central clinical features *i.e.* central obesity and insulin resistance (IR). Obesity is fundamental to MetS as it appears to precede the emergence of the other MetS risk factors [5]. The defining components of MetS that cluster together are obesity/central obesity, IR, hypertension and circulating hypertriglyceridemia (dyslipidemia) [6].

Although the increased consequential risk of T2DM and CVD is recognised by many organisations including the World Health Organisation (WHO), the European Group for the study of Insulin Resistance (EGIR), the National Cholesterol Education Program-Third Adult Treatment Panel (NCEP:ATPIII), the American Association for Clinical Endocrinology (AACE) and the International Diabetes Federation (IDF), a single precise definition and the defined contributions of the underlying components of MetS are of much debate. As detailed in Table 1.1, each of these groups has developed their own (granted, typically overlapping) criteria for defining MetS, with regard to thresholds for each of the variables of MetS in order to accurately identify people at a higher than average risk of developing T2DM, CVD and cancer. The first attempt to define MetS was made by the WHO in 1998, which proposed IR as central to the definition of MetS, along with at least 2 of raised blood pressure (BP), hypertriglyceridemia and/or low HDL-cholesterol, obesity (waist circumference (WC) or body mass index (BMI)) and microalbuminuria [7]. Shortly thereafter, the EGIR excluded microalbuminuria as a component of MetS and required impaired glucose tolerance to be present. Additionally, WC and not BMI was regarded as the main indicator of obesity and new cut-off points for the other parameters of MetS [8]. Subsequently, in 2001, the NCEP:ATPIII introduced a new set of criteria in which WC, hypertriglyceridemia, BP and fasting glucose

were included. Interestingly, IR was not integral to the definition of MetS, which was integral to the WHO and EGIR definitions [9]. Finally, in 2005, the IDF published criteria in an attempt to precisely define MetS, so it could be a better predictor of CVD and T2DM. The IDF introduced abdominal obesity as a prerequisite of MetS. They also included IR, hypertriglyceridemia and BP [10].

In 2009, however, a joint statement by the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute (NHLBI); American Heart Association (AHA); World Health Federation; International Atherosclerosis Society; and International Association for the Study of Obesity was published. This joint definition stated that obesity and IR are not pre-requisites for MetS but that three of the five components (Table 1.2 A) would suffice for a diagnosis of MetS, with the thresholds for measuring waist circumference requiring ethnic and nation specificity, with a recommendation that the IDF cut-off points be used for non-Europeans and either the IDF or AHA/NHLBI cut-off points for Europeans (Table 1.2 B) [11].

Table 1.1: Criteria for the diagnosis of MetS according to a number of organisations.

IDF	WHO	NCEP:ATP III	EGIR	AACE
Central Obesity = WC (Ethnic and Gender Specific) + Two of the Following: Triglycerides ≥150 mg/dL	High Insulin Level + Two of the Following: Abdominal Obesity WC >37'' BMI >30 kg/m ²	Any Three of the Following: WC > 40'' (Male) >35'' (Female)	Impaired Glucose Tolerance + Two of the Following: WC ≥94cm (Male) ≥80cm (Female)	Central Obesity = WC (Ethnic and Gender Specific) + Two of the Following: Triglycerides ≥150 mg/dL
Cholesterol – HDL <40 mg/dL (male) <50 mg/dL (Female)	Triglycerides >150 mg/dL	Triglycerides ≥ 150 mg/dL	Triglycerides >2 mmol/L	Cholesterol – HDL <40 mg/dL (Male) <50 mg/dL (Female)
BP ≥ $\frac{130}{85}$ mm Hg	Cholesterol –HDL <35 mg/dL (Male) <39 mg/dL (Female)	Cholesterol –HDL <40 mg/dL (Male) <50 mg/dL (Female)	Cholesterol – HDL <1 mg/dL	BP ≥ $\frac{130}{85}$ mm Hg
Fasting Glucose ≥5.6 mmol/L or T2DM	BP ≥ $\frac{140}{90}$ mm Hg Microalbuminuria >30 mg/g	BP ≥ $\frac{130}{85}$ mm Hg Fasting Glucose ≥110 mg/dL	BP ≥ $\frac{140}{90}$ mm Hg or Hypertensives Fasting Glucose ≥6.1 mmol/L	

AACE: American Association of Clinical Endocrinology, EGIR: European Group for the study of Insulin Resistance, WHO: World Health Organisation, NCEP
ATPIII: National Cholesterol Education Program-Third Adult Treatment Panel, IDF: International Diabetes Federation, WC: waist circumference, BMI: body
mass index, BP: blood pressure, HDL: high density lipoprotein.

Table 1.2: Definition of MetS according to the Joint Scientific Statement.

A.

Component of MetS	Cut-Off Point
Elevated WC	Table 1.2 B
Elevated triglycerides (or drug treatment)	≥150 mg/dL
Reduced HDL-cholesterol (or drug treatment)	<40 mg/dL (males), <50 mg/dL (females)
Elevated BP (anti-hypertensive medication)	≥ $\frac{130}{85}$ mm Hg
Elevated fasting glucose	≥100mg/dL

B.

Ethnicity	Male	Female
Europid (Caucasian)	≥102cm	≥88cm
South Asian	≥90cm	≥80cm
Central & South America	≥90cm	≥80cm
Middle Eastern Mediterranean	≥94cm	≥80cm
Sub-Saharan Africa	≥94cm	≥80cm
Chinese	≥90cm	≥80cm
Japanese	≥90cm	≥80cm

A. Components of MetS required for a diagnosis of MetS and B. Ethnic specific WC cut-off points.

Elevated triglyceride and HDL-cholesterol treatment are with fibrates and nicotinic acid. A patient taking 1 of these is presumed to have high triglycerides and low HDL-cholesterol. High dose ω-3 fatty acid presumes high triglycerides. WC-waist circumference, HDL-high density lipoprotein, BP-blood pressure

1.1.1 Prevalence

The most widely accepted and clinically used definitions for MetS are those set out by the WHO, NCEP:ATPIII and IDF (Table 1.1) [12]. The existence of these three somewhat varying definitions –even though they differ only in small details and defining values- impedes the determination of the true prevalence of MetS worldwide, as well as within specific countries, genders, and ethnicities. Regardless of the details of each specific definition, however, it is generally accepted by all groups that the prevalence of MetS is increasing, in accordance with increasing BMI and age [13]. Kaur *et al.* [14] reported that the world-wide prevalence of MetS to be between 10% and 84%, depending on the ethnicity, age, gender and race of the population; while the IDF estimates that one-quarter of the world’s population has MetS. According to Pal and Ellis [15], 20% of adults in the western world have MetS.

Despite ambiguity on the precise definition of MetS, a number of studies have been undertaken to determine– as accurately as possible– the incidence of MetS within specific countries, ethnic backgrounds and between genders. Although this thesis cannot claim to be comprehensive for all countries and ethnicities, a number of studies have been reviewed (as exemplified in Table 1.3) for Australia, China, Denmark, India, Ireland, South Korea and the US. Evidently, MetS is a serious global issue.

Table 1.3: Prevalence of MetS in a selection of countries worldwide

Country	n	Age (yrs)	NCEP:ATP III	IDF	Ref
<u>United States</u>	3601	≥20	33.7% Male 32.4% Female	39.9% Male 38.1% Female	[16]
<u>Australia</u>	11247	≥25	24.4% Male 19.9% Female	34.4% Male 27.2% Female	[17]
<u>Europe –</u>					
Ireland	1716	32.78	21.8% Male 21.5% Female	26.4% Male 14% Female	[18]
Denmark	2493	41.72	18.6% Male 14.3% female	23.8% Male 17.5% Female	[19]
<u>Asia-</u>					
China	15540	≥ 35-74	9.8% Male 17.8% Female	N/R N/R	[20]
India	2350	> 20	17.1% Male 19.4% Female	N/R N/R	[21]
Korea	40698	20-82	5.2% Male 9.05% Female	N/R N/R	[22]

Prevalence of MetS according to age, gender and NCEP ATPIII or IDF definitions. N/R = not reported.

1.1.1.1 Prevalence of MetS in the United States

In the US, data taken from the 2000 census estimated that 47 million of the US adult population (accounting for 22.48% of the adult population) had MetS, according to the NCEP:ATPIII definition [23]. This figure could be an underestimation, as the IDF method is now proposed to be superior at diagnosing MetS compared to the NCEP:ATPIII definition, as detailed below.

In 2011, Mozumdar *et al.* [24] compared MetS prevalence between the National Health and Nutrition Examination Survey (NHANES) III data (1988-1994) (6423 participant's ≥ 20 years) and NHANES 1999-2006 data (6962 participant's ≥ 20 years), according to the NCEP:ATPIII definition. The data showed that the prevalence of MetS had significantly increased between the time of the first and the second surveys, considering both the unadjusted ($27.9 \pm 1.1\%$ to $34.1 \pm 0.8\%$) and age-adjusted ($29.2 \pm 1.0\%$ to $34.2 \pm 0.7\%$) data. The unadjusted ($p=0.012$) and age-adjusted (0.046) prevalence of MetS in males had significantly increased. For females, the unadjusted and age-adjusted ($p<0.001$) prevalence were also significantly increased from the time of the first survey to the second survey. From the NHANES 1999-2006 data, Mozumdar *et al.* [24] estimated that approximately 68 million US adults had MetS (*i.e.* 32.4 million men; 35.3 million women).

Ford [16] in 2005, identified a discrepancy between the NCEP:ATPIII and IDF definitions for diagnosing MetS. A total of 3,601 men and women aged ≥ 20 years were recruited from the NHANES 1999-2002 survey. In this cohort, the NCEP:ATPIII definition identified $34.5 \pm 0.9\%$ ($33.7 \pm 1.6\%$ men, $35.4 \pm 1.2\%$ female) of the cohort with MetS. In contrast, the IDF definition identified $39.0 \pm 1.1\%$ ($39.9 \pm 1.7\%$ male, $38.1 \pm 1.2\%$ female) of the same population to have MetS.

In order to determine the prevalence of MetS by group, age, race and gender, a US study similar to the one described above used data from the NHANES 2003-2006 cohort for a total of 3,423 adults sub-divided into 3 cohorts based on age *i.e.* aged 20-39 years, 40-59 years and >60 years. Here, the overall prevalence of MetS was reported as 34% based on the NCEP:ATPIII definition. Specifically, for the 20-39 years age group, 20% of males and 16% of females were identified as having MetS. For the 40-59 years age group, 41% of males and 37% of females

had MetS; and for the >60 years age group, 52% of males and 54% of females had MetS. These results show that MetS prevalence increases with age, but with no significant difference between genders. However, when the cohort was divided into race and ethnicity *i.e.* non-Hispanic white, non-Hispanic black, and Mexican-American, a difference in MetS prevalence was apparent. Specifically, 25% of non-Hispanic black males were considered to have MetS compared to 37% of non-Hispanic white males but no significant differences in MetS prevalence for the female sub-groups were reported. Non-Hispanic black and Mexican-American females were approximately 1.5 times more likely to have MetS than non-Hispanic white females [13] .

1.1.1.2. Prevalence of MetS in Australia

An Australian population-based survey (n=11247; 5049 male and 6198 female), termed AusDiab, was conducted. Participants had anthropometry, blood pressure and fasting glucose levels measured and subsequently their 10-year CVD risk was calculated [25]. In 2005, Zimmet *et al.* [26] identified 29.1% of this Australian population older than 24 years as having MetS according to the IDF criteria and 19.3% of the same population when evaluated according to the NCEP:ATPIII criteria.

Using the same AusDiab data, Cameron *et al.* [17] compared MetS prevalence using four definitions, *i.e.* the IDF, NCEP:ATPIII, EGIR and WHO. The NCEP:ATPIII definition identified 22.1%, the WHO definition identified 21.7%, the IDF definition identified 30.7% and the EGIR definition identified 13.4% of the cohort as having MetS. Regardless of the definition of MetS considered, the prevalence of MetS increased with increasing age and was significantly higher in men than women in all groups ($p<0.001$). It was also determined that the WHO definition was associated with greater CVD risk [17] (Table 1.3). Again, in both Australian studies, the IDF definition identified a higher percentage of the population with MetS than the NCEP:ATPIII definition, Furthermore, the IDF criteria identified an apparently higher prevalence of MetS than the WHO and EGIR definitions.

1.1.1.3 Prevalence of MetS in Europe – Ireland and Denmark

In 2009, Waterhouse *et al.* [18] compared the prevalence of MetS by the NCEP:ATPIII and IDF criteria in an Irish population. The study enrolled 1,716 participants aged 32-78 years. The IDF definition identified 21.4% (26.4% male, 14% female) of individuals as having MetS, while the NCEP:ATPIII comparative figure was 13.2% (15.8% male, 9.3% female). The NCEP:ATPIII apparently did not recognise 8.2% of MetS patients that the IDF definition identified (Table 1.3).

In a Danish study, termed Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA-1) health survey, 2493 participants of 41-72 years were asked to take part in the MetS study. MetS was, again, diagnosed according to the IDF and NCEP:ATPIII definitions. The IDF definition identified 23.8% of males and 17.5% of females as having MetS, while the NCEP:ATPIII definition identified 18.6% of males and 14.3% of females as having MetS [19] (Table 1.3).

1.1.1.4. Prevalence of MetS in Asia – China, India, South Korea

Zhao *et al.* [27] aimed to determine the prevalence of MetS among North-western, rural Chinese. A cohort of 2990 participants aged 18-80 years were enrolled and MetS was identified according to the NCEP:ATPIII, IDF definitions, and the NCEP:ATPIII modified for an Asian population. MetS was identified in 7.9% (male 5.4%, female 10.4%), 10.8% (male 8.1%, female 13.6%) and 15.1% (male 12.8%, female 17.4%) according to each of the three definitions, respectively. MetS had a higher prevalence in women than men, increasing with age. In this study it was found that hypertension was the most common MetS component in this population. In a separate study, Gu *et al.* [20] identified MetS in a Chinese population according to the NCEP:ATPIII definition. Of the 15,540 participants, aged 35-74 years, the prevalence of MetS was determined to be 9.8% in men and 17.8% in women (Table 1.3). The data illustrates that the percentage of MetS individuals identified by the NCEP:ATPIII method were somewhat different in both studies for the same ethnicity. This discrepancy may be due to the differences in sample size for each study (*i.e.* 2990 for Zhao *et al.* [27] and 15540 for Gu *et al.* [20]) or differences in age groups studied (*i.e.* 18-80 years for Zhao *et al.* [27] and 35-74 years for Gu *et al.* [20]). Gu *et al.* [20] reported that the prevalence of MetS was higher in Northern China compared to Southern China

and was also higher in urban areas than rural areas. As Zhao *et al.* [27] studied a Northwest, rural area, this may explain the differences in MetS prevalence between the two studies, although both studies do show a trend that Chinese women have a higher prevalence of MetS than Chinese men.

Sawant *et al.* [28] conducted a study in the urban city of Mumbai, India using data from the CARDIAC evaluation camp on 548 individuals who are ≥ 20 years. MetS was identified in 19.52% (male 25.16%, female 12.6%) of the cohort by the NCEP:ATPIII criteria. MetS was significantly ($p=0.008$) higher in males than females. Interestingly in 95% of the cohort, at least one component of MetS was identified according to the modified NCEP:ATPIII criteria. Considering 2,350 individuals aged ≥ 20 years, Deepa *et al.* [21] identified MetS in 23.2% of the subjects according to the WHO definition, 18.3% according to the NCEP:ATPIII definition and 25.8% by the IDF definition, again, as previously observed in other cohorts *e.g.* in the US [16] the IDF criteria identified the highest MetS prevalence (Table 1.3).

Lim *et al.* [29] compared the prevalence of MetS in a South Korean population based on data from the Korean Health and Nutrition Examination Surveys from 1998, 2001, 2005, and 2007 for a total of 6907, 4536, 5373, 2890 individuals ≥ 20 years from each survey respectively. The age-adjusted prevalence of MetS was 24.9% in 1998, 29.2% in 2001, 30.4% in 2005 and 31.3% in 2007, according to the revised NCEP:ATPIII definition. Therefore, the prevalence of MetS increased with time and LDL-cholesterol was the most common abnormality, increasing by 13.8% in the 10-year period. This was followed by obesity (8.7%) and hypertriglyceridemia (4.9%). In a study [22] of 40698 South Korean participants, MetS was identified in 6.8% (5.2% male, 9.0% female) using the NCEP:ATPIII definition, while the definition adjusted specifically for the Asian population and WC identified 10.9% (9.8% male, 12.4% female), while the same definition modified for BMI identified 13.1% (13.2% male, 13.1% female) with MetS (Table 1.3). The discrepancies in the MetS prevalence figures between the two Korean studies may be due to the huge difference in sample sizes (*i.e.* 2890-6970 for Lim *et al.* [29] and 40698 for Lee *et al.* [22]). Lee *et al.* [22] studied a Korean population based in Seoul, while Lim *et al.* [29] was a national survey, again the different regions of study may explain the discrepancy in MetS prevalence for the Korean population.

Collating these many studies from around the world demonstrates that MetS is a substantial international problem, regardless of the specific criteria/definition selected to determine MetS. Differences in prevalence within a given population are apparent, depending on which specific definition is used, indicating a level of agreement but also that results from different studies should be collated and compared with caution and in full knowledge of the definition that was used. To truly determine the problem of MetS throughout the world, ideally one international definition would be agreed upon and consistently used.

1.1.2. Pathophysiology

1.1.2.1. Obesity

Obesity is an excess of body fat and is measured by BMI or by WC. A BMI $\geq 25\text{kg/m}^2$ and WC $\geq 94\text{cm}$ in men and $\geq 80\text{cm}$ in women is considered overweight, while a BMI $\geq 30\text{kg/m}^2$ and WC $\geq 102\text{cm}$ in men and WC $\geq 88\text{cm}$ in women is considered obese [30]. Central obesity is considered the most common manifestation of fundamental importance for the diagnosis of MetS (Figure 1.1) [31]. The prevalence of obesity worldwide is reaching epidemic levels. In 2005, the WHO estimated that 1 billion people world-wide had a BMI $\geq 25\text{kg/m}^2$ and 300 million had a BMI $\geq 30\text{kg/m}^2$. They also estimated that by 2015, 1.5 billion people will have a BMI $\geq 25\text{kg/m}^2$ [32]. By 2008, Finucane *et al.* [33] had demonstrated that this figure of 1 billion had risen to an estimated total of 1.46 billion people world-wide with a BMI $>25\text{kg/m}^2$; this correlated with 898 million overweight individuals and 502 million obese individuals. A recent report from the American Heart Association stated that 154-157 million adults in the US are overweight or obese [34]. MetS is, therefore, of substantial concern to the medical field and to society in general, due to the worldwide increase in obesity.

It should be noted that although obesity leads to an increased risk of mortality, studies show that the risk of mortality is only slightly but not significantly increased for an overweight person (BMI: 25-29). The risk of mortality increases significantly for the moderately obese (BMI: 30-34.9), increasing again for the severely obese (BMI: 35-39.9) and increasing again for the very severely obese (BMI: >40) [35].

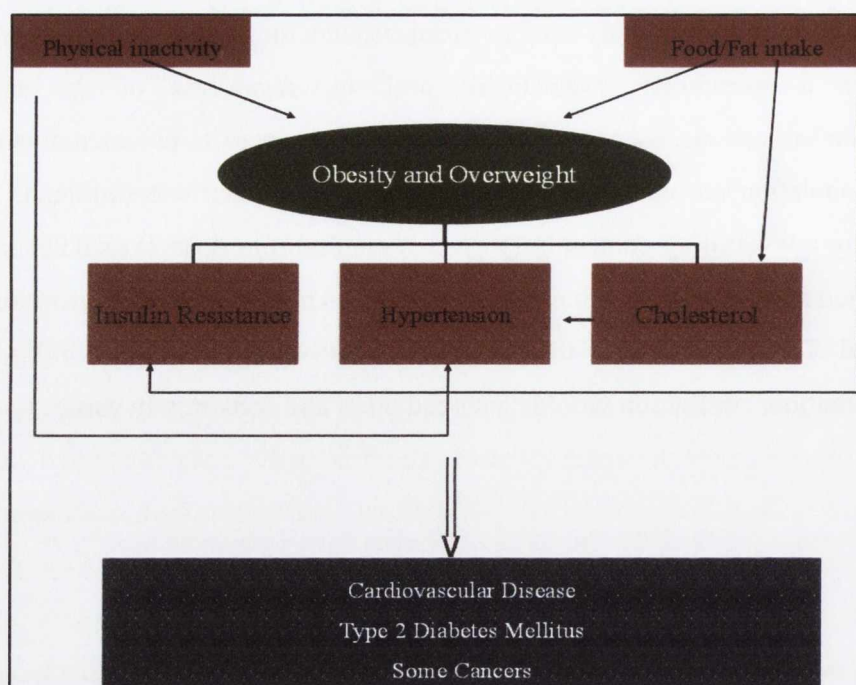


Figure 1.1: Interplay between the risk factors for metabolic disease.

Overweight and obesity are central to the risk of MetS, both predisposing to IR, hypertension and dyslipidemia, all of which are risk factors of MetS. Arrows indicate that physical inactivity and excess food/fat intake lead to overweight and obesity, excess food/fat intake leads to hypercholesterolemia and IR, overweight and obesity lead to IR, hypertension and hypercholesterolemia.

1.1.2.2. Insulin Resistance

In 1988, Reaven proposed the notion of Syndrome X to describe the clustering of components of MetS, with IR as the common denominator [36]. While obesity is the predominant risk factor for IR and T2DM, environmental and genetic factors also contribute to the pathogenesis [37], contributing to 171 million people being diagnosed with diabetes in 2000 and projected to increase to 366 million by 2030 [38]. 30-40% of the population are reported to be IR [39]. In addition to contributing to insulin resistance (by an, as of yet, unidentified mechanism), elevated levels of fatty acids (FAs) impair β -cell function [38]. IR is linked to hyper-triglyceridemia and is a driver of T2DM and also CVD [40]. IR is related to several CVD risk factors, such as obesity, hyperglycaemia, hyper-triglyceridemia, low HDL cholesterol and hypertension [39]. Therefore, as with obesity, IR is also an important factor in MetS. IR is measured using the homeostatic model assessment to quantify insulin resistance and beta-cell function.

1.1.2.3. Dyslipidemia

In addition to obesity and IR, dyslipidemia (an abnormal amount of lipids *e.g.* cholesterol and/or fats in the blood) is a hallmark of MetS and includes the flux of fatty acids, Apolipoprotein B (Apo B), high triglycerides, low HDL cholesterol and high small dense-low density lipoprotein (LDL) [41]. An association between obesity, hypertension and hyper-triglyceridemia was proposed by Albrink *et al.* [42] in the 1980s. From there, Albrink along with others, such as Reaven, began to demonstrate a link between dyslipidemia and MetS [40]. After several decades, the IR Atherosclerosis study illustrated a direct link between IR and atherosclerosis. In addition to FAs playing a substantial role in the onset of IR in obese individuals, it was established that such FAs also have a role in the increase in triglyceride levels that lead to hyper-triglyceridemia [40]. In MetS patients, dyslipidemia may be caused by a combination of the over-production of very low density lipoprotein (VLDL); over-production of apolipoprotein B (ApoB); decreased breakdown of ApoB and increased catabolism of HDL cholesterol. All of these may be a consequence of IR [41]. Overall, the three major components of dyslipidemia associated with MetS are increased fasting and postprandial triglyceride-rich lipoproteins, decreased HDL, and increased LDL (Figure 1.2) [43].

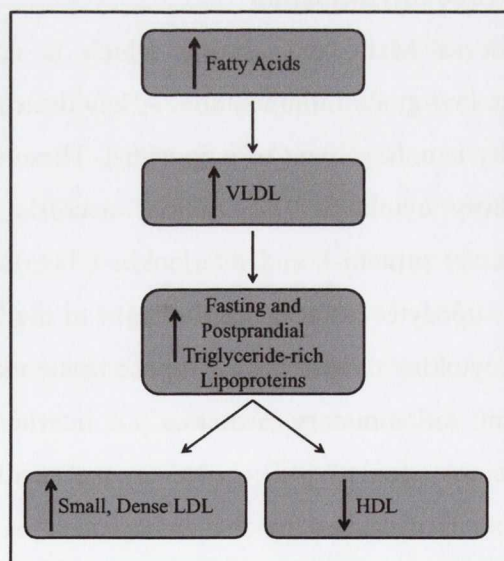


Figure 1.2: Process of progression from obesity and insulin resistance to dyslipidemia.

Arrows indicate that increased fatty acids lead to increased VLDL, which leads to increased fasting and postprandial triglyceride-rich lipoproteins, which leads to both increased small, dense LDL and decreased HDL. VLDL-very low density lipoprotein, LDL-low density lipoprotein, HDL-high density lipoprotein.

1.1.2.4. Hypertension

As illustrated in Figure 1.1 hypertension (abnormally high blood pressure (BP)) is also a central component of MetS, with approximately 85% of MetS patients suffering with this condition [44]. Hypertension is usually diagnosed at a late stage in the disease, at which point consequential life-threatening conditions, such as kidney damage and heart failure may occur [45]. IR and obesity have been recognised as the leading cause of hypertension [45]. 50% of hypertensive people are IR [40]. Obesity and IR contribute to the development of hypertension, both independently and collectively. Under normal, healthy circumstances, introduction of insulin into the bloodstream causes a release of nitric oxide and subsequent vasodilation. This is not seen in obese, insulin-resistant individuals [40]. Instead, IR and thus compensatory hyper-insulinemia activate the rennin-angiotensin-aldosterone system (RAAS) with consequential vasoconstriction and hypertension [44].

The NHANES 1999-2000 study in the US, including a total of 1677 US adults aged ≥ 20 years, demonstrated that hypertension is the most common component of MetS in men (41%), but was the third most common component in women (37%; while 52% had high WC and 43% had low HDL levels) [46].

1.1.2.5. Low-Grade Inflammation

Another aspect of MetS and obesity which is not included in the definitions for MetS is low-grade inflammation. A key determinant of low-grade inflammation in obesity is enlargement of adipocytes. These enlarged adipocytes release pro-inflammatory cytokines (*i.e.* tumour necrosis factor- α , IL-6, monocyte chemoattractant protein-1 and interleukin-1 β) into the circulation [47]. This release of adipocytes attracts macrophages to the enlarged adipocyte, resulting in increased cytokine release [48]. Adipose tissue macrophages are also capable of secreting anti-inflammatory mediators (*i.e.* interleukin-10, adiponectin and interleukin-1 receptor agonist) [48]; however, the secretion of these anti-inflammatory mediators is disturbed by the enlargement of the adipocyte and interestingly diet-induced obesity leads to a shift in the adipose tissue macrophages to a more pro-inflammatory state [49]. This adipose tissue inflammation contributes to IR [50], therefore, low-grade inflammation is thought to link obesity to IR, hypertension, dyslipidemia and atherosclerosis [51].

1.1.3. Consequential risks associated with Metabolic Syndrome

It has been established that those with MetS have a five times higher risk of developing T2DM, a three times higher risk of developing CVD [2] and an increased risk of developing cancer [52]. So far, however, our understanding is only as an indicator of risk and it has not yet been possible to develop an algorithm determining the personalised relative risk for a given individual developing CVD and/or T2DM and/or cancer [53].

1.1.3.1. Cardiovascular Disease/ Chronic Heart Disease

One purpose of accurately diagnosing MetS is to at least get an indication of the increased risk of CVD [54]. While the component factors of MetS that cluster together increase an individual's risk of CVD, it is apparent that each individual component also increases this risk [55]. CVD is the leading cause of death in the western world, accounting for 17 million deaths per year. In a Danish study focused on MetS, IR and CVD and which included 2493 participants, 21% were defined as having MetS according to the IDF criteria and 16% were reported as having MetS according to the NCEP:ATPIII definition. After 9.4 years of follow-up, 233 deaths from the original 2493 participants had resulted from cardiovascular events (CV death, ischaemic heart disease and stroke). This demonstrated that the incidence of CVD deaths was 14.6% according to IDF criteria and 16.6% according to NCEP:ATPIII criteria. These results taken together showed that NCEP:ATPIII definition was a significant predictor of CVD and the IDF definition was not when adjusted for IR. Also, IR was an independent risk factor for CVD and MetS and IR were both significant predictors of CVD in the non-diabetic population. The authors did not look at risk of CVD from each component of MetS [19]. The authors indicate that the NCEP:ATPIII definition of MetS has a higher predictive power for CVD than the IDF definition. However, in a Greek study including 9669 individuals, focused on comparing the predictive power of both definitions, MetS was defined at 43.4% and 24.5% according the IDF and NCEP:ATPIII definitions, respectively. The NCEP:ATPIII definition predicted a higher prevalence of CVD (23.3%) than did the IDF definition (18.3%) [56].

Population-based research studies have analysed the increased risk of CVD among MetS patients, across ethnicities. A US study spanning 8 years and involving 3323 middle-aged individuals of mainly Caucasian ethnicity (participants were part of the Framingham offspring study, examination 4) showed that the prevalence of MetS (by the NCEP:ATPIII definition), in both men and women who did not have CVD or T2DM at baseline, increased over an 8 year period. This increase was from 21.4% to 33.9% in men and 12.5% to 23.6% in women; an increase of approximately 50% for both genders. The risk of CVD and T2DM increased in correlation with this increase in the prevalence of MetS. After 8 years 174 individuals had CVD, 107 had chronic heart disease (CHD) and 178 individuals had T2DM [57]. The relative risk of CVD was 2.88 in men and 2.25 in women.

1.1.3.2. Type 2 Diabetes Mellitus

T2DM, like MetS, is a complex heterogeneous constellation of metabolic disorders including IR, unique dyslipidemia (*i.e.* characterized by hypertriglyceridemia; elevated blood levels of apolipoprotein B; small, dense LDL-cholesterol; and low levels of HDL cholesterol), obesity and hyperglycaemia [58] with the most common etiological factor being obesity [59], resulting in elevated blood glucose concentrations [6]. IR, dyslipidemia, obesity and hyper-insulinemia all precede T2DM in 75-85% of patients [59]. There are approximately 150 million people worldwide with the condition and this figure is projected to double by 2020 [58]. In a US population of 20-74 year olds, a meta-analysis incorporating studies from 1976-1980 and 1999-2000 showed an increase in prevalence of total diabetes (*i.e.* both Type 1 and Type 2) from 5.3% to 8.2% and the number diagnosed with diabetes between the two time periods increasing from 41% to 83%. The increase in the prevalence of T2DM was reported to be due to the rise in obesity. An increase in mean BMI from 25.4kg/m² to 26.7kg/m² from 1980-1962 to 1999-2000 was also seen. This correlated with an increase in mean obesity from 14.5% to 26.7% during the same periods and these increases correlated with an increase in T2DM from 1.8% to 5.8% [60].

The pancreatic hormone glucagon is of interest in T2DM. Glucagon is secreted by the alpha (α) cells of the pancreas and dysregulation of the α -cells is

proposed to precede beta (β)-cell dysregulation in T2DM [61]. Glucagon functions to increase glucose levels in the blood which in turn, is antagonistic to insulin. Glucagon is deregulated in both Type 1 diabetes and T2DM, but the effect is more subtle in T2DM. There is evidence of increased secretion and/ or incomplete suppression of α -cells in the majority of T2DM patients. In T2DM, the levels of glucagon are inappropriately high and appear to contribute to hyperglycaemia and abnormal glucose regulation [62]. Appropriate studies relating glucagon to MetS are lacking. However, of note is glucagon-like peptide-1 (GLP-1). GLP-1 is an integrin produced in the intestines that regulates both insulin and glucagon levels. In T2DM, GLP-1 is also increased in the serum of patients. In a study of a Japanese population of n=60 (MetS) and n=37 (pre-MetS [1-2 MetS components]), serum GLP-1 was measured. GLP-1 was significantly ($p<0.001$) increased in the MetS group compared to the pre-MetS group. The levels of GLP-1 correlated with the number of MetS components present. Of further note, MetS patients with a high level of GLP-1 were at a higher risk for CVD, independent of diabetes [63]

1.1.3.3. Cancer as a co-morbidity

Over the last several years the prevalence of cancer and its association to MetS has been documented. From studies that have been reported on so far, MetS or its components may play a role in the etiology, progression or prognosis of certain cancers. The American Cancer Society estimates that there are approximately 1.5 million new cancer cases in the United States per year accounting for 500,000 cancer deaths [64]. Overweight and obesity account for approximately 20% of all cancer cases [65]

1.1.3.3.1. Cancer and obesity

Considering the components of MetS, obesity is reaching epidemic proportions world-wide (Section 1.1.2.1) with 68% (72.3% male, 64.1% female) of the US population being overweight or obese [66]. Obesity is associated with a high incidence of cancer risk at multiple sites and is both a risk factor and poor prognostic factor for many malignancies including renal, breast, ovarian, pancreatic, endometrial, and oesophageal cancers [67, 68]. An association

between obesity and cancer has been established, but the mechanism(s) for this association has not been elucidated. It is known, however, that obesity contributes to IR; this leads to increased insulin levels correlating with increased levels of the growth factor insulin-like growth factor receptor (IGF-1) [68]. IR or oxidative stress may, therefore, be a contributing mechanism.

A study performed in the US from 1982-1998 involving approximately 900,000 individuals (404,576 men, 495,477 women), who were free from cancer at baseline, found that at the time of the 16-year follow-up 57,415 deaths had occurred. The results of the study showed that men with a BMI ≥ 40 had a 52% higher death rate from all cancers and women with a BMI ≥ 40 had a 62% higher death rate from all cancers compared to men and women of normal weight, respectively. From this data it was concluded that, in the US, 14% and 20% of all cancer deaths in men and women respectively could be prevented if normal weight was maintained [69].

Of note, brown adipocyte differentiation may play a key role in breast cancer progression. In a study of 96 breast cancer patients and 96 age- and weight-matched control female patients. Data on the distribution and intensity of active brown adipose tissue (BAT) as determined by FDG PET/CT (see Section 1.3) was evaluated by 2 nuclear medicine physicians. Results showed the prevalence of BAT to be significantly higher in the breast cancer group compared to the control group. Additionally, the frequency of BAT activity was 3-fold higher in breast cancer patients as compared to controls [70]. Furthermore, the master regulator of adipocyte differentiation, PRDM16, has been found to be expressed in a subset of mammary cancer stem cells in xenographs from mice [71].

1.1.3.3.2. Cancer and insulin resistance

There is growing epidemiological and clinical evidence implicating IR to cancer risk, but the mechanism(s) by which IR increases cancer risk is yet to be fully elucidated. However, many theories have been proposed, including genetic and/or environmental factors that lead to IR, *e.g.* obesity and hyper-insulinemia. Obesity leads to a number of pathophysiologies including an increase in FAs and triglycerides in the blood resulting in IR. Once IR is established, hyper-insulinemia, hyperglycaemia and increased gluconeogenesis result. A model for

the role of IR in cancer progression has been proposed: in insulin-resistant patients, hyper-insulinemia leads to mitosis of cancer cells; reactive oxygen species (ROS) produced by hyperglycaemia and also increased FAs. ROS overproduction results in DNA mutagenesis and carcinogenesis. Increased gluconeogenesis results in hyperglycaemia, decreased sex hormone binding globulin, increased IGF-1 that leads to increased IGF-1 feed-back loop and anti-apoptosis of cancer cells, altogether resulting in tumour initiation. Hyper-insulinemia and increased availability of IGF-1 in insulin resistant patients apparently have a role in tumour initiation and progression. Specifically, they stimulate ovarian synthesis of sex steroids and, in the breast and endometrium, they promote cellular proliferation and inhibit apoptosis [72]. Although the exact mechanism linking cancer and IR is not known, hyperinsulinemia and increased IGF-1 availability appear to play a role in tumour initiation and progression in insulin resistant patients.

1.1.3.3.3. Cancer and dyslipidemia

Dyslipidemia includes high levels of LDL-cholesterol and low levels of HDL-cholesterol and has also been associated with increased cancer risk. During the US Atherosclerosis Risk in communities (ARIC) study from 1987-2000, 259 of 14,547 individuals were diagnosed with lung cancer. The individuals diagnosed with lung cancer were more likely than controls to have low levels of HDL-cholesterol and to have a higher prevalence of CVD at baseline [73].

A one year study performed in the Edith Wolfson Medical Centre, Israel, included 204 patients aged 66.6 ± 18.9 years (107 male, 97 female) and divided according to low and high HDL-cholesterol levels. Cohort 1 included $n=108$ with $\text{HDL} \leq 20$ mg/dl, while cohort 2 included $n=96$ individuals with $\text{HDL} \geq 65$ mg/dl. HDL levels in cohort 1 were found to inversely correlate with odds of death. Low HDL was seen more so in men (67.7%) than women (36.5%). Of the 204 individuals, 50 had been previously diagnosed with a cancer malignancy which correlated to 37% and 10.4% in cohort 1 and cohort 2, respectively. The researchers deduced that an increase of 1mg/ml serum HDL correlated with a 2.3% decrease in cancer risk. In agreement with this notion, low HDL-cholesterol has been demonstrated in colorectal, gastric, breast and prostate cancer [74], while

a high LDL-cholesterol is associated with a 15-times increase in haematological cancer risk [75].

1.1.4. Treatment of MetS

Currently there is no single therapy for directly treating MetS; instead treatment of MetS requires the treatment of each individual component separately. The primary treatment for MetS is lifestyle modification and increased physical activity as these can target each component of MetS. The principle goal of most MetS interventions is weight reduction. Unlike the definitions of MetS there is complete agreement on the benefits of weight loss to the clinical abnormalities of MetS. Weight loss has been shown to improve blood glucose, dyslipidaemia and blood pressure [76], IR and adiposity [6]. Interestingly a modest reduction in weight can improve the metabolic profile. A 2-year study of 14 obese individuals who were assigned to a long-term diet and life-style modification regime illustrated that 75% of individuals who achieved a weight loss of 10% or more showed regression of the MetS phenotype. Reducing the caloric intake is the leading method for weight loss, however bariatric surgery [77] and pharmaceutical intervention *e.g.* orlistat [78] and sibutramine [79] may also be considered, although sibutramine is no longer on the market. Although the reduction of calories is vital to weight loss, the macronutrient intake is vital for the health of MetS individuals [76] and certain milk proteins have been shown to be beneficial for treating MetS [80] (Section 2.1). As MetS becomes better understood a single therapy for its direct treatment may be developed.

1.2. Biomarkers

Blood-based biomarkers for MetS [81-83] could offer an improved minimally-invasive diagnostic tool, a method for prognosing patients' outcome, a means for predicting response to therapeutic intervention, and, importantly, also an opportunity for earlier intervention to avoid the development of more life-threatening pathologies, such as T2DM, CVD, and cancer. An ideal biomarker for MetS would be an entity that is consistently dysregulated in MetS compared to healthy controls and would be quantifiable, quick to assess, specific and sensitive

(Figure 1.3). Of course, many of the tests currently contributing to a MetS diagnosis, regardless of the specific definition used (be it WHO, EGIR, NCEP-ATPIII, AACE or IDF), are blood-based e.g. glucose tolerance. However, additional tests are also required. As will be outlined in this section (Section 1.2) increasing evidence suggests extracellular blood-based biomarkers such as, nucleic acids and proteins isolated directly from plasma/serum, extracellular vesicles (EVs) and EV-contained nucleic acids and proteins all have potential to form useful biomarker panels for MetS.

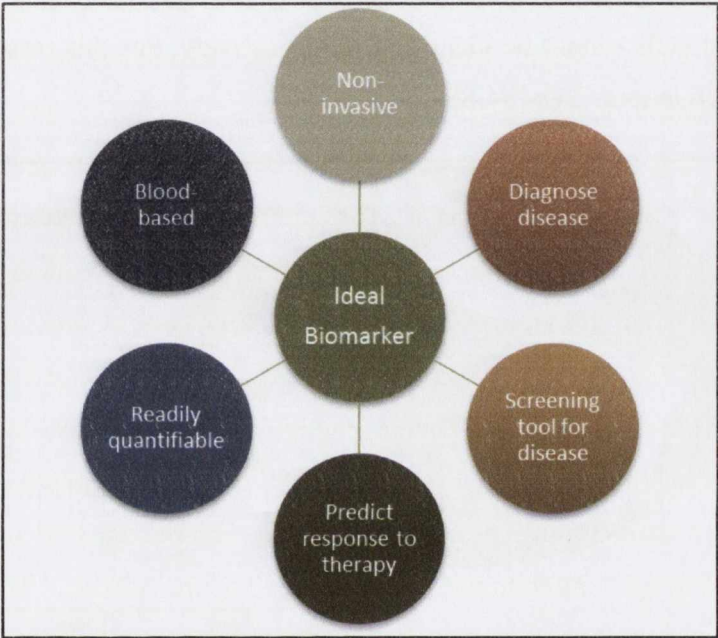


Figure 1.3: Characteristics of an ideal biomarker for MetS

Ideal Characteristics of a biomarker

1.2.1. Extracellular Vesicles/Exosomes

Although it has long been known that eukaryotic cells release complex vesicular structures into their environment, only in recent years has it been established that these entities, namely exosomes, microvesicles/ ectosomes/ membrane vesicles/ microparticles (collectively termed EVs) are not merely junk or debris, but tailor-made specialised mini-maps of their cell of origin. EVs were described by two independent studies, in 1983 [84] and 1984 [85], when it was observed that reticulocytes could discharge the transferrin receptor by its incorporation into EVs that were then released by cells. These vesicles were termed exosomes as they originated from the cells endosome/multivesicular

bodies [86]. The term microvesicles/ectosomes is typically used if the vesicles originate from pinching of the cell membrane [87]. The umbrella term EVs includes exosomes and microvesicles/ectosomes that are often defined and sub-grouped first-and-foremost on size and proposed origin (exosomes are ~30nm-120nm and are of endosomal origin; microvesicles/ectosomes are >120-1000nm and originate from pinching of the cell membrane) (Figure 1.4). However, there is no reason to believe that microvesicles of smaller sizes cannot also exist, thus the relevance of size *per se* is questionable. Furthermore, as unique markers for the different vesicle types have not yet been fully defined, the origin of vesicles released from cells cannot be identified retrospectively. For this reason, here we use the collective term EVs [84-88].

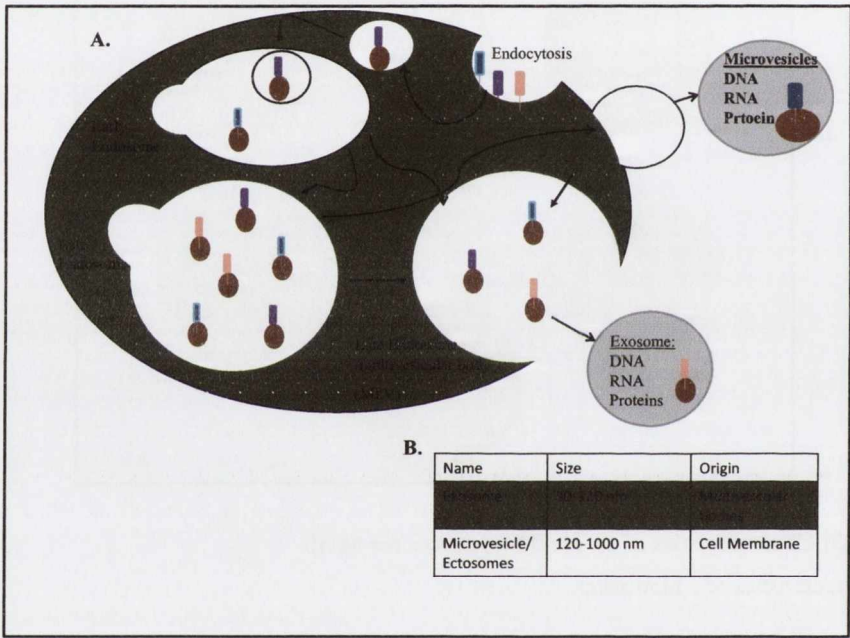


Figure 1.4: Biogenesis of extracellular vesicles and their classification.

A. Exosome formation starts at the cell membrane when the membrane is pinched inwards, this creates an endosomal-like structure. Intraluminal-vesicles (ILV) develop capturing bioactive molecules, such as proteins, lipids and nucleic acids. The ILV matures to form multi-vesicular bodies (MLV). The MLV then takes one of two paths i.e. they are transported to the: 1. lysosome for degradation, or 2. cell membrane, fuse to this and, subsequently, release the ILVs into the extracellular space. Released ILVs are termed exosomes. Alternatively, budding at the cell membrane, capturing cytosolic fragments, results in the formation of microvesicles (MV); also known as ectosomes/ membrane vesicles/microparticles. These vesicles have been reported to contain DNA, RNAs and proteins, although the mechanism by which DNA could get incorporated into exosomes is as of yet unknown. **B.** Some characteristics of EVs as exosomes and microvesicles/ectosomes based on size and origin

Studies of circulating EVs as biomarkers for MetS are still in their infancy. Trends are pointing towards a relationship between the quantities of blood-based EVs and MetS. When plasma specimens from individuals (Table 1.4 A) with MetS were compared to those from healthy controls, a significant ($p<0.001$) increase in quantities of EVs (as determined by flow cytometry following labelling of EVs with antibodies against surface antigens) was observed with MetS [89]. A follow up study (Table 1.4 B) confirmed these results and found circulating quantities of plasma EVs (as determined by flow cytometry) to be significantly ($p<0.05$) higher in MetS compared to healthy individuals [90]. Thus, these studies support the hypothesis that basic quantification of circulating EVs may offer potential as diagnostic biomarkers for MetS. Although further appropriately designed studies are required, this would suggest that EV quantities may prove to have benefit for screening those at increased risk of developing MetS.

Table 1.4: Details of studies investigating blood-based biomarkers for MetS

	Individuals (n)	Age (yrs)	MetS Definition	Controls (n)	Age(yrs)	Ref
A	MetS, n=43 M-74%, F-26%	52±1.7	NCEP:ATPIII	n=37 M-57%, F-43%	52.7±1.7	[89]
	MetS, n=21 M-81%, F-19%	56±2	NCEP:ATPIII	n=17 M-71%, F-29%	54±2	[90]

M-male, F-female, n=number of individuals, NCEP:ATPIII-National cholesterol education program third adult treatment panel

Freely circulating miRNAs (see also Section 1.2.4) and those carried in EVs are also under investigation as biomarkers for MetS [91-93]. A particular interest in miRNAs has emerged from the fact that they appear to be causally and mechanistically involved in events that contribute to MetS. Regulation of miRNAs is tightly controlled and each miRNA may, in turn, target multiple mRNAs. This is necessary for regulating and fine-tuning different aspects of metabolism. However, when miRNAs become disrupted, pathology may follow, as can be seen in the case of metabolic diseases [92]. Changes in miRNA expression have now been directly associated with many components of MetS.

For example, cholesterol is regulated at the cellular level by both classical transcription factors and also by miRNAs. With regards to their relevance as blood-based biomarkers for MetS, Karolina *et al.* [94] investigated both total miRNAs isolated from whole blood and EV-contained miRNAs isolated from plasma of individuals with MetS, T2DM, hypercholesterolemia, hypertension and healthy controls (Table 1.5). Five miRNAs were identified to be present in EVs from all groups. From those, four (miR-17, miR-197, miR-509-5p, miR-92a) were found to be at higher quantities in the MetS and hypercholesterolemic groups and lower in the T2DM and hypertensive groups. miR-320a was increased in the MetS and T2DM groups and decreased in the hypercholesterolemic and hypertensive groups. In this study, the detection patterns of these miRNAs was reported to be similar in EVs and in whole blood.

miRNA	MetS	T2DM	Hypercholesterolemia	Hypertension	Healthy controls
miR-17	↑	↓	↑	↓	↓
miR-197	↑	↓	↑	↓	↓
miR-509-5p	↑	↓	↑	↓	↓
miR-92a	↑	↓	↑	↓	↓
miR-320a	↑	↑	↓	↓	↓

Table 1.5: Details of individuals recruited to the Karolina *et al.* [94] study and detailed results of that study

MetS (n)	MetS-Age (yrs)	Results	MetS (vs controls)	T2DM (vs controls)	HCL (vs controls)	HPT (vs controls)
Cohort #1: 18	37±8.1	miR-17	Increased (1.93±0.19-fold)	Decreased (1.55±0.07-fold)	Increased (1.72±0.08-fold)	Decreased (1.63±0.23-fold)
Cohort #2: 29	45.7±11.3					
Healthy Controls– Age (yrs)		miR-197	Increased (1.77±0.28-fold)	Decreased (1.49±0.09-fold)	Increased (1.62±0.15-fold)	Increased (1.14±0.23-fold)
Cohort #1: 17	39±8.1	miR-509-5p	Increased (1.96±0.23-fold)	Decreased (1.39±0.25-fold)	Increased (1.66±0.06-fold)	Decreased (1.85±0.21-fold)
Cohort #2: 29	45.7±11.3					
T2DM (n)		miR-92a	Increased (1.52±0.13-fold)	Decreased (1.47±0.11-fold)	Decreased (1.63±0.04-fold)	Increased (1.77±0.15-fold)
Cohort #1: 21	39±15.1	miR-320a	Increased (1.56±0.23-fold)	Increased (1.41±0.11-fold)	Decreased (1.16±0.13-fold)	Decreased (1.35±0.16-fold)
Cohort #2: 29	44.2±8.4					
HCL (n)		HCL-Age (yrs)				
Cohort #1: 41	39±6	HPT-Age (yrs)				
Cohort #2: 48	40.7±11.7					
HPT (n)						
Cohort #1: 14	14±11					
Cohort #2: 16	43±8.1					

miR-17, miR-197, miR-509-5p and miR-92 has been shown to be decreased in the T2DM group compared to the MetS cohort suggesting these miRNAs possibly have stronger implications in fully manifested diabetes. miR-320a may have stronger implications in triglyceridemia.

HPT – hypertension, HCL - hypercholesterolemia

Whether or not extracellular miRNAs are predominant in EVs is still under debate and is very likely to differ with different miRNAs and different cellular/clinical conditions. For example, it has been reported that in serum and saliva, the majority of miRNAs are concentrated in the exosomes rather than freely-circulating [95] while a study of plasma miRNAs found them to be mainly concentrated in microvesicles [96]. Conversely, an analysis of three miRNAs in plasma found them to be predominantly (>97%) exosome-free [97]. Furthermore, Arroyo *et al.* [98] claimed that 90% of miRNAs in the circulation are not present in a membrane-bound form. However, regardless of whether or not miRNAs are contained within EVs, it is apparent that blood-based miRNAs have promise as biomarkers for full-blown MetS and for many of its contributing components (See Section 1.2.4).

1.2.2. Circulating Protein Biomarkers for MetS

Adiponectin is an anti-atherogenic, anti-diabetic [99] and anti-hyperlipidemic adipokine and its low circulating levels ($<4 \mu\text{g/mL}$) are associated with MetS. Plasma adiponectin levels were found to be significantly (<0.0001) decreased in obese ($3.7 \pm 3.2 \text{ mg/mL}$) compared to non-obese ($8.9 \pm 5.4 \text{ mg/mL}$) individuals [100]. A strong inverse correlation was also found between plasma adiponectin protein concentration and BMI for both men and women [100]. Plasma adiponectin protein concentrations were also reported to be lower in individuals with hypertension compared to normotensive individuals [101]. Thus, the association of low adiponectin protein levels with MetS was subsequently investigated in a cohort of individuals with T2DM compared to healthy controls; with and without MetS (Table 1.6 A). This study found that serum adiponectin protein levels were significantly lower ($p=0.001$) in the T2DM cohort. Of the individuals with MetS, adiponectin protein was decreased in both males and females, when compared to individuals without MetS, inversely correlating with MetS. Interestingly, adiponectin protein was found to decrease progressively with increasing number of MetS components present. Furthermore, for those individuals with diabetes and MetS, compared to individuals with only MetS, the plasma concentration of adiponectin was again much lower [102].

A study of Japanese men and women (Table 1.6 B) showed plasma levels of adiponectin in both men and women to inversely correlate with BMI, WC, visceral fat, triglycerides and positively correlated with HDL levels [103]. Studies of each component of MetS independently showed that an adiponectin concentration of $<4\mu\text{g/ml}$ of plasma correlated with a higher prevalence of each of the MetS components, when compared to adiponectin protein levels for healthy individuals. For example, a concentration of $<4\mu\text{g/ml}$ was observed in a higher percentage of obese compared to non-obese males and females. Furthermore, MetS had a significantly higher prevalence in both males ($p<0.001$) and female ($p<0.01$) who had adiponectin plasma concentrations $<4\mu\text{g/ml}$ compared to those who had higher circulating levels. [103]. While more extensive double-blinded studies are warranted, data produced to date suggests that low level circulating adiponectin protein (*i.e.* below a $4\mu\text{g/ml}$ threshold) has potential as a biomarker that could contribute to a biomarker panel for MetS diagnosis. In fact, based on its associated activities in the body (anti-atherogenic, anti-diabetic and anti-hyperlipidemic), it could be cautiously extrapolated that introducing adiponectin may have therapeutic benefit. However, this would have to be thoroughly investigated, initially in pre-clinical models.

Interestingly, lipid-lowering agents affect the bioavailability of adiponectin. Specifically, statins exert variable effects and this is influenced by patient characteristics such as diabetes status or IR. Statins affect adiponectin levels by enhancing the peroxisome proliferator-activator receptor (PPAR)- γ activation and they also exhibit antioxidant and anti-inflammatory potential. Fibrates such as niacin can increase adiponectin levels. Niacin, along with plant sterols, ω -3 fish oils and bezafibrate increase PPAR- γ activity, possibly by reducing oxidative stress or inflammation [104]. Additionally, anti-hypertensive drugs ramipril, valsartan, doxazosin and amlodipine have been shown to increase plasma adiponectin levels significantly in individuals previously untreated for hypertension. The Ramipril and valsartan are both RAAS inhibitors and they increased plasma adiponectin levels better than doxazosin and amlodipine. Metoprolol had no effect on plasma adiponectin levels [105]. Leptin, a satiety hormone [106], also has potential as a blood-based biomarker for MetS. This is supported by a study from the Iranian Third National Surveillance of Risk Factors of Non-Communicable Diseases (SuRFNCD) involving 3045 participants (Table

1.6 C) where MetS was diagnosed according to the IDF definition. Individuals with MetS had higher serum level of leptin compared to non-MetS individuals. Leptin levels were independent of overall obesity or abdominal/central obesity and indicated an independent role in the development of MetS [107]. In parallel, a study including 367 Mexican-American individuals from the Cameron County Hispanic Cohort (CCHC) (Table 1.6 D) showed that both decreased adiponectin protein and increased leptin protein correlated with MetS [108]. Again, this data suggests that more extensive analysis of leptin protein, *e.g.* via a double-blinded study, would help to confirm its relevance as a biomarker. It would make sense to study adiponectin and leptin -and indeed any other entities holding promise as biomarkers- in the same blood (plasma/serum) specimens. Of course to be useful as a biomarker, a robust threshold for leptin assessment would also need to be established.

Neuromedin U (NmU) is a neuropeptide involved in the gut-brain axis. NmU is just one of a number of neuromedin peptides, including neuromedin B, C, K, L, N, S and U. These neuropeptides have been found in a number of tissues and have a plethora of functions, including appetite regulation and tumour promotion. NmU is present in many species as 8 or 25 amino acids in length (NmU-8 and NmU-25), however other forms do exist [109]. NmU is of interest in the field of obesity research as the prevalence of obesity is increasing globally and the link between obesity and cancer is starting to be investigated, therefore resulting in the growing interest in understanding the molecular basis of energy homeostasis. Many neuropeptides involved in energy expenditure and feeding behaviour have been identified. Among these is NmU [110], which has been shown to have potent effects on feeding behaviour, body weight and metabolic activity in mice. Transgenic mice with a knockout of both NmU genes (NmU^{-/-}) exhibit hyperplasia (larger and more fat cells), reduced energy expenditure, increased feeding and they eventually become obese. Intracerebroventricular administration of NmU to these mice resulted in decreased feeding, increased metabolic rate and weight loss in a dose-dependent manner [111]. However, intracerebroventricular administration of NmU to mice lacking the NmU receptor 2 (NmU R2) does not have an effect on the obese phenotype in these mice.

In a study of obese Czech children and obese Danish adults (Table 1.6 E) children had a mean Z-BMI $4.3 \pm 1.7 \text{ kg/m}^2$, while adults had a mean BMI

36±5kg/m². This study investigated the NmU position on chromosome 14 as a biological candidate gene in the pathogenesis of human obesity. The researchers identified a novel Ala19Glu polymorphism that, in its homozygous form, associates with the combined phenotypes of overweight, obesity and WC in middle-aged white people. Also, increased BMI and increased WC in men were associated with this gene variant in its homozygous form. Of note there was also a hereditary polymorphism identified in a Czech family – Arg165Trp – which segregated with childhood obesity. Data suggests the Trp165 allele disrupts the anorexic function of NmU [110].

Recently, our laboratory proposed NmU as a biomarker for HER2-overexpressing breast cancer [112]. Here, NmU was investigated as a potential extracellular biomarker for HER2-overexpressing cancer cells resistant to HER-targeting drugs (lapatinib, trastuzumab, neratinib). NmU mRNA levels and protein levels were significantly higher intracellularly and extracellularly in cell lines resistant to HER-targeting drugs when compared to age-matched control cells. Interestingly, levels of NmU were dose-dependent on the resistance of the cell line to the HER-targeting drugs. They also found, through mining of publically-available datasets, representing a total of approximately 3500 tumours, that NmU expression was associated with a poor outcome in breast cancer patients, this particularly associated with a poor outcome in HER-overexpressing tumours. Also of note, increased NmU expression through direct transfection of the HER2-overexpressing cell lines, SKBR3 and HCC1954 parent cells resulted in a significantly compromised anti-proliferative effect of the HER-targeted drugs compared to mock transfected cells. Furthermore, knock down of NmU in drug-resistant cell lines resulted in an increase in the anti-proliferative effects of the drugs. This suggests that NmU is a potential biomarker for prognosing drug resistance in HER2-overexpressing breast cancer patients and determining patient outcome [112]. However, despite the association of obesity and breast cancer and the apparent role of NmU in controlling obesity and cancer plasma or serum NmU protein levels, to the best of our knowledge, have never been investigated in obese individuals or in the NmU^{-/-} mice.

Our group has also demonstrated that NmU-overexpressing cells (SKBR3 and HCC1954) release more interleukin 6 (IL-6) into their conditioned media than their mock transfected counterparts. Also, short term treatment of these cells with

NmU-25 peptide induced IL-6 secretion into the extracellular medium. Interestingly, the HER-targeted resistant HCC1954 cells which have higher levels of NmU than their drug sensitive parental cell line also showed increased IL-6 secretion. These results suggest that increased secretion of IL-6 in these drug resistance HER2-overexpressing cells is mediated by NmU (Martinez *et al*, Submitted).

IL-6 is a cytokine with a plethora of functions inside and outside the immune system. It functions like a hormone with both pro- and anti-inflammatory functions [113]. IL-6 has been linked to both obesity and cancer. In obesity, IL-6 is one of the most abundant cytokines. A hallmark of obesity and MetS is a chronic, low grade inflammatory state. As obesity progresses so too does the adipose tissue mass, there is also a concomitant increase in IL-6 production [114]. In clinical studies IL-6 has been shown to trigger c-reactive protein (CRP) (an inflammatory biomarker linked to IR, obesity and MetS [115]) and thus correlates with the development of T2DM [114]. Specifically, in a study of 27628 women 188 women developed T2DM over a four year period and were considered the case subjects. These case subjects were then age-matched to 362 control individuals. In the case subjects compared to controls IL-6 ($p<0.001$) and CRP ($p<0.001$) were both elevated significantly and it was concluded IL-6 and CRP could predict T2DM [116]. In another study of MetS (Table 1.6 F), CRP ($p<0.0001$) and IL-6 ($p<0.0001$) were significantly associated with MetS, however adjustment for each component of MetS eliminated this association, the authors concluded that the components of MetS highly contribute to the inflammation associated with MetS [117]. Conversely, others have proposed IL-6 to have a beneficial role in obesity [118, 119]. IL-6 is also one of the most abundant cytokines in cancer, it is associated with tumour growth, progression and relapse, and has been associated with many cancers [114]. Park *et al* [120], investigated the implications of obesity on hepatocellular carcinoma (HCC) development in mice, either with dietary (mice fed high fat diet) or genetic obesity (leptin deficient mice). They found dietary obese mice developed HCC tumours that were larger in size and had a higher incidence of HCC, the genetic obesity mice also had an increased development of HCC. The authors found circulating IL-6 to be significantly ($p<0.05$) elevated in the obese mice. Interestingly, the authors subjected IL-6 knockout mice to a high fat diet and found that there was

no difference in the HCC tumour multiplicity, size or incidence compared to IL-6 knockout mice on a normal chow diet. These results concluded that obesity-induced expression of IL-6 is an important contributing factor to HCC. IL-6 has been shown to be required for TNBC cell growth. Hartman *et al* [121] found that in TNBC IL-6 is both highly expressed and secreted from analysis of 12 TNBC cell lines (BT20, BT549, HCC1143, HCC1806, HCC1937, HCC70, HCC38, Hs578T, MDA-MB-231, MDA-MB-436, MDA-MB-468, SUM159) compared to ER+/luminal breast cancers (MCF7, MDA-MB-175-VII, MDA-MB-361, MDA-MB-451, T470, zR-75-1). They also found that IL-6 release is required for autocrine signalling.

1.2.3. Circulating DNA Biomarkers for MetS

A genomic DNA (gDNA) polymorphism in the adiponectin (*AdipoQ*) gene is also a potential biomarker for MetS. Genome-wide scan studies have identified a susceptibility locus for obesity, T2DM and CHD in the same chromosomal region as *AdipoQ* (i.e. 3q27). Forty-two single nucleotide polymorphisms (SNPs) in the linkage disequilibrium blocks of the adiponectin gene were found to exist and these SNPs confer a risk of obesity, T2DM and diabetic nephropathy [122]. Therefore, not only do the protein levels of adiponectin show potential as a biomarker, the gDNA polymorphisms in the *AdipoQ* gene could also be of relevance. A study that recruited 936 Chinese adolescents diagnosed MetS based on the IDF definition (Table 1.6 G) extracted gDNA from peripheral blood and found that the frequency of a SNP in the adiponectin gene at position 45 with 2 G-alleles (i.e. SNP+45 GG) was significantly increased in the MetS cohort compared to the frequency of the same SNP at position 45 but with either two T-alleles (i.e. SNP +45 TT) or with T- and G-alleles (i.e. SNP+45 TG). It was also determined that the risk of MetS was significantly higher in subjects with the SNP+45 GG genotype compared to those with the SNP+45 TT or TT +TG genotypes [123], suggesting that the SNP+45 GG genotype is a biomarker/risk factor for MetS. However, SNP+45 GG genotype had no effect on phenotype *i.e.* on serum adiponectin protein concentrations [123]. Ohashi *et al.* [124] also genotyped gDNA isolated from whole blood specimens procured from 383 coronary artery disease (CAD)

individuals and 368 controls (Table 1.6 H). MetS was diagnosed if total cholesterol $>5.6\text{mmol/L}$, triglycerides $>1.69\text{mmol/L}$, HDL-cholesterol $<1.03\text{mmol/L}$ and blood pressure of systolic $\geq 140\text{mmHg}$ and diastolic $\geq 90\text{mmHg}$ were present. The presence of SNP-276, SNP-94 and missense mutation I164T in *AdipoQ* were assessed. The frequency of the I164T mutation compared to wild type was significantly higher ($p<0.001$) in individuals with three abnormalities (MetS, T2DM, CAD). Furthermore, the prevalence of MetS was significantly higher in CAD individuals with the I164T mutation than in CAD individuals without this mutation. Therefore, this study suggests that the I164T mutation in the *AdipoQ* gene is associated with MetS-related CAD. This emerging data, although limited so far, suggests that assessing DNA mutations could add further value to forming a panel of biomarkers for MetS diagnosis, rather than assessing proteins alone.

1.2.4. Circulating miRNA Biomarkers for MetS

Micro-RNAs (miRNAs) are small endogenous ~20-23 nucleotide long non-coding RNAs that are widely distributed throughout all eukaryotic organisms [91]. miRNAs are encoded in the genome [125] and their biogenesis involves two processes that lead to the formation of mature miRNAs. The first step involves the transcription of the miRNA gene in the nucleus to form a primary transcript (pri-miRNA) from intergenic, intronic or polycistronic loci by RNA polymerase II [92]. This pri-miRNA forms the stem-loop structure that is subsequently processed by the enzyme DROSHA into precursor miRNA (pre-miRNA). Pre-miRNA is exported to the cytoplasm by exportin-5. In the cytoplasm Dicer removes the stem-loop and separates the double helix. One strand enters the RISC complex and binds in an anti-sense manner to the complementary mRNA. If perfect complementarity results the mRNA is repressed from being translated into a protein (Figure 1.5). miRNAs function to regulate gene expression post-transcriptionally and participate in a diverse collection of regulatory events via their association with target sites on mRNAs and association with effector complexes [126].

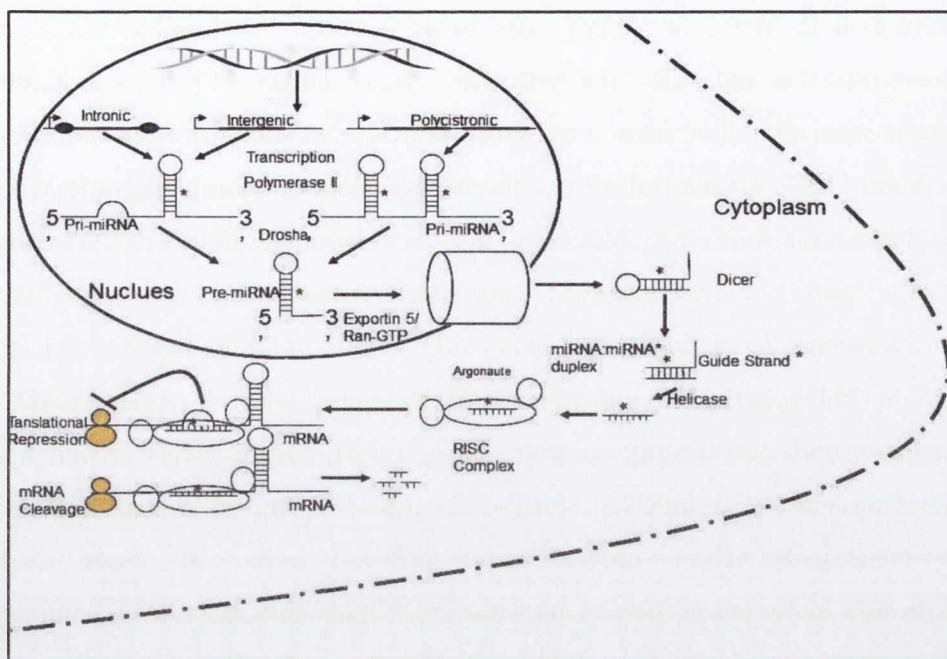


Figure 1.5: miRNA biogenesis

The stepwise biogenesis of miRNAs from the intronic, intergenic and polycistronic loci in the nucleus. miRNAs are usually transcribed by Polymerase II to form a stem-loop structure, this is processed by Drosha to produce Pre-miRNA, which is exported into the cytoplasm by Exportin 5. Once in the cytoplasm, the stem-loop structure is processed by Dicer and helicase to produce single stranded miRNA that enters the RISC complex and binds to complementary mRNA in an antisense manner to cleave the mRNA or repress translation.

A number of miRNAs have been identified that regulate components of MetS (

Table 1.7 1.7), cholesterol and FA metabolism. These include miR-122 and let-7g that were found elevated in the serum of an Asian cohort with MetS (Table 1.6 I) [94]. Levels of both miRNAs increased in parallel with increasing number of MetS components presented. For individuals presenting with 4-5 components of MetS, let-7g and miR-122 were both significantly increased. Moreover, the elevation of serum let-7g was significantly associated with a low level of HDL cholesterol and high blood pressure [94]. miR-122 has been shown to regulate FA metabolism, cholesterol synthesis and cholesterol levels [125]. As outlined above, Karolina *et al.* [94] conducted a profiling study including 265 individuals comprised of five sub-groups (i.e. MetS, T2DM, HCL, HPT, and controls) and found three miRNAs to be increased in MetS (miR-197, miR-23a, miR-509-5p) as potential contributors of dyslipidaemia in MetS, correlating with BMI. From this study, miR-130a and miR-195 were proposed as potential contributors of

hypertension in MetS, correlating with blood pressure. A plausible association between miR-27a and miR-320a with MetS and T2DM were proposed, as both miRNAs were altered in these conditions. miR-370 is another miRNA associated with MetS [127]. To the best of our knowledge, data on circulating miR-370 in individuals with full-blown MetS has not been reported. However, circulating levels of miR-370 and miR-122 have been reported for individuals with hyperlipidaemia, a risk factor for MetS [127]. Gao *et al.* [128] reported that in a cohort of 255 hyperlipidaemia individuals presenting with or without CAD in comparison with 100 healthy controls (Table 1.6 J), plasma levels of four lipid metabolism-associated miRNAs (miR-122, miR-370, miR-33a and miR-33b) were investigated. Here, miR-122 and miR-370 were both found to be significantly increased in the plasma from hyperlipidaemia individuals compared to that of healthy controls, while miR-33a and miR-33b were undetected. Interestingly both miR-122 and miR-370 correlated positively with levels of total cholesterol, triglyceride, and LDL-cholesterol in the plasma of both patient and control cohorts. Of note, miR-122 and miR-370 were both found to be decreased with statin treatment [128], suggesting that these miRNAs have potential as both diagnostic biomarkers for hyperlipidaemia and predictive biomarkers for response to statins. As management of MetS in practice involves treatment of each component individually and so many individuals diagnosed with MetS are on statins, this highlights the potential importance of miR-122 as a biomarker for MetS and supports an investigation of miR-370.

Additionally, miRNAs expression can be altered by medications. For example many MetS patients are treated with statins. Statins have been shown to significantly alter the expression of miRNAs [129]. Furthermore, miR-126 has been identified as a potential biomarker for endothelial dysfunction in T2DM and CAD however; de Boer *et al* [130] have shown that aspirin administration to T2DM patients results in decreased miR-126 levels.

Table 1.6: Details of studies investigating blood-based biomarkers for MetS

	Individuals (n)	Age (yrs)	MetS Definition	Controls (n)	Age(yrs)	Ref
A	Diabetic, n=100 MetS, n=88 M-50%, F-50%	M-48±12 F-48±10	NCEP:AT PIII (modified for Asians)	Non-diabetic, n=100 MetS, n=18 M-50% F-50%	M-47±11 F-45±12	[102]
B	n=661 M-479, MetS 22.8% F-182, MetS 3.6%	M-53±10 F-54±10	NCEP:AT PIII (modified)			[103]
C	MetS – 1215 M-561 F-654	M- 47.19±10.95 F-48.2±10.3	IDF	n=1830 M-907 F-923	M-41±12 F-40±11	[107]
D	MetS=984 M-58%, F-52%	62±9	NCEP:AT PIII	n=1586 M-40%, F-60%	58±1.9	[117]
E	n=367, MetS IDF: M-127, F-52 MetS NCEP:ATPII: M-121, F-41	11-16	IDF + NCEP:AT PIII	_____	_____	[108]
F	n=289 Czech children: M-132, F-157 84 Danish adults: M-44, F-44 n=5851	1-18 50±15 46.1-64.4±7.9	_____	n=53 M-25, F-28	5-44	[110]
G	n=939 M-53.5%, F-46.7%	11-16	IDF	_____	_____	[123]
H	n=383 M-70% F-30%	63±0.4	See Section Circulating DNA biomarkers for MetS – used own set of criteria	n=368 M-65% F-35%	62.3±0.6	[124]
I	MetS – 31 M-16, F-15	M-55±8 F-58±9	NCEP:AT PIII	n=71 M-36, F-35	M-56±9 F-55±6	[131]
J	CAD, n=155 M-105, F-100 Non-CAD, n=100 M-57, F-43	CAD – 65.3±11 Non-CAD – 65.2±10.2	_____	n=100 M-35 F-65	63±10.7	[128]

Table 1.7: miRNAs associated with components of MetS

Metabolic Process	miRNAs Regulating the Metabolic Process	Targets of each miRNA and area of inhibition	Ref
Lipid Metabolism + Cholesterol Efflux	miR-122	Targets <i>ALDOA</i> and <i>CS</i> in primary mouse hepatocytes	[132]
	miR-33a, miR-33b	Targets <i>ABCA1</i> in liver cells and macrophages	[132]
	miR-758	Targets <i>ABCA1</i> in human and mouse macrophages	[132]
	miR-106b	Targets <i>ABCA1</i> in human hepatocytes	[132]
Glucose Homeostasis + Insulin Signalling	miR-132	Targets <i>SIRT1</i> in adipocytes	[133]
	miR-29a, miR-29b	Expressed in pancreatic islet cells of obese mice and target <i>MCT1</i>	[92]
	miR-126	Inhibits <i>IRS1</i> to promote insulin resistance. Targets <i>IRS2</i> in liver cells and macrophages	[92]
	miR-33a, miR-33b	Targets <i>SIRT6</i> and <i>AMPKα1</i>	[134]
	miR-223	Expressed in skeletal muscle and targets <i>GLUT 4</i>	[92]
	miR-103, miR-107	Expressed in liver and adipose tissue and targets <i>CAVI</i> and <i>DICER</i> .	[135]
Adipocyte Differentiation	let-7	Targets <i>IRS2</i> , <i>IGF1R</i> , <i>INSR</i> in muscle and adipose tissue	[92]
	miR-143	Increased during adipocyte differentiation and targets <i>ERK5</i>	[136]
	miR-204	Increased in hADSCs during adipogenesis in concert with <i>C/EBPα</i> , <i>PPARα</i> , <i>FABP4</i> and targets <i>DVL3</i>	[137]
	miR-200	Expression is up in mouse <i>ST</i> mesenchymal stem cells during adipocyte differentiation and targets Wnt signaling	[138]
	miR-17/92 cluster	Cluster shown to target Rb2 and p130 in 3t3 L1 cells during differentiation. miR-92a was found to be higher in MetS and hypercholesteraemic individuals compared to controls	[138]
	miR-130	Expressed in human adipose tissue. Down-regulated in adipose tissue of obese women, targeting <i>PPARα</i>	[139]

ABCA1, cholesterol efflux regulatory protein; *ALDOA*, aldolase A; *AMPK α 1*, anti-AMP activated protein kinase alpha 1; *CAVI*, caveolin 1; *C/EBP α* , enhancer binding protein; *CS*, citrate synthase; *DVL3*, dishevelled segment polarity protein 3; *ERK5*, extracellular-signal-regulated kinase 5; *FABP4*, fatty acid-binding protein 4; *GLUT 4*, glucose transported type 4; hADSCs, human adipose-derived stem cells; *IGF1R*, Insulin-like growth factor receptor 1; *INSR*, insulin receptor; *IRS1* and *2*, insulin receptor 1 and 2; *MCT1*, monocarboxylate transporter 1; *PPAR α* , peroxisome-proliferator-activated receptor alpha; Rb2, retinoblastoma-like protein 2; *SIRT1*, Sirtuin 1; *SIRT6*, Sirtuin 6

1.2.5. Circulating messenger RNA (mRNA)

mRNAs represent a class of RNAs that could be used as minimally-invasive tools for the diagnosis and monitoring of MetS [140]. As previously explained (see Sections 1.2.2 and 1.2.3), adiponectin gDNA and protein both have potential as biomarkers for MetS. Although it seems that adiponectin mRNA levels have only been studied in adipose tissue thus far, the results warrant investigation of adiponectin mRNA in the circulation of MetS individuals. Specifically, Lihn *et al.* [141] recruited 13 obese female (37.8 ± 2.8 yrs) and 30 healthy control females (46.3 ± 0.4 yrs) and collected visceral and subcutaneous abdominal adipose tissue biopsies. mRNA analysis revealed a 6-fold decrease in adiponectin mRNA levels in the obese cohort compared to the healthy controls. Additionally, adiponectin mRNA levels were 33% lower in visceral adipose tissue compared to subcutaneous adipose tissue. This result is of interest as visceral fat is a substantial player in MetS.

In a similar study, Neprilysin (NEP), a zinc metallo-endoropeptidase was investigated. NEP is expressed by adipocytes [142], plays a role in inhibiting peptide signalling events and is involved in the metabolism of a number of regulatory proteins [143]. This study showed that the circulating levels of NEP mRNA in a mouse model of obesity were higher after 15 weeks on a high-fat diet, with a progressive increase from week 7 to week 15 [142]. This suggests a potential of adipose tissue-derived NEP mRNA in the plasma as a biomarker for MetS.

1.3. Metabolism of Cancer Cells

MetS and obesity are the result of disruptions to the metabolic homeostasis of an individual. Not only does altered metabolism result in disorders such as MetS, cancer cells also undergo metabolic changes in order to proliferate and expand throughout the body [144]. Carcinogenesis is a complex disease culminating from the ability of the cancer cell to overcome normal cellular barriers, such as evading growth suppressors, resisting cell death, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and sustaining proliferative signalling, which together have been termed hallmarks of cancer [145]. Interestingly, re-programming of energy metabolism

has also been established as an emerging hallmark of cancer [145]. Cancer cells adjust their metabolism in order to fuel growth and division. Under normal cellular conditions when oxygen is available, cells convert glucose to pyruvate in the cytosol. Pyruvate enters the Krebs cycle in the mitochondria to produce adenosine triphosphate (ATP) energy in a process known as oxidative phosphorylation. Under anaerobic conditions, glycolysis takes place forming lactic acid and ATP but at a rate 18 times less than oxidative phosphorylation [146] (Figure 1.6 A).

Alteration of cancer cell metabolism was first identified in 1924 by Otto Warburg, when he reported that cancer cells re-programme their metabolism to glycolysis even in the presence of oxygen (Figure 1.6 B). It was proposed that cancer cells do this so to increase the uptake and incorporation of nutrients to increase growth rates (see Section 4.1). This results in an increase in the consumption of glucose, which can now be visualised by positron electron tomography (PET) [147].

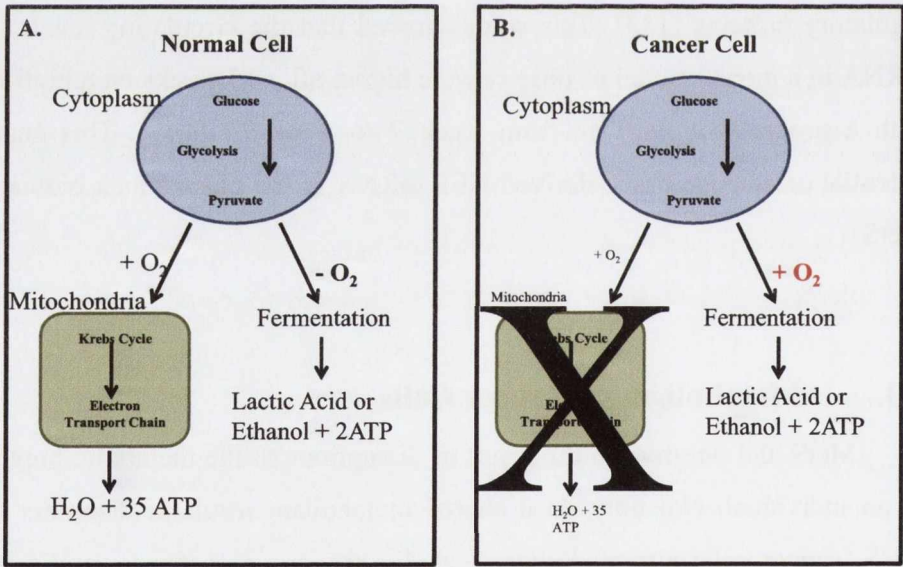


Figure 1.6: Cellular respiration of normal cells and cancer cells

A. Normal cells, in oxygen rich environments process glucose by oxidative phosphorylation to meet the energy demands of the cell and generate ATP energy. When oxygen is not available, cells switch to glycolysis to generate ATP. **B.** Cancer cells in oxygen rich environments process glucose at a high level by glycolysis to produce ATP energy but this is inefficient compared to oxidative phosphorylation.

The PET technique involves the use of the glucose analogue tracer fluorodeoxyglucose (FdG). This type of imaging, used for thousands of cancer patients has shown that most primary and metastatic lesions have substantially increased glucose consumption [147]. The specificity of FdG PET as an identifier for both primary and metastatic lesions is almost 90% for many cancers. However, the sensitivity of this method is lowered in lesions less than 0.8cm^3 , and by the trapping of FdG in other tissues. This indicates that the altered glucose metabolism of cancer cells has been used as a biomarker for cancer. For example, the non-invasive breast cancer cell line MCF-7 has much lower aerobic glucose consumption compared with highly invasive MDA-MB-231 cell line which shows much higher rates of aerobic glucose consumption [147].

One of the main processes undergoing alterations in the switch of a normal cell to a cancer cell is cellular energy metabolism [144]. Many hypotheses for this switch have been postulated:

1. Solid tumours have a significantly decreased oxygenation compared to normal non-ischemic tissues. This decreased oxygen availability to tumours results in hypoxia. Hypoxia is a negative prognostic and predictive factor, this is due to its role in chemoresistance, angiogenesis, vasculogenesis, invasion, metastasis, resistance to cell death [148] and importantly here altered metabolism [147]. Although the switch from oxidative phosphorylation to glycolysis precedes the emergence of hypoxia in cancer cells, the hypoxia microenvironment in pre-malignant tumours favours a switch to anaerobic metabolism [147]. Analysis of hypoxia in rat mammary adenocarcinomas using recessed-tip oxygen microelectrodes [149] and in mice fibrosarcomas using T2*-weighted gradient-echo magnetic resonance imaging [150] has shown that oxygen levels in tumours fluctuate [147]. Therefore it has been postulated that the glycolytic phenotype of cancer cells initially arises as an adaptation to local hypoxia, persistent or fluctuating hypoxia lead to upregulation of glycolysis [147]. Interestingly, analysis of MCF-7 and MDA-MB-231 cells under normoxic and hypoxic conditions found both cell lines to have higher glucose metabolism under hypoxic conditions [147].
2. Proliferating mammalian cells are exposed to a continual supply of glucose and nutrients in the circulation; therefore the inefficient production of ATP

via glycolysis is not an issue for these cells. There is evidence that in proliferating cells ATP is never limiting [146].

3. In order to proliferate a normal cell must receive signals, known as growth factors, in order to uptake nutrients for cell division and proliferation. Cancer cells have overcome this requirement through genetic alterations that alter their growth factor receptors, this allows for the constant uptake of nutrients for cell growth and proliferation [146]. This evasion of growth factor signalling is considered a hallmark of cancer [145].

In triple negative breast cancer (TNBC) especially, there is an urgent requirement for the development of effective therapies as TNBCs are not responsive to hormonal therapies or other targeted therapies [151]. Dissection and understanding the metabolic phenotypes of TNBC cells may aid in the development of effective therapies. An interesting study investigated the metabolic phenotypes of a number of breast cancer cell lines, including receptor positive (e.g. BT474 [ER+, PR+, HER2+], MCF-7 [ER+, PR+], T470 [ER+, PR+], ZR751 [ER+]), HER2-overexpressing (e.g. SKBR3) and TNBC (e.g. BT20, MDA-MB-231, MDA-MB-436, MDA-MB-468). This study found that the rate of aerobic glycolysis was higher in the TNBC cell lines compared to the receptor positive cell lines and the HER2-overexpressing cell line SKBR3 showed a similar trend as the TNBC cell lines [151]. This is an interesting result as SKBR3 and MDA-MB-468 have been shown to be more invasive *in vitro* than the BT474 cell line [152]. Of note, the TNBC cell lines investigated required glucose for their proliferation as culturing of all breast cancer cell lines in high glucose, low glucose and galactose (this requires functional mitochondria for ATP production) showed TNBC cell lines to rapidly proliferate in high glucose. The rate of proliferation was reduced in low glucose conditions and the cells failed to proliferate, even showing decreased proliferation in the presence of galactose without glucose. Conversely, the receptor positive cell lines continued proliferating in low glucose and galactose. Importantly, analysis of ATP levels in all cell lines showed a similar level in all, this indicates that the glycolysis of TNBC cell lines are undergoing produces enough ATP to compensate for the lack of oxidative phosphorylation in these cells [151].

Obesity triggers systemic and metabolic alterations that can influence carcinogenesis and tumorigenesis [52]. Recently, obesity was shown to promote

the glycolysis of prostate cancer cells. In this study prostate cancer cells (LNCaP) were cultured with serum procured from obese mice and pyruvate kinase activity was measured [153]. They found pyruvate kinase activity to be increased in the LNCaP cells cultured with serum from obese mice compared to those cultured with serum from lean mice. Obesity is associated with IR and thus a pre-diabetic state. IR is characterised by altered glucose metabolism which may aid cancer cells and provide the glucose required for glycolysis [154] and increased glucose has been observed in TNBC [151]. Most cancer cells including TNBC cells alter their metabolic phenotype in order to proliferate when oxygen is present or not. Obesity may contribute to this altered metabolic phenotype [52]

1.4. Project Aims

The primary aim of this PhD research project was to investigate if minimally-invasive freely-circulating biomarkers exist for MetS that may have potential to distinguishing MetS from obesity. The objective of identifying this biomarker or panel of biomarkers is to aid in the diagnosis of MetS in a clinical setting, thus, to reduce the incidence of subsequent T2DM, CVD and cancer. The secondary aim was to identify mRNA targets for any miRNA(s) identified as potential MetS biomarkers using *in vitro* cell culture techniques.

- In relation to freely-circulating biomarkers for MetS, the initial aim was to seek miRNA biomarkers in the plasma of participants with MetS (as defined by the IDF definition) that could distinguish MetS from obesity.
 - Secondly, to identify a biomarker for MetS suitable for monitoring response of participants to dietary intervention.
 - Thirdly, to determine the effect of 4 dietary interventions on miRNA levels in the plasma of obese and MetS participants.
 - Finally, to analyse miRNA levels in the plasma of obese and MetS participants' pre- and post- 12-week diets following a high fat meal test to determine the postprandial effect on miRNA levels.
- To evaluate EVs isolated from the plasma of control, obese and MetS participants as biomarkers for MetS.
 - Additionally, the EV-cargo, *i.e.* miRNA content was investigated for their potential as EV-contained miRNA biomarkers for MetS.
 - To compare the freely-circulating miRNA levels and the EV-contained miRNAs to determine if one method of biomarker discovery is superior or if both are required.
 - To investigate the functional effects EVs from obese and MetS participants may have on TNBC cells (*i.e.* induce proliferation or *anoikis* in the TNBC cell line Hs578T).
- To investigate the metabolic phenotype of two TNBC cell line variants- Hs578T and its more aggressive subclone Hs578Ts(i)₈- as TNBC is commonly associated with obesity and, therefore, MetS.

- To investigate the oxygen consumption and glucose consumption of the TNBC cell line variants.
- To investigate NmU as a biomarker for MetS and the effect of exogenous NmU on the metabolism of the Hs578T and Hs578Ts(i)₈ cell line variants.
- To determine if the anti-IL-6 antibody has any effect on the metabolism of these cell lines and to investigate IL-6 levels in the conditioned medium of the Hs578T and Hs578Ts(i)₈; as IL-6 is known to be increased in TNBC cell lines and therefore, may be a biomarker for more aggressive TNBC. Similarly, IL-6 is known to be increased in obesity, and therefore, we hypothesises it is increased in MetS, therefore, there may be a link between obesity, MetS and cancer through IL-6.
- To assess the potential of NmU and IL-6 as plasma protein biomarkers for MetS.

This project was part of an international collaboration called DairyHealth, which focused on MetS, and was taking place in Denmark, The Netherlands, and here in Ireland. The entire collaboration was divided into four groups (Figure 1.7) with seperate aims to address an overall common goal, which was to investigate MetS at a molecular level and to determine patients' response to dietary intervention.

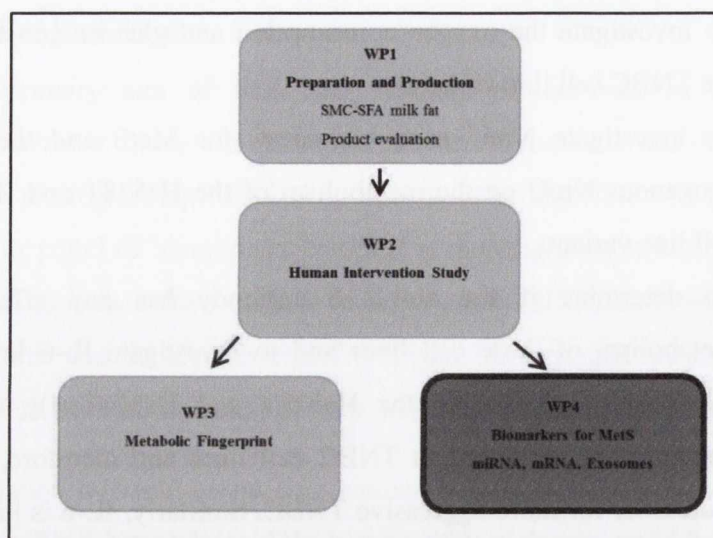


Figure 1.7: Working groups forming the DairyHealth research cluster

Diagram of DairyHealth working groups involved in MetS research and the area of MetS these groups are researching. WP4 is highlighted in a black border represents the area of MetS research we are undertaking.

Dairy products constitute a significant part of our diet and it is, therefore, of critical importance to understand the health impact of crude dairy products in general, as well as specific macronutrients. The overall aims of the DairyHealth study were to:

1. explore and understand the influence of medium chain saturated fatty acids (MC-SFA) and proteins (whey and casein) on human health, and
2. study the interaction and synergy of these macronutrients on MetS; focusing on MC-SFAs and proteins, and their potential positive synergy on MetS and CVD.

Components of this research are published in:

Bohl M, Bjørnshave A, Rasmussen KV, Schioldan AG, Amer B, Larsen MK, Dalgaard TK, Holst JJ, Herrmann A, O'Neill S, et al. Dairy proteins, dairy lipids, and postprandial lipemia in persons with abdominal obesity (DairyHealth): a 12-wk, randomized, parallel-controlled, double-blinded, diet intervention study. Am J Clin Nut (2015) 101(4):870-878.

Our part of this research consortium came under work package (WP) 4 (Figure 1.7) and our initial specific aims are highlighted above.

Chapter Two: MicroRNAs as Biomarkers in the Plasma of Participants with Clinical Obesity and Metabolic Syndrome.

2. Abstract

Introduction: Metabolic syndrome (MetS) is the culmination of a number of risk factors, of which obesity is central. Blood-based miRNAs have potential as diagnostic biomarkers for MetS and biomarkers of response to dietary intervention in MetS. miRNA biomarkers may also aid in identifying MetS, independently of obesity. Obesity and MetS have both been linked to an increased risk of triple negative breast cancer (TNBC). TNBC cell lines offer a method for investigating targets of miRNAs identified as potential biomarkers for MetS.

Methods: 52 participants, including those with clinical obesity (n=26) and those with MetS (n=26) as defined by the international diabetes federation (IDF) definition (Section 1.1) enrolled in a dietary intervention study conducted in Aarhus University Hospital, Denmark. Participants attended a clinic, fasting, before (visit 2) and after (visit 7) a 12-week dietary intervention. Blood specimens were procured. At both visits additional blood specimens were procured, *i.e.* at 30, 120 and 240mins following a meal high in fat. The 12-week diets consisted of differing milk proteins (whey or casein) and milk fats (low and high amounts of small and medium chain-fatty acids [MC-SFA]). RNA was subsequently isolated from 250µl of plasma (n=416, 8 specimens/participant) and custom miRNA profiling (n=300 specimens, 38 participants) for a total of 25 miRNAs was performed. Identification of potential miRNA biomarkers for MetS led to the validation of these miRNAs in a sub-cohort of participants (n=28 specimens, 14 participants). Subsequently, targets of the most interesting miRNA identified following validation (miR-758-3p) were assessed *in silico* using miR-walk (publically-available prediction software) and *in vitro* using two TNBC cell line variants (isogenic pairs- Hs578T and Hs578Ts(i)₈). miR-758-3p target proteins were assessed by immunoblotting of protein from the cell line variants and subsequent transfection of the cell lines with an inhibitor or a mimic of miR-758-3p to determine any functional association was performed.

Results: Specifically, of the 25 miRNAs assessed miR-758-3p was identified as potentially of most interest. We demonstrated that miR-758-3p were absent in plasma from MetS participants however, it was detected in plasma from obese participants ($C_T=33\pm0.3$). This was discovered using custom miRNA profiling panels and validated (obese $C_T=33\pm1.6$) in a sub-cohort, for a total of 52

participants. Analysis of miRNA levels following the 12-week diets and meal high in fat indicated all 25 miRNAs assessed were influenced by dietary intervention. For example, miR-758-3p was detected in the low MC-SFA-whey group in the MetS cohort after the 12-week diet (visit 7 $C_T=33.5\pm0.6$). However, this result was not significant. miR-walk predicted ABCA1/CERP (cholesterol efflux regulatory protein) and ABCG2/BCRP (breast cancer resistance protein) as targets of miR-758-3p. ABCA1 was absent in the Hs578T cell line but present in the Hs578Ts(i)₈ cell line variant and ABCG2 expression was significantly ($p=0.006$) higher in the Hs578Ts(i)₈ cell line variant compared to the Hs578T cell line. Modulation of ABCA1 and ABCG2 in the Hs578T and HS578Ts(i)₈ isogenic pair was not achieved following manipulation of miR-758-3p.

Conclusion: Our findings suggest miR-758-3p as a potential biomarker for MetS independent of obesity. Interestingly, investigation of miR-758-3p as a biomarker for response to dietary intervention showed this miRNA to be detected in the low MC-SFA-whey diet, indicating this diet affects miR-758-3p levels, which may be of interest as the whey protein had a positive clinical effect on these participants. Whey protein decreased the postprandial chylomicron load in these participants, as shown by collaborators. Although modulation of miR-758-3p did not result in alterations of ABCA1 or ABCG2 proteins in our cell line variants, we predict miR-758-3p to target ABCA1 in MetS, but the effect may be specific to cells known for their ABCA1 cholesterol efflux function, such as macrophages or hepatocytes.

2.1. Introduction

Type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD) and cancer are among the leading causes of morbidity globally. MetS has been identified as a risk factor for these three conditions; with a diagnosis of MetS increasing the individuals risk of T2DM five-fold and the risk of CVD three-fold [155] and cancer [3] (see Section 1.1.3) . In 2009, a joint statement by the International Diabetes Federation (IDF) and the American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI), stating that the IDF cut-off points should be used for non-Europeans and the AHA/NHLBI cut-off points be used for Europeans, with specific waist circumference (WC) cut-offs (see Section 1.1) was published [11]. However, much controversy on the precise definition still remains. It is, for this reason, that a method for diagnosing MetS in conjunction with these definitions is identified. A class of small non-coding RNAs (*i.e.* microRNAs (miRNAs)) have potential as diagnostic biomarkers for MetS.

miRNAs (see Section 1.2.4) regulate gene expression post-transcriptionally and participate in a diverse collection of regulatory events via their association with target sites on mRNAs and association with effector complexes [126]. miRNAs function to post-transcriptionally regulate gene expression, which has been primarily down-regulation of the target protein through translational repression (binding or cleavage) of the target mRNA untranslated regions. However, miRNAs can function by post-transcriptionally stimulating gene expression, by working in conjunction with their associated microribonucleoproteins via a direct or indirect mechanism [126]. It is, therefore, not surprising that aberrant miRNA expression is associated with numerous disorders, including obesity [156, 157], diabetes [157], and various cancer subtypes [158, 159].

Currently there is no nutritional strategy [160] other than caloric restriction and increased physical activity for the treatment of MetS and obesity [161] (see Section 1.1.4). Recent findings on the effectiveness of bioactive peptides derived from milk proteins have generated considerable interest. Specifically, the use of these peptides either through incorporation into food or nutraceuticals in the prevention and treatment of MetS via several mechanisms: satiety response, regulation of insulinemia and blood pressure and alteration of lipid profile may be

beneficial for the treatment of MetS [80]. The beneficial effect of milk components such as MC-SFA have been established [162]. Many of the beneficial peptides in milk such as whey and casein can be released by enzymatic hydrolysis. Milk and other dairy products offer the best precursors of bioactive peptides. These bioactive peptides may reduce the risk of MetS by 1) anti-obesity effect, *i.e.* whey and casein both have satiety effect; 2) anti-lipidemic effect, *i.e.* whey derived milk proteins decrease serum cholesterol; and 3) anti-hypertensive effect, *i.e.* β -casein has been shown to be a potent inhibitor of the renin-angiotensin (RAAS) pathway in blood pressure (see Section 1.1.2.4) [80].

The aim of this chapter was to investigate total plasma miRNAs in obesity and MetS and to try to identify a miRNA(s) that could distinguish MetS from obesity. Also, we aimed to determine whether such miRNA(s) are altered by a 12-week dietary intervention differing in milk derived proteins (whey or casein) and milk derived fats (low or high MC-SFAs). For this purpose, we performed custom miRNA profiling using Exiqon pick-n-mix miRNA profiling arrays and identified miR-758-3p as a potential biomarker for MetS independent of obesity. miR-758-3p was then chosen for further study in TNBC cell line variants as obesity and MetS have been linked to an increased risk of TNBC (see Section 1.1.3.3 and Section 1.3). We manipulated miR-758-3p in these cell line variants to determine if its manipulation had an effect on its predicted target proteins. Analysis of miR-758-3p after the 12-week diet identified an increase of the miRNA after the whey supplementation and this result complemented the positive clinical findings of WP2 [163].

2.2. Methods

2.2.1. Study Design and Participants

Sixty-three Caucasian participants were recruited to the study at the Department of Endocrinology and Internal Medicine, Aarhus University Hospital, Denmark between October 2011 and December 2012. After receiving written and oral information, all participants gave their written informed consent prior to part-taking in the study. Participants were screened on the basis of their medical history and a physical examination. Inclusion and exclusion criteria are presented in Table 2.1 below. Participants were divided into 2 groups, obese (n=26) and MetS (n=26) (Table 2.2), as defined by the IDF definition (Section 1.1). Eleven participants were being treated with lipid-lowering drugs, nine with anti-hypertensive drugs (three were treated with the combination of angiotensin II-receptor antagonist (AT2-inhibitor) and thiazide diuretics, three were treated with AT2-inhibitor, one was treated with β -blocker, one was treated with the combination of angiotensin-converting enzyme and thiazide, and one was in dual therapy with calcium antagonist and AT2-inhibitor). All participants continued taking their regular medication without dose changes throughout the study period.

Healthy control specimens were not procured, as the primary focus of the collaboration was the effect of the 12-week dietary supplementation with dairy proteins and dairy lipids on post-prandial lipidemia. Also the aim was to determine if there were synergistic effects of the dairy proteins and dairy lipids. However, healthy control specimens would greatly improve the quality of the study.

Table 2.1: Inclusion and exclusion criteria for participants in the study.

Inclusion Criteria	1. ≥ 18 years 2. Abdominal circumference: ≥ 94 cm (Male) ≥ 80 cm (Female) 3. Weight stable (± 5 kg) for at least 3mths prior to inclusion
Exclusion Criteria	1. T2DM 2. Severe cardiovascular, renal, endocrine or psychiatric disease 3. Drug abuse 4. Pregnancy or lactation 5. Blood donation prohibited for 3 months prior to inclusion

Upon enrolment in the study, participants attended the clinic for baseline (*i.e.* at visit 2 before any meal test or dietary intervention) testing (Table 2.3) at visit 2. The participants attended the clinic after an overnight fast and a blood specimen was procured. Following this each participant was given a high fat meal and blood specimens were subsequently procured 30mins (T_{30}), 60mins (T_{60}) and 120mins (T_{120}) following the high fat meal. Participants were then assigned to one of four 12-week diets (Table 2.4) with varying protein and MC-SFA contents. These diets were termed White A (whey protein-high MC-SFA), White B (casein protein-high MC-SFA), Green A (whey protein-low MC-SFA) and Green B (casein protein-low MC-SFA) as developed by WP1 and WP2 (See Section 1.4 and Appendix I, Section A). During the 12-week intervention participants attended the clinic four times (*i.e.* visits 3, 4, 5, 6) for clinical assessment. Following the 12-week dietary intervention, the participants attended the clinic fasting and blood specimens were procured as is described for visit 2.

Plasma specimens for the obese ($n=26$) and MetS ($n=26$) participants (Table 2.2) before and after the 12-week dietary intervention and following procurement 30mins, 120mins and 240mins following the meal high in fat were received from Aarhus University, thus a total of eight plasma specimens for each participant were procured (Figure 2.1).

It should be noted that the overall statistical power for the cohort is calculated as 52 participants and there were 52 participants in the study. However, the number of participants in each diet group is quite low.

Table 2.2: I.D. for each participant in the study and their classification as clinically obese or having MetS

Obese Participants	MetS Participants
Specimen I.D.	Specimen I.D.
003-DF	004-JE
009-KL	006-BH
013-JS	007-ST
015-UV	008-DCS
022-SS	014-TGJ
025-AM	019-DB
029-JP	023-HT
030-ABV	024-TM
037-SDO	034-BGI
038-EK	036-KAC
039-CH	040-DHP
041-LS	042-BJ
048-SC	045-AB
049-KA	052-EAS
050-MA	053-HKS
064-EGJ	063-SL
065-HKS	068-BST
074-AAT	071-SM
075-HD	077-HK
088-SW	079-MBJ
089-HSP	082-INA
091-IH	086-TM
092-JK	087-BP
095-EPL	091-VGP
097-BDH	096-LBM
098-KH	099-EGJ

Table 2.3: Overview of 8 visits to the clinic and procedures taking place at each visit

Week 1	Week 2	Week 1-2	Week 3+5+9	Week 12	Week 12
Visit 1	Visit 2	Visit 3	Visits 4 +5+6	Visit 7	Visit 8
Blood screening specimens + BP (Electrolytes, TSH, Hgb, alanine transaminase) OGTT	6 hr high fat meal test Blood specimens (0, 15, 30, 60, 120, 180, 240, 360mins)	Dexa Scan Diurnal BP	Compliance to test diet Anthropomet ry (Weight, waist to hip ratio)	6 hr high fat meal test Blood specimens (0, 15, 30, 60, 120, 180, 240, 360mins)	Blood screening 0specimens + BP OGTT
Anthropometry (Weight, waist to hip ratio)	Indirect calorimetry (0 + 180mins)			Indirect calorimetry (0 + 180mins)	Anthropometry (Weight, waist to hip ratio).
Diet Diary	Fat biopsy (0 +240mins) Urine specimens (0-360 min) Follow up of diet diary+ instruction test diet Follow up of Visit 1 blood specimens			Fat biopsy (0 +240mins) Urine specimens (0-360 min) Diet diary	Dexa Scan Diurnal BP Follow up of diet diary

BP – blood pressure, TSH – Thyroid stimulating hormone, Hgb - hemoglobin, OGTT –oral glucose tolerance test 0+180=baseline and 180mins following meal test, 0–360=0, 15, 30, 60, 120, 180, 240, 360mins following meal test. Sections highlighted in light grey are relevant to specimens analysed in this thesis, 0-360 included our specimens at 30, 120, 240, the other time-points were for analysis in Denmark.

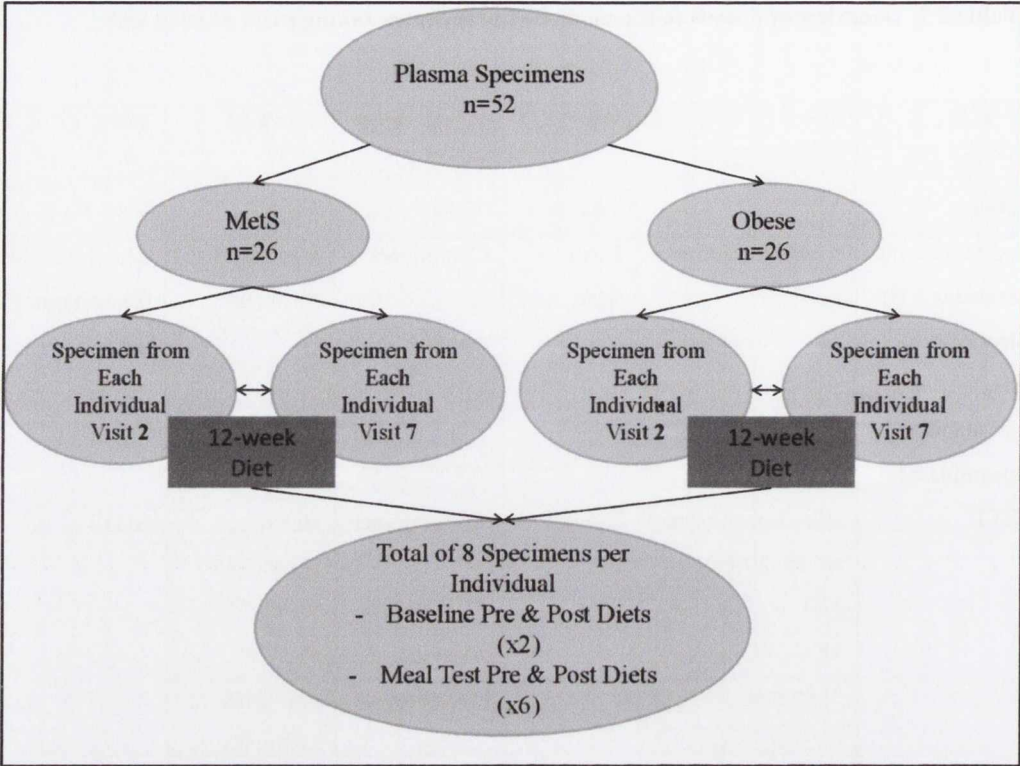


Figure 2.1: Flow diagram of the experimental design and plasma specimens procured

Flow diagram of the experimental design and plasma specimens procured from participants at visit 2 and following the 12-week diets at visit 7 in Aarhus University Hospital, Denmark.

Table 2.4: Dietary group to which each participant was randomised.

	A–Obese Participants	B–MetS Participants
Diet	Specimen I.D.	Specimen I.D.
White A	009-KL	004-JE
	013-JS	007-ST
	025-AM	014-TGJ
	029-JP	024-TM
	092-JK	068-BST
	097-BDH	082-INA
		099-EGJ
White B	038-EK	036-KAC
	039-CH	042-BJ
	049-KA	079-MBJ
	050-MA	087-BP
	064-EGJ	091-VGP
	065-HKS	096-LBM
	088-SW	
Green A	003-DF	006-BH
	030-ABV	023-HT
	041-LS	040-DHP
	074-AAT	052-EAS
	095-EPL	071-SM
	98-KH	077-HK
		086-TM
Green B	015-UV	008-DCS
	022-SS	019-DB
	037-SDO	034-BGI
	048-SC	045-AB
	075-HD	053-HKS
	89-HSP	063-SL
	91-IH	

Subject participants I.D. **A.**–Obese Subjects, **B.**–MetS Subjects. **Diets**–the diets the participants were placed on for a 12-week period from visit 2 to visit 7.

2.2.2. RNA Extraction and Quantification

Total RNA was extracted from plasma specimens (n=416) using TriReagentTM (Sigma-Aldrich, Cat. #: T9424) with optimisation of the manufacturer's instructions. In brief, 250µl of plasma and 750µl of TriReagentTM were combined, mixed and incubated at room temperature for 10mins. 200µl of chloroform (Sigma-Aldrich, Cat. #: C2432) was added, mixed vigorously for 15secs and incubated at room temperature for 10mins. The resulting solution was centrifuged (Eppendorf, model: 5415 R) at 13,200g, 4°C for 15mins to separate the protein, DNA and RNA layers. The upper aqueous layer was collected and placed in a fresh Eppendorf tube with 1.2µl glycogen (final concentration 120µg/ml) (Sigma-Aldrich, Cat. #: G8751) and 500µl of ice-cold isopropanol (Sigma-Aldrich, Cat. #: I9516). This was incubated overnight at -20°C to maximise RNA precipitation.

Samples were removed from -20°C and allowed to rest at room temperature for 5-10mins. Samples were centrifuged at 13,200g, 4°C for 30mins to pellet the precipitated RNA. The isopropanol supernatant was decanted and the pellet was washed with 750µl of 75% ethanol (Sigma-Aldrich, Cat. #: E7148). The pellet and ethanol were vortexed and centrifuged at 7500g, 4°C for 5mins to remove residual isopropanol (this wash step was repeated, but centrifuged at 13,200g, 4°C for 5mins). The pellet was allowed to air-dry until translucent (~20mins), and was resuspended in 10µl of DEPC-treated H₂O (Ambion, Cat. #: AM9916). The resuspended RNA was incubated on ice for 30mins to ensure complete homogeneity.

The quantity and quality of the extracted RNA was assessed using the NanoDrop ND-1000 Spectrophotometer (Labtech International). Readings were taken at 260nm, 280nm and 230nm to assess the quality and purity of the isolated RNA. A 260/280 ratio of ~2.0 is generally accepted as pure RNA. A lower ratio may indicate the presence of contaminants such as phenol. The 260/230 is a secondary measure of RNA purity, a ratio of ~2.0-2.2 indicates a pure RNA sample again, a lower value indicates contaminants that absorb at 230nm.

2.2.3. Quality Assessment of RNA – Agilent Characterisation

2.2.3.1. RNA 6000 Pico Kit

Assessment of the quality of the RNA isolated was performed using the Agilent RNA 6000 Pico Kit on the 2100 Bioanalyser (Agilent Technologies, Cat. #:G2939AA) as per the manufacturer's instructions.

2.2.3.2. Small RNA Kit

To assess the miRNA content of the small RNA peak detected using the RNA 6000 Pico kit, the Small RNA kit (Agilent Technologies Cat. #: 5067-1548) was performed as per the manufacturer's instructions. (Of note: there was no difference in the electropherograms for the specimens analysed at random, therefore, only a selection of specimens, randomly chosen, were analysed).

2.2.4. Analysis of miRNAs in the Plasma of Obese and MetS

Participants

In order to perform miRNA profiling, a serial dilution of RNA to determine optimal RNA input for reverse transcription (RT) reaction was performed. A pre-screen to determine endogenous control RNAs for this cohort of specimens was also performed.

2.2.4.1. Serial Dilution

Due to inhibitors of the RT reaction possibly being present in the plasma RNA, the possible contamination of the RNA with phenol resulting in unreliable NanoDrop readings and for possible transition to clinical use, it was determined that an input volume of RNA would be superior to an input concentration. In order to determine the optimal input volume a serial dilution was performed.

2.2.4.1.1. Reverse Transcription (cDNA Synthesis) of Test Specimens for Determination of RNA Input

The RT reaction was performed according to Exiqon's miCURY LNATM Universal RT miRNA PCR instruction manual v5.2 March 2013 for Serum/Plasma and other biofluids. RNA was isolated from one obese (050-MA) participant and one MetS (087-BP) participant. The RNA from each specimen was diluted 1:2, 1:4, 1:8 and 1:16. The RT reaction was set up using the cDNA synthesis kit (Exiqon, Cat. #: 203301) as follows:

Table 2.5: Set up of Exiqon universal cDNA synthesis kit for the RT reaction

Reagent	RT Reaction Volume (µl)
5X Reaction	2
Enzyme Mix	1
Nuclease-free Water	4.9
Spike-in (optional)	0.5
Template RNA	1.6

UniSp6 spike-in was used as a positive control for the qPCR reaction and to determine the RT reaction was successful. Water (H₂O) was the negative control for all qPCR reactions. A master mix of the reagents (Table 2.5) was prepared without the template RNA and 8.4µl was added to the MicroAmp fast optical 96-well reaction plate (Applied Biosystems, Cat. #: 43469062). RNA was then added to its appropriate well. The plate was sealed with optical sealing film (Applied Biosystems, Cat. #: 4306311) and centrifuged (Eppendorf Centrifuge, model: 5804R) on a short spin to bring reagents to bottom of wells. The plate was then placed in the Thermocycler (Applied Biosystems, model: Veriti 96-well thermal cycler) and the reaction was conducted using thermal cycling conditions shown below:

- Incubate at 42°C for 60mins
- Heat-inactivate the reverse transcriptase at 95°C for 5mins
- Cool to 4°C

The cDNA was then stored at -20°C.

2.2.4.1.2. PCR Amplification of Test Specimens for Determination of RNA Input

Real-time (qPCR) was performed according to Exiqon’s protocol as named in Section 2.2.4.1.1. miR-103a-3p primer was selected for serial dilution analysis as miR-103-3p is recommended by Exiqon as a miRNA whose levels are believed to be abundant in serum/plasma. The cDNA was diluted 40X in DEPC-H₂O. Each cDNA sample was run in triplicate and set up as in Table 2.6:

Table 2.6: Set up of Exiqon Exilent SYBR green master mix for qPCR reaction

Reagent	RT-qPCR Reaction Volume (µl)
SYBR green PCR Master Mix (Exiqon, Cat. #: 203420)	5
Primer miR-103a-3p (Exiqon, Cat. #: 204063)	1

6µl of master mix and primer was added to each well and 4µl of the appropriate diluted cDNA was added. This was performed in triplicate wells for each specimen. The plate was sealed (Applied Biosystems, Cat. #: 4360954) and centrifuged to bring reagents to bottom of wells. The plate was then placed in the Applied Biosystems ViiA7 (Life Technologies) instrument under the following conditions:

- Hold Stage at 95°C for 10mins
- PCR Stage (x 40 cycles)
 - 95°C for 10secs
 - 60°C for 1min
- Melt Stage
 - 95°C for 15secs
 - 60°C for 1min
 - 95°C for 15secs

2.2.4.2. Pre-screen of Plasma RNA to Determine Endogenous Control RNAs

A pre-screen of 192 miRNAs (Appendix I, Section B, Table B.1) using the RNA from 20 plasma specimens (Table 2.7) for determination of endogenous control RNAs for this cohort of plasma specimens was performed.

2.2.4.2.1. RT-qPCR Reaction for Specimens Selected for Pre-screen

cDNA synthesis was performed similarly to that in Section 2.2.4.1.1. Specifically, as 20µl RT reaction was prepared as per Table 2.5, except that reagent volumes were doubled. Cel-miR-39-3p (Exiqon, Cat. #: 203203) spike-in was combined with UniSp6 and added as per Table 2.5 (Cel-miR-39-3p is also a spike-in RNA used to ensure the RT reaction was successful, *i.e.* RT efficiency and positive control for the qPCR reaction). The reagents in Table 2.5 were added together and 18.4µl of this was added to wells of 96-well reaction plate. 3.2µl of RNA was then added to its appropriate well.

Table 2.7: Plasma specimens chosen for pre-screen to determine optimal endogenous control RNAs for miRNA profiling

Obese Participants	Mets Participants
Specimen I.D.	Specimen I.D.
079-MBJ	050-MA
007-ST	013-JS
023-HT	030-ABV
034-BGI	022-SS
091-VGP.1 (T ₀)	029-JP
091-VGP.2 (T ₁₂₀)	038-JK
024-TM	037-SDO
036-KAC	075-HD
006-BH	
040-DHP	
096-LBM.1 (T ₀)	
096-LBM.2 (T ₁₂₀)	

The PCR amplification was performed as per Section 2.2.4.1.2. The cDNA synthesised was diluted 50X in nuclease-free water. cDNA is diluted 50X as opposed to 40X (Section 2.2.4.1.2) as the primers for this assay are dried down on the profiling plate, whereas the primers are resuspended before use for individual RT-qPCR. The final cDNA concentration is 50X for both. 20µl of each diluted cDNA sample was mixed with the following reagents (Table 2.8):

Table 2.8: Set up of Exiqon Exilent SYBR green master mix for qPCR reaction.

Reagents	RT-qPCR Reaction Volume (µl)
SYBR green PCR Master Mix	1000
Nuclease-free Water (part of PCR kit)	980

10µl of the PCR master mix combined with the cDNA was pipetted into half of a 384 well Serum/Plasma Focus miRNA PCR Panel (Exiqon, Cat. #: 203843). Therefore, cDNAs from the RNA of 2 participants were loaded on each plate. The plate was sealed, centrifuged and placed in the Applied Biosystems 9500 HT Sequence Detection System (Applied Biosystems) instrument under the conditions described in Section 2.2.4.1.2.

2.2.4.2.2. RT-qPCR for U6 snRNA

U6 snRNA is routinely used as an endogenous control RNA for RT-qPCR of RNA isolated from cultured cells. RT-qPCR was performed using U6 snRNA primer (Exiqon, Cat. #: 203907) on 20 RNA specimens (Table 2.7) as described in Section 2.2.4.1.

2.2.4.2.3. Data Analysis of Pre-screen for 192 miRNAs in 20 Specimens

The pre-screen was performed using the Applied Biosystems ABI7900 HT sequence detection system. The SDS software was set up for absolute quantification (AQ). This method determined the input copy number, by relating the PCR signal from the SYBR green to a standard curve. The ABI SDS v2.1 was, therefore, used to obtain raw cycle threshold (C_T) values and the threshold for each plate was set at 1.00021 in ABI RQ Manager v1.2.1. The raw C_T values were then exported into Excel and normalised using the interpolate calibrator and following formula in Excel:

- Specimen C_T – (Mean C_T of interpolate calibrator (IPC) for specimen - mean C_T of IPC for all specimens and all plates).

From these C_T values the endogenous control RNAs were chosen. The mean values of these control RNAs for each specimen were used to normalise the raw data and the fold change was calculated using the delta delta C_T ($\Delta\Delta C_T$) method:

- Step 1: $\Delta C_T = C_T$ value of specimens – mean C_T of endogenous control RNAs
- Step 2: $\Delta\Delta C_T = \Delta C_T$ – average of all obese specimens (control group)
- Step 3: $2^{(-\Delta\Delta C_T)} = \Delta\Delta C_T$ plugged into formula $2^{(-\Delta\Delta C_T)}$.
-

2.2.4.3. Custom MicroArray Panels – Discovery Phase

Custom 384-well profiling arrays (Exiqon, Cat. #:203802) were designed. Each plate could profile 12 specimens for a total of 32 miRNAs per specimen. The miRNAs consisted of 25 selected study miRNAs, 5 endogenous control RNAs (Table 2.9), UniSp6 and UniSp3IPC (this is added to the plate to allow for normalisation of raw data obtained from a number of panels/plates).

Table 2.9: miRNAs selected for analysis and endogenous control RNAs

miRNAs Selected for Profiling	Reason For Selection from Pre-screen	Endogenous Control RNAs
miR 223-3p	Substantially decreased in MetS vs Obese (-6.6±0.9-fold, p=0.1)	miR 128
miR 126-3p	>2-fold decreased in MetS vs Obese (-2.29±0.89, p=0.50)	miR 486-5p
miR 107	>2-fold decreased in MetS vs Obese (-2.51±0.69, p=0.61)	miR 320a
miR 106a-5p	Substantially decreased in MetS vs Obese (-4.56±1.16, p=0.25)	miR 150-5p
miR 29b-3p	>2-fold decreased in MetS vs Obese (-3.04±0.7-fold, p=0.4)	U6 snRNA
miR 29a-3p	>2-fold decreased in MetS vs Obese (-2.86±0.98-fold, p=0.15)	
miR 122-5p	>2-fold increased in MetS vs obese (2.86±0.94-fold, p=0.2)	
miR 103a-3p	Substantially decreased in MetS vs Obese (-7.31±1.2, p=0.17)	
miR 92a-3p	>2-fold decreased in MetS vs Obese (-2.43±0.80-fold, p=0.12)	
miR 106b-5p	Substantially and significantly decreased in MetS vs Obese (-7.7±0.95-fold, p=0.05)	
let-7d-5p	Substantially decreased in MetS vs Obese (-11.20±0.94-fold, p=0.06)	
let-7f-5p	>2-fold decreased in MetS vs Obese (-3.5±0.64-fold, p=0.1)	
miR 26b-5p	Substantially decreased in MetS vs Obese (-5.19±0.84, p=0.06)	
miR 23a-3p	>2-fold decreased in MetS vs Obese (-3.76±0.79, p=0.319)	
miR 23b-3p	>2-fold decreased in MetS vs Obese (-2.76±0.7, p=0.08)	
miR 15a-5p	>2-fold decreased in MetS vs Obese (-3.4±1.03, p=0.19)	
miR 33a-3p	Substantially and significantly decreased in MetS vs Obese (-6.88±0.7-fold, p=0.04)	
miR 130a-3p	Substantially decreased in MetS vs Obese (-7.31±1.2, p=0.17)	
miR 204-5p	Significantly increased in MetS vs Obese (1.70±0.16, p=0.02)	
miR 200a-3p	Substantially increased in MetS vs Obese (17.26±2.18, p=0.27)	
miR-200c-3p	<2-fold increased in MetS vs Obese (3.72±0.8, p=0.16)	
miR-423-5p	Increased 1.6±0.7-fold, p=0.67)	
miR-33b-5p, miR-33b-3p	Highly cited [134] and miR-33s-3p was significantly decreased.	
miR-758-3p	Also highly cited [164]	

Specimens (from each time point/ visit 2 and visit 7) from the 38 participants termed the discovery cohort were randomly chosen for analysis (n=300 specimens). RNA from these specimens was analysed in the discovery phase of the miRNA profiling. From the MetS cohort–9 females, 10 males, aged 25-72 were chosen. Similarly from the obese cohort–8 females, 11 males, aged 29-78 were chosen for analysis (Table 2.10). *p<0.05, Student’s T-test.

Table 2.10: Specimens chosen from the Obese and MetS cohorts for the discovery phase of the miRNA profiling

Obese Participants			MetS Participants		
Specimen I.D.	Gender	Age (yrs)	Specimen I.D.	Gender	Age (yrs)
009-KL	F	29	014-TGJ	F	25
097-BDH	M	47	068-BST	M	56
092-JK	M	61	099-EGJ	M	58
013-JS	F	57	007-ST	F	55
029-JP	M	64	004-JE	M	68
038-EK	F	61	042-BJ	F	62
064-EGJ	M	65	087-BP	M	65
065-HKS	M	69	091-VGP	F	72
050-MA	M	40	096-LBM	M	52
074-AAT	F	42	023-HT	M	64
003-DF	M	65	086-TM	M	64
041-LS	M	65	077-HK	F	57
098-KH	F	52	040-DHP	F	46
075-HD	M	61	053-HKS	M	63
089-HS	M	69	063-SL	M	65
015-UV	M	48	034-BGI	M	35
093-IH	F	78	045-AB	F	65
037-SDO	F	34	008-DCS	F	57
030-ABV	F	60	071-SM	F	67

2.2.4.3.1. RT-qPCR Reaction for 25 Selected miRNAs and 7 Controls

cDNA was synthesised as per Section 2.2.4.1.1. cDNA was diluted 50X in nuclease-free H₂O, and the cDNA was amplified as described in Section 2.2.4.1.2 using the Applied Biosystems ABI7900 HT sequence detection system and the data was captured and analysed as per Section 2.2.4.2.3.

2.2.4.3.2. Data Analysis

To normalise the resulting raw data from Section 2.2.4.3.1, all plates were set to the same threshold and the average of the two endogenous control RNAs (*i.e.* miR-486-5p and miR-150-5p, they were selected as they had the most consistent levels) was used to normalise each C_T value, resulting in the ΔC_T value. The fold change was then calculated as described in Section 2.2.4.2.3.

Hierarchical clustering using normalised fold changes for both obese and MetS specimens was performed with the help of Dr. Karsten Hokamp, School of Microbiology and genetics, Trinity College Dublin. Specifically, several steps were performed to generate the heatmaps:

- C_T values of > 35 were set as NA, *i.e.* not detected
- miRNAs that contained >80% NAs per specimen were omitted
- The C_T values were linearised by calculating $2^{(-\text{intensity})}$
- Intensity values were transformed into ranks
- The heatmap was then generated using these rank values with default parameters, *i.e.* 'euclidean' distance measure and hierarchical clustering with the 'complete' agglomeration method for that the 'heatmap.2' command in the R package 'gplots'.

2.2.4.3.3. Statistical Analysis

Statistical analysis was performed using unpaired Student’s t-test and one-way *ANOVA* to generate p-values in Microsoft Excel 2003 and Graphpad 5.0 (GraphPad Software Inc, La Jolla, USA). Graphs were generated in GraphPad. Statistical analysis performed on the data was verified by Dr. Bahman Honari, statistical consultant, the Centre for Support and Training in Analysis and Research (CSTAR), University College, Dublin for a selection of the data obtained. A p value <0.05 was considered statistically significant, *i.e.* * p<0.05, ** p<0.01, *** p<0.001.

2.2.4.4. Individual RT-qPCR – Validation Stage

Individual RT-qPCR was performed using the ABI VIIA 7 instrument. Five miRNAs were identified in the discovery stage of the study from the custom miRNA profiling as possible biomarkers for MetS compared to obesity at baseline. RT-qPCR was performed to validate these miRNAs in a sub-cohort of the specimens, *i.e.* n=14 specimens (baseline specimens). In the MetS cohort – 5 females, 2 males, aged 34-79 were chosen. Similarly, in the obese cohort – 5 females, 2 males, aged 20-78 were chosen for analysis (Table 2.11). Primers for five miRNAs; miR-15a-5p (Exiqon, Cat. #: 204066), miR-106b-5p (Exiqon, Cat. #: 205884), miR-200a-3p (Exiqon, Cat. #:204707), miR-204-5p (Exiqon, Cat. #: 205708), and miR-758-3p (Exiqon, Cat. #: 204353) and miR-150-5p (Exiqon, Cat. #: 204660) and miR-486-5p (Exiqon, Cat. # 204001) endogenous control RNAs and UniSp6 internal control primers were used.

Table 2.11: Specimens chosen from the MetS and Obese cohorts for the validation phase of miRNA profiling.

Obese Participants			MetS Participants		
Specimen I.D.	Gender	Age (yrs)	Specimen I.D.	Gender	Age (yrs)
025-AM	F	29	024-TM	M	34
088-SW	F	62	082-INA	F	67
049-KA	M	68	036-KAC	F	64
039-CH	F	22	079-MBJ	F	65
095-EPL	F	66	006-BH	F	67
048-SC	F	20	052-EAS	M	79
022-SS	M	78	019-DB	F	64

2.2.4.4.1. RT-qPCR for Validation Stage

cDNA was synthesised as per Section 2.2.4.1.1. cDNA was diluted 40X in DEPC- H₂O, and the cDNA was amplified as described in Section 2.2.4.1.2 with miR-15a-5p (Exiqon, Cat. #: 204066), miR-106b-5p (Exiqon, Cat. #: 205884), miR-150-5p (Exiqon, Cat. #: 204660), miR-200a-3p (Exiqon, Cat. #:204707), miR-204-5p (Exiqon, Cat. #: 205708), miR-486-5p (Exiqon, Cat. # 204001) and miR-758-3p (Exiqon, Cat. #: 204353) primers.

2.2.4.4.2. Data Analysis

Data analysis was performed as described in Section 2.2.4.3.2. However, hierarchical clustering was not performed.

2.2.4.4.3. Statistical Analysis

Statistical analysis was performed as described in Section 2.2.4.3.3.

2.2.5. miR-758-3p mRNA Target Identification

2.2.5.1. Bioinformatics Prediction of mRNA targets of miR-758-3p

miR-walk 2.0 (<http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/>) [165], a publically available database of predicted miRNA targets was used to identify several hundred potential targets of miR-758-3p.

2.2.5.2. *In vitro* analysis of miR-758-3p, ABCA1 and ABCG2.

2.2.5.2.1. Cell Culture

Hs578T (TNBC, carcinoma; derived from human mammary gland tumour) and its isogenic sub-clone, Hs578Ts(i)₈, were gifts from Dr. Susan Mc Donnell [166]. Both cell line variants were grown in Dulbecco's Modified Eagles Medium (DMEM) (Sigma-Aldrich; Cat. #: D5671) containing 10% fetal bovine serum (FBS) (Gibco, Cat. #: 10270106, 2mM L-Glutamine (Sigma-Aldrich; Cat.

#: I0516) and 10µg/ml insulin (Sigma-Aldrich; Cat. #: I9278). MDA-MB-231 (TNBC, adenocarcinoma; mammary gland/breast; derived from human metastatic site: pleural effusion) and HCC1954 (Her2/neu overexpressing; TNM stage IIA, grade 3, ductal carcinoma; derived from human mammary gland; breast/duct) and EFM192A (adenocarcinoma) were obtained from the American Tissue Culture Collection (ATCC). MDA-MB-231 cells were grown in DMEM supplemented with 10% FBS, 2mM L-Glutamine and 10µg/ml insulin. HCC1954 and EFM192A cells were grown in Roswell Park Memorial Institute medium (RPMI)-1640 (Sigma-Aldrich; Cat. #: R0883) containing 10% FBS and 2mM L-Glutamine. All cells were grown at 37°C and 5% CO₂

Hs578Ts(i)₈ is a more aggressive cell line variant than its parental cell line Hs578T. Hs578Ts(i)₈ were isolated from the Hs578T population by Hughes *et al.* [166] by a series of eight successive passages of the cells through matrigel coated cell culture inserts with 8µm pores (Figure 2.2). This process resulted in the generation of the Hs578Ts(i)₈ cell line variant, which is 3-fold more invasive, 2.5-fold more migratory, produced 25 times more colonies in soft agar than the Hs578T cells and can produce tumours in nude mice [166]. Therefore, a cell line model for aggressive TNBC was established.

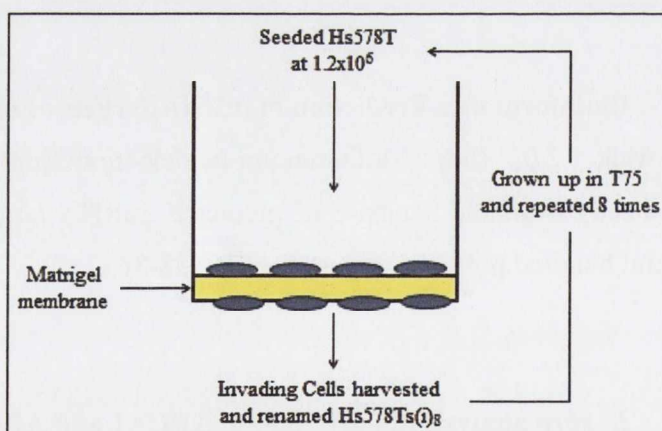


Figure 2.2: A model for the selection of more invasive subclones using BD Matrigel™ invasion chambers.

This is the process used by Hughes *et al.* [166] in UCD to develop the Hs578Ts(i)₈ cell variant from Hs578T cells. Briefly, 1.2x10⁶ parental cells are seeded into the chamber; cultured for 5 hrs; invading cells collected from the bottom of the membrane by trypsinisation and subsequently cultured. Eight successive rounds of this process allowed for the selection of more invasive cells from the parental cells. Diagram adapted from Hughes *et al.* [166].

Routine *Mycoplasma* analysis was performed by qPCR (Promokine; Cat. #: PK-CA91-1048, ATCC; Cat. #:30-1012K) every three months. Performing of the *mycoplasma* test rotated around our group.

2.2.5.2.2. Cell Pellet Collection

All cells lines were seeded at at 2×10^6 cells/ T75/10 ml medium. A minimum of triplicate flasks for each cell line at different passage numbers were prepared. When approximately 80% confluent, cells were:

1) For RNA, trypsinised, counted and centrifuged (900, for 5mins) to pellet the cells for RNA isolation. The pellet was washed twice with Phosphate Buffered Saline (PBS) (Sigma-Aldrich, Cat. #: D8537), and resuspended in 1ml TriReagent™ (Sigma-Aldrich, Cat. #: C2432) and were incubated on ice for 10mins and stored at -80°C until required) or

2) For immunoblotting, scrapped using cell scrappers (Fisher Scientific; Cat. #: 08-100-241) and centrifuged at 13,000g, 4°C for 5mins. Pellets were washed with 1ml PBS and centrifuged again. PBS was removed and cell pellets were stored at -80°C until required.

2.2.5.2.3. RNA Isolation and Quantification

Total RNA was extracted as per Section 2.2.2

2.2.5.2.4. miR-758-3p Analysis

Analysis of miR-758-3p levels in Hs578T and HS578Ts(i)₈, HCC1954, MDA-MB-3231 and EFM192A was performed. Endogenous control RNAs, miR-150-5p, miR-486-5p, UniSp6 and U6 snRNA were also analysed in all cell pellets. Each sample was analysed in triplicate.

2.2.5.2.4.1. RT-qPCR

cDNA was synthesised from 1.6µl input RNA as described in Section 2.2.4.1.1. cDNA was diluted 40X in DEPC-H₂O, and the cDNA was amplified by PCR as described in Section 2.2.4.1.2.

2.2.5.2.5. Protein Quantification and Immunoblotting

Cell pellets were lysed using Cell Extraction Buffer (Invitrogen, Cat. #: FNN0011) supplemented with 1X protease inhibitor cocktail (Roche, Cat. #: 05892970001). Cell pellets were combined with 30-50µl of lysis buffer/protease inhibitor, vortexed and incubated on ice for 30mins with vortexing every 10mins for 10secs. Cell lysates were sonicated (Misonix, microson ultrasonic cell disruptor, model: XL2000, setting 3) for approximately 10secs. Following sonication cell lysates were centrifuged at 13,200g, 4°C for 10mins to pellet cell debris; the resulting supernatants were transferred to fresh Eppendorf tubes.

Protein was quantified using the Bio-Rad protein assay dye reagent (BioRad, Cat. #: 500-0006). Specifically, BSA (Bovine Serum Albumin, Sigma Aldrich, Cat. #: A9418) standards were prepared in ddH₂O (800mg/ml, 400mg/ml, 200mg/ml, 100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml, 0mg/ml) and 10µl was pipetted in duplicate into the wells of a 96-well plate. Protein lysates were diluted 1:10 in ddH₂O and 10µl was again pipetted in duplicate into the wells of the 96-well plate containing the BSA standards. Bio-Rad protein dye reagent was diluted 1:5 in ddH₂O and 200µl of this solution was added to each well containing either BSA standards or protein lysates. The plate was incubated in the dark for 5mins and subsequently read at 595nm using the FlouStar Optima microplate reader (BMG Labtech). Total cellular protein concentrations were determined using Excel, regression analysis applied to absorbance readings, R² values were calculated and the protein concentrations were extrapolated from the standard curve. 4X Loading buffer was prepared (Table 2.12), added to a final concentration of 1X and samples were heated to 95°C for 10mins.

Table 2.12: Reagents for preparing loading buffer

Reagent	Volume
Stacking Buffer (see Table 2.13)	4ml
β -mercaptoethanol (Sigma-Aldrich, Cat. #: M7154)	2ml
Glycerol (Sigma-Aldrich, Cat. #: 5516)	6ml
Sodium Dodecyl Sulfate (SDS) (Sigma-Aldrich, Cat. #: L4390)	1g
Bromophenol Blue (Sigma-Aldrich, Cat. #: B5525)	50mg

50 μ g total cellular proteins were resolved on NuPage® Novex® 4-12% Bis-Tris for protein SDS-PAGE electrophoresis (Biosciences, Cat. #: NP0323) or resolved on 7% and 10% gels (as prepared in Table 2.13). Proteins were resolved along with a molecular weight marker, SeeBlue Plus2 Pre-stained standard (Invitrogen; Cat. #: LC5925) and Spectra™ Multicolor High Range Protein Ladder (Fisher Scientific; Cat. #: 26625) for ABCG2 and ABCA1 respectively.

Separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (BioRad, Cat. #: 162-0177) using semi-dry transfer at 20V for 1 hour. Ponceau (Sigma-Aldrich; Cat. #: P7170) stain was used to ensure successful transfer of proteins onto the membranes. Following transfer, blots were incubated in blocking buffer (5% BSA [Sigma-Aldrich, Cat. #: A9413] in PBS/0.01% Tween 20 [Sigma-Aldrich, Cat. #: P2287]) at room temperature for 1 hour. Blots were then washed in PBS/0.01% Tween 20 for 5mins (x3 times). Primary antibodies were prepared in 3% BSA in PBS/0.01% Tween 20 and diluted as follows: ABCA1 (Abcam, Cat. #: Ab18180, 1:200 dilution), ABCG2 (Santa Cruz, Cat. #: sc-58224 Clone: BXP-53, 1:500 dilution) and β -actin (Sigma-Aldrich, Cat. #: A5316, 1:1000 dilution). Membranes were incubated in primary antibodies overnight at 4°C under constant rocking. Membranes were subsequently washed in PBS/0.01% tween 20 for 5mins (x3 times) and incubated in secondary horseradish peroxidase-conjugated secondary antibodies *i.e.* anti-mouse (Cell Signalling, Cat. #: 7076s, 1:1000 dilution) for ABCA1 or anti-rat (Santa Cruz, Cat. #: sc-2006, 1:1000 dilution) for ABCG2 for 1 hour at room temperature under constant rocking. Membranes were again washed in PBS/0.01% Tween 20 for 5mins (x3 times) and proteins were visualised by chemiluminescence (Thermofisher, Cat. #: 34080). Proteins were detected using a chemidoc exposure system (BioRad laboratories). Densitometry on the bands of protein was

performed using NIH ImageJ software and normalised to loading control, β -actin (Sigma, Cat. #: A1978).

Table 2.13: Reagents and quantities for preparation of 7% and 10% SDS-PAGE gels

	7%	10%	Stacking
30% Bis-Acrylamide	3.75mls	5mls	670 μ l
4 X Resolving Buffer	4mls	3.8mls	4 X Stacking Buffer 500 μ l
ddH ₂ O	8mls	6mls	2.7mls
10% Ammonium Persulfate	160 μ l	100 μ l	40 μ l
10% SDS	160 μ l	150 μ l	40 μ l
TEMED	16 μ l	16 μ l	4 μ l

2.2.5.2.6. Transient miR-758-3p Inhibition or Mimic into Cells.

[Confocal analysis was performed with the help of Dr. Gavin McManus]

Transient transfection of TNBC cell line variants with inhibitors or mimics of miR-758-3p was performed with the use of miR-758-3p inhibitors from Exiqon and Dharmacon. Exiqon inhibitors and mimics were labelled with FITC for visualisation of successful transfection using confocal analysis, Exiqon negative control inhibitors and mimics and Dharmacon reagents were not labelled.

As miR-758-3p was found to be increased in the Hs578T cells they were transfected with 30nm of miR-758-3p inhibitor (Exiqon, Cat. #: 4100323-021) or miRNA inhibitor negative control (Exiqon, Cat. #: 199006-011) or 40nm miR-758-3p inhibitor (Dharmacon Cat. #: 15541670) or miRNA inhibitor negative control (Dharmacon Cat. #: 12610636).

As miR-758-3p was decreased in the Hs578Ts(i)₈ cells they were transfected with 5nm of miR-758-3p mimic (Exiqon, Cat. #: 479997-611) or miRNA mimic negative control (Exiqon, cel-miR-39-3p, Cat. #: 479997-611) or 40nm miR-758-3p mimic (Dharmacon Cat. #: 15475264) or miRNA mimic negative control (Dharmacon Cat. #: 11587170). The concentration of Exiqon inhibitors or mimics used was determined by the manufacturer as the highest concentration of these reagents to be used and we used the Dharmacon reagents as described by Ramirez *et al* [164].

For both Exiqon and Dharmacon reagents, cells were seeded in 6-well plates at 1.5×10^5 cells/well in 2mls of medium. Cells were allowed to attach overnight at 37°C/5% CO₂. Cells were then transfected with 30nm/40nm inhibitor and negative

control inhibitor or 5nm/40nm mimic and negative control mimic. Transfection was performed according to manufacturer's instructions for both the inhibitors and mimics for Exiqon and Dharmacon. In brief, two master mixes were prepared:

1. 5µl Lipofectamine 2000 (Invitrogen, Cat. #: 11668-027) and 250µl Opti-MEM (Invitrogen, Cat. #: 11058-021) per well were combined and incubated for 5mins at room temperature in the dark.
2. 30nm/40nm inhibitor or negative control inhibitor and 250µl opti-MEM per well or 5nm/40nm mimic or negative control mimic and 250µl opti-MEM per well were combined and incubated 5mins.

Mixes 1 and 2 were combined and incubated for 20mins. After this, 565µl of inhibitor/negative control inhibitor was added to 1.45ml complete growth medium or 515µl of mimic/negative control mimic were added to 1.75ml complete growth medium. Cells were incubated for 24 hours, after which lipofectamine, inhibitors and mimics were removed, cells were washed with complete growth medium (x2 times) and 2mls fresh complete growth medium was added.

Confocal Analysis was performed at 24 hour incubation to visualise the inhibitors and mimics inside the cells, in order to confirm successful transfection of Exiqon inhibitor and mimic. Cell protein was collect as described in Section 2.2.5.5.5 and 2.2.5.2.5 for immunoblotting with anti-ABCA1 and anti-ABCG2 antibodies for cells transfected with both Exiqon and Dharmacon reagents.

2.2.5.2.7. Statistical Analysis

Statistical analysis was performed in Excel. p-values were generated using Student's t-tests, with $p < 0.05$ considered as statistically significant Graphpad Prism 5.0 was used to generate graphs (Graphpad Software Inc, La Jolla, USA). Linear regression analysis and calculation of R^2 for protein quantification were performed in Excel.

2.3. Results

2.3.1. RNA was Successfully Extracted from Plasma Specimens

Total RNA was successfully isolated from 250µl of plasma from both obese and MetS participant. Quantification of RNA using the NanoDrop at 260/280nm determined no significant ($p=0.63$) differences between the levels of RNA obtained from obese (mean= $4.85\pm0.28\mu\text{g/ml}$ plasma) or MetS (mean= $4.64\pm0.32\mu\text{g/ml}$ plasma) participants plasma specimens (Figure 2.3 A) at visit 2 baseline. To determine RNA purity the 260/280 ratio was measured simultaneously to the RNA concentration (Figure 2.3 B). The difference in the 260/280 for the MetS and obese cohort was not significant ($p=0.1$) at visit 2 baseline.

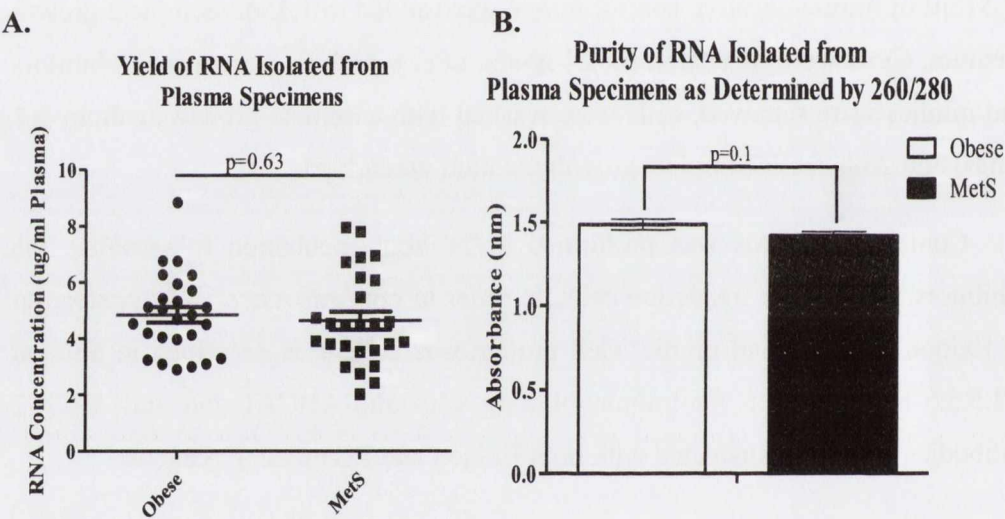


Figure 2.3: Yield of RNA isolated from plasma specimens

RNA, as assessed by NanoDrop ND-1000 spectrophotometer. RNA was:

A. Concentration of RNA isolated per ml of plasma. **B.** Purity of RNA isolated per ml of plasma as determined by the 260/280. Data for B are expressed as the mean $\pm$ SEM for each RNA specimen for each cohort. Statistical analysis was performed using the Students t-test, results were not significant.

Total RNA was also successfully isolated from 250µl of plasma from both obese and MetS participants following the 12-week diets. Following the 12-week diets at baseline, quantification of RNA using the NanoDrop determined the differences between the levels of RNA obtained from the plasma from obese (mean=5.05±0.34µg/ml plasma) and MetS (mean=4.16±0.32µg/ml plasma) participants (Figure 2.4 A) was not significant (p=0.06). To determine RNA purity the 260/280 ratio was measured again using the NanoDrop (Figure 2.4 B). The differences between the obese and MetS cohorts for the 260/280 (p=0.81) was not significant. Comparing the quantity of RNA obtained from the obese cohort for each diet was not significant (p=0.08) (Figure 2.4 C). Tukey post-hoc analysis found the difference in RNA quantity, isolated from the high MC-SFA-whey diet and the high MC-SFA-casein diet to be significantly different (p=0.04) and the high MC-SFA-casein and low MC-SFA-casein diet to again be significantly different (p=0.01) (Figure 2.4 C) in the obese cohort. However, the quantity of RNA obtained from the MetS cohort for each diet at visit 7 found no significant difference (p=0.88) in the quantity for all 4 diets (Figure 2.4 D).

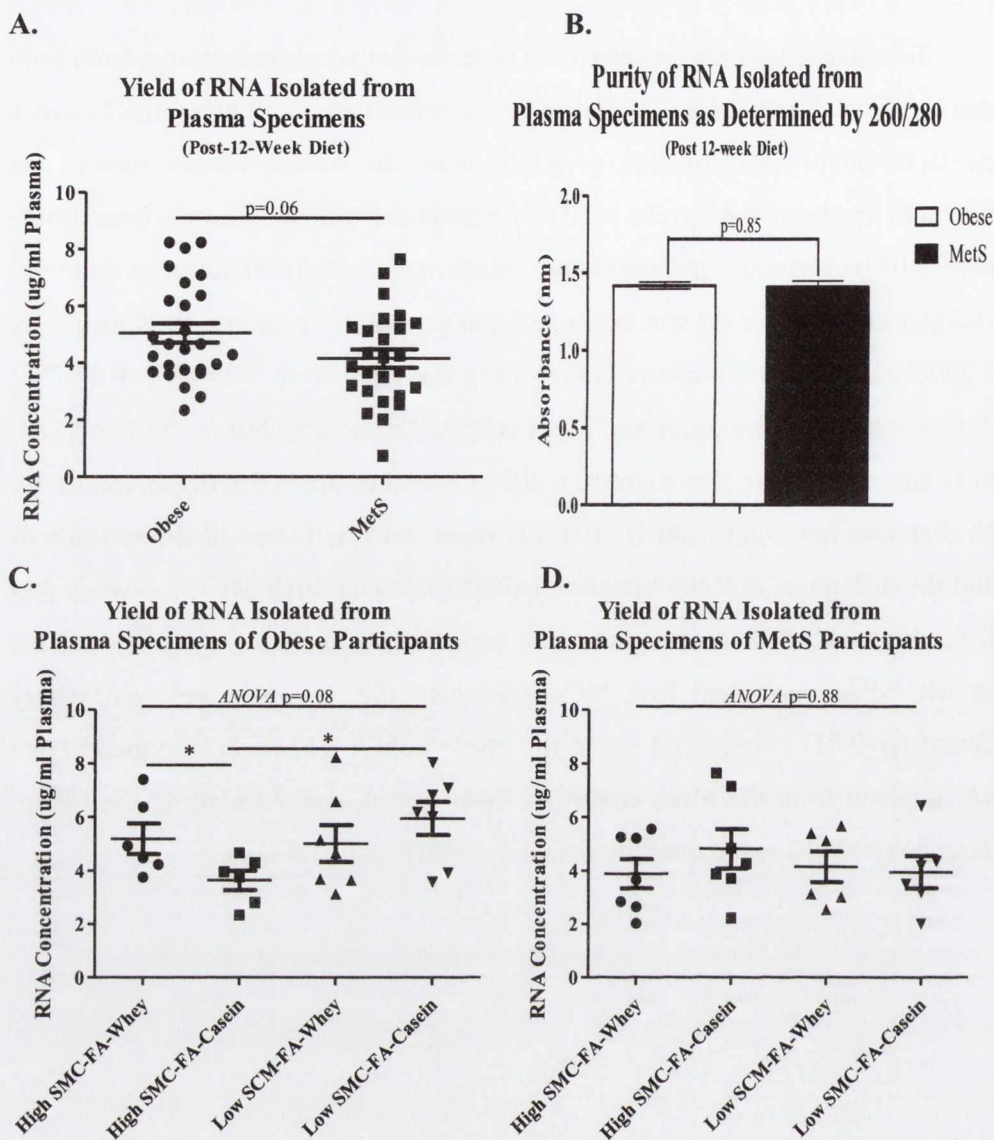


Figure 2.4: Yield and purity of RNA isolated from plasma specimens

RNA, as assessed by NanoDrop ND-1000 spectrophotometer.

A. Concentration of RNA isolated per ml of plasma. **B.** Purity of RNA isolated per ml of plasma as determined by absorbance values at 260/280. **C.** Concentration of RNA isolated per ml of plasma for each of the 12-week diets for the MetS cohort. **D.** Concentration of RNA isolated per ml of plasma for each of the 12-week diets for the obese cohort. Data for B are expressed as the mean $\pm$ SEM for all RNA specimens in each cohort. Statistical tests used: Student's t-test for A+B, one-way *ANOVA* analysis with post-hoc Tukey analysis for C+D. * $p<0.05$. MC-SFA-small and medium chain-saturated fatty acids.

Following the high fat meal test, analysis of the quantity of RNA in the obese specimens compared to the MetS specimens at all time-points 30 (T₃₀), 120 (T₁₂₀) and 240 (T₂₄₀)mins following the meal test, there was no significant difference in the levels of RNA either pre- or post-12-week diets (Table 2.14) Analysis of the RNA quantity following the 12-week diets for each of the diets separately, again there was no significant difference in the quantity of the RNA when analysed by one-way *ANOVA* (Table 2.15).

Table 2.14: Quantity of RNA isolated from plasma of Obese and MetS participants after a high fat meal before (visit 2) and after (visit 7) 12-week dietary intervention

T ₃₀	Obese (ng/ml plasma)	MetS (ng/ml plasma)	p
Visit 2	3.9±0.4	3.7±0.4	0.44
Visit 7	3.8±0.4	4.0±0.4	0.92
T ₁₂₀	Obese (ng/ml plasma)	MetS (ng/ml plasma)	p
Visit 2	2.7±0.2	3.1±0.3	0.23
Visit 7	3.3±0.3	2.5±0.2	0.06
T ₂₄₀	Obese (ng/ml plasma)	MetS (ng/ml plasma)	p
Visit 2	2.8±0.3	3.1±0.3	0.45
Visit 7	3.1±0.3	3.1±0.2	0.9

Values represent the average RNA quantity isolated for each cohort ±SEM for the whole cohort. Data are expressed at the mean ± SEM for all RNA specimens for each cohort. Statistical analysis was performed using one-way *ANOVA*, results were not significant.

Table 2.15: Quantity of RNA isolated from plasma of Obese and MetS participants after a high fat meal after (visit 7) 12-week dietary intervention, for each diet group.

	High MC-SFA- Whey (ng/ml plasma)	High MC-SFA- Casein (ng/ml plasma)	Low MC-SFA- Whey (ng/ml plasma)	Low MC-SFA- Casein (ng/ml plasma)	
T₃₀					p
Obese	2.9±0.5	5.7±1.3	4.0±0.5	3.9±0.8	0.15
MetS	4.5±0.8	4.4±0.6	5.1±0.9	2.5±0.3	0.09
T₁₂₀					p
Obese	4.3±0.7	3.0±0.5	2.9±0.6	3.2±0.8	0.23
MetS	2.1±0.23	2.6±3.4	3.2±0.5	2.5±0.3	0.45
T₂₄₀					p
Obese	2.8±0.5	3.2±0.6	3.2±0.5	3.3±0.7	0.14
MetS	3.1±0.4	2.6±0.4	2.3±0.6	3.8±0.5	0.92

Values represent the average RNA quantity isolated for each cohort ±SEM for the whole cohort. Data are expressed at the mean ± SEM for all RNA specimens for each cohort. Statistical analysis was performed using Students t-test, results were not significant.

2.3.2. MicroRNA Content and Quality of Total RNA was Determined by Agilent RNA 6000 Pico and Small RNA Kits

Electropherograms from the RNA 6000 Pico kit analysis of the total RNA showed plasma contains little or no ribosomal RNAs (18s and 28S were not detected), as is expected. A small peak was detected at 24-29secs [s]. This small peak is assumed to be 5S, 5.85S subunits, transfer RNAs or small RNA fragments less than 100 base pairs (Figure 2.5 A). The small RNA kit analysis of the total RNA showed the presence of small RNAs of approximately 10-40 nucleotides *i.e.* the miRNA content of the total RNA (Figure 2.5 B). The kit also determined the percentage of miRNAs detected in the total RNA sample (Obese=59.91±3.65% miRNAs, MetS=62.67±3.60% miRNA) (p=0.59; obese vs MetS) (Appendix I, Section C, Figure C.1 A and B, Table C.1). The miRNA content of the total RNA was also determined following the high fat meal at each time-point. There was no difference in the miRNA content in the obese or MetS cohorts pre- or post-12-week diets following the meal high in fat at all time-points (Appendix I, Section C, Table C.1).

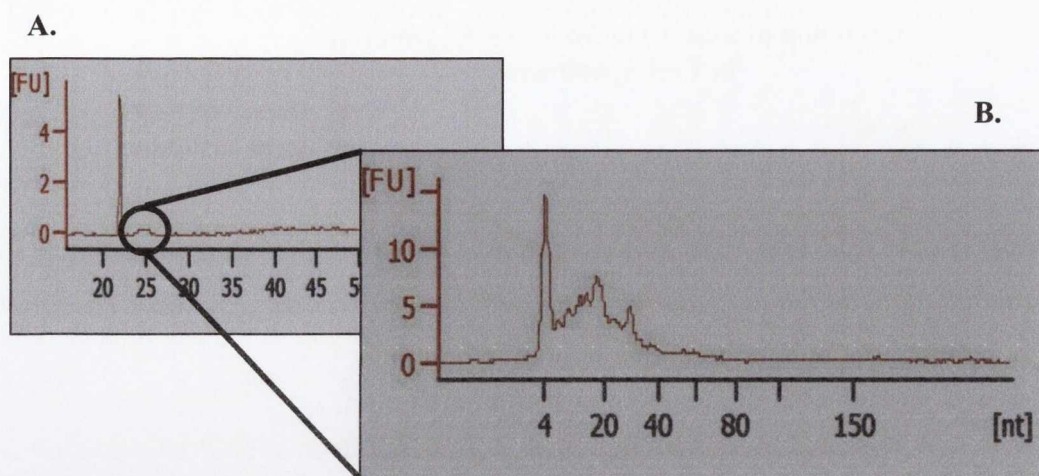


Figure 2.5: RNA, characterisation using the Agilent 2100 Bioanalyser.

A. RNA 6000 Pico kit – shows the presence of 5S and 5.8S subunits, tRNAs and small RNA fragments (<100bp). **B.** Small RNA kit – shows the presence of small RNAs and indicates the percentage of miRNAs in the total RNA analysed. Plasma RNA specimens are known not to contain the 18S and 28S ribosomal RNAs.

2.3.3. Determination of RNA Input Volume and Endogenous Control RNA for miRNA Profiling

2.3.3.1. Serial Dilution of Input RNA determined the ideal RNA Input Volume.

Due to possible phenol contamination interfering with NanoDrop readings, possible inhibitors in the plasma and for ease of possible transition to the clinic a constant input volume of RNA for the RT reaction was deemed to be the optimum method for cDNA synthesis. RNA from one participant with clinical obesity (050-MA) and one participant with MetS (087-BP) was diluted 1:2, 1:4, 1:8 and 1:16 and an RT-qPCR reaction for miR-103a-3p (a possible endogenous control RNA as determined by Exiqon) was performed. An input volume of 0.8-1.6µl was determined as an appropriate input volume, as C_T values of 24-25 (Figure 2.6) were obtained. In order to avoid pipetting error 1.6µl was selected for all RT reactions.

Detection of miR-103a-3p by Serial Dilution in Test Specimens

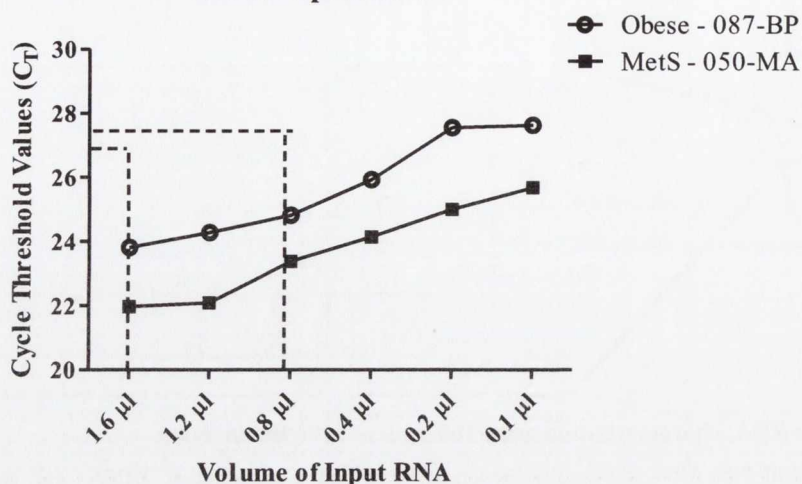


Figure 2.6: Serial dilution to determine RNA input volume for RT reactions.

RT-qPCR was performed using miR-103a-3p and 6 RNA input volumes (1.6, 1.2, 0.8, 0.4, 0.2, 0.1 µl) in order to determine the appropriate RNA input volume to avoid inhibition of the RT reaction. Data are expressed as the mean $\pm$ SEM of 3 technical repeats.

2.3.3.2. Endogenous Control RNAs were selected from the Pre-screen

Normalisation of C_T values obtained during the PCR reaction is a standard step in the analysis of miRNA profiling data and other PCR applications, such as gene expression analysis. Endogenous control RNAs often used for gene expression and miRNA profiling from cells are GAPDH and U6 snRNA, respectively. However, for plasma specimens' conventional endogenous control RNAs are often not at a constant level. Therefore, it is difficult to normalise data. In this study we performed a pre-screen of 192 miRNAs per specimen in order to seek a single or panel of endogenous control RNAs for this cohort of specimens. miR-128, miR-150-5p, miR-320a, miR-486-5p and U6 snRNA were chosen as they were the most uniformly detected miRNAs, however, the average of all five miRNAs was most consistent in all specimens (Figure 2.7) and all five were detected in all specimens. All five miRNAs were then placed on the custom miRNA panels as potential endogenous RNA controls.

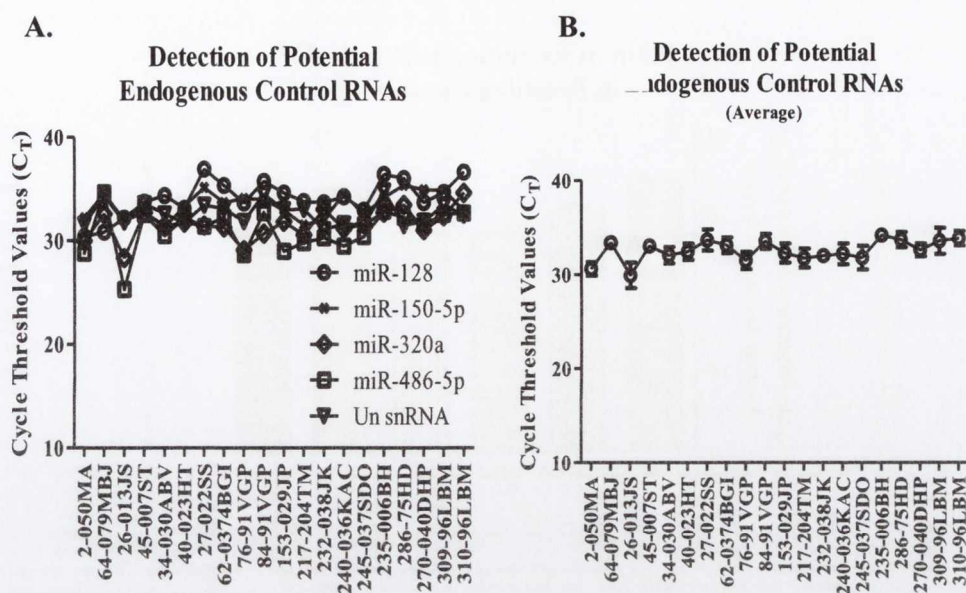


Figure 2.7: Endogenous control RNAs detected from a pre-screen.

A. Five RNAs identified from a pre-screen of 192 miRNAs as potential endogenous control RNAs for this study cohorts. **B.** Average of these 5 RNAs as a potential endogenous control RNA, mean for each specimen for 5 miRNAs $\pm$ SEM.

2.3.4. Custom miRNA Profiling (Discovery Phase) Identified Five miRNAs as Potential Biomarkers for MetS

2.3.4.1. Selection of Endogenous Control RNAs miR-150-5p and miR-486-5p

As detailed in Section 2.2.4.3, custom miRNA profiling of the RNA extracted from the plasma of obese and MetS participants was performed for 25 miRNAs, 5 endogenous control RNAs, UniSp6 spike in and UniSp3IPC. Raw C_T data was exported from the ABI SDS v2.1 software and normalised using UniSp3IPC for interpolate variation as detailed in Section 2.2.4.3.2. Subsequently, the five endogenous control RNAs were analysed and the mean of miR-486-5p (C_T=27.82) and miR-150-5p (C_T=26.73) was chosen as the endogenous controls for normalisation of the study (Figure 2.8).

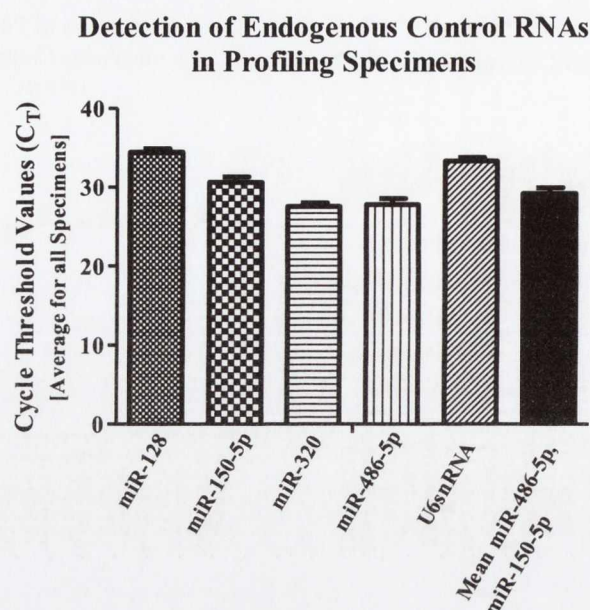


Figure 2.8: Selection of miR-486-5p and miR-150-5p as endogenous control RNAs

Following normalisation for interplate differences, five miRNAs were analysed as potential endogenous control RNAs for the study. miR-150-5p and miR-486-5p were selected as endogenous control RNAs and their values averaged for all specimens. Data represent the mean $\pm$ SEM for each miRNA.

2.3.4.2. Analysis of miRNAs in Plasma Specimens from Obese and MetS Participants

A cut-off point of $35C_T$ was taken, where miRNAs detected $\leq 35C_T$ were considered as ‘present’ where as those with a C_T value $>35C_T$ were considered ‘undetected’. The detection of miRNAs in the specimens from those with obesity and MetS is shown in Figure 2.9. Of the 25 miRNAs analysed, 18 were detected in plasma from both the obese and MetS cohorts, miR-29b-3p and miR-758-3p were only detected in plasma from the obese cohort and miR-204-5p was only detected in the plasma of the MetS cohort. Four miRNAs (miR-33a-3p, miR-33b-3p, miR-33b-5p and 200a-3p) were undetected in both cohorts at visit 2 baseline (Figure 2.9).

C_T values and have a lower detection. miRNAs surrounded by black boxes, red boxes, green boxes are grouped and miR-758-3p boxed is orange is only detected in the obese cohort.

2.3.4.3. Identification of Potential miRNA Biomarkers for MetS

Following normalisation of the custom miRNA profiling data to the mean C_T of miR-150-5p and miR-486-5p, analysis of dysregulated miRNAs between obese and MetS was performed. Taking a cut-off point of ≥ 2 -fold increased or decreased, in MetS specimens compared to obese specimens five potential miRNA biomarkers for MetS at baseline were identified. Fold changes for each miRNA (MetS vs Obese) were calculated using the $2^{(-\Delta\Delta C_T)}$ method. Specifically, the levels of miR-15a-5p (-2.8 ± 1.4 -fold, $p=0.4$), miR-106b-5p (-3.1 ± 1.2 -fold, $p=0.3$), and miR-200a-3p (-2.1 ± 0.4 -fold, $p=0.4$) were decreased in the MetS cohort, miR-204-5p (2.04 ± 0.6 -fold, $p=0.3$) was increased in the MetS cohort (Figure 2.11 A). However, for each of these there was a >2 -fold difference, but the results were not statistically significant.

Much more interestingly, miR-758-3p, was present in the obese cohort ($C_T=33 \pm 0.3$) but undetected in the MetS cohort (Figure 2.11 B).

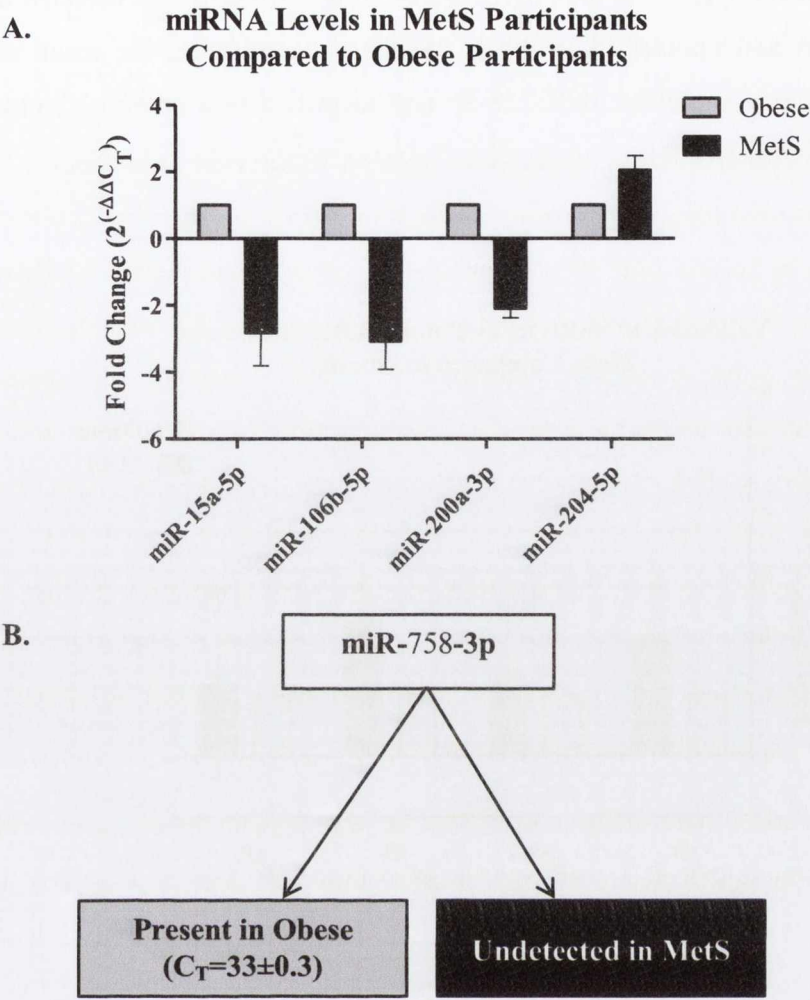


Figure 2.11: Change of miRNA levels in plasma of MetS participants compared to Obese participants

A. Fold change in the level of miRNAs in the plasma of MetS participants compared to obese participants and **B.** miR-758-3p detected in plasma of obese participants and undetected in the plasma of MetS participants. Data represent mean $\pm$ SEM for each miRNA. Results are not significant.

2.3.4.4. Validation Phase of miRNAs as potential biomarkers for MetS

Five miRNAs were identified in the discovery phase of this study as potential biomarkers for MetS compared to obesity at baseline. Individual RT-qPCR of these five miRNAs on a sub-cohort of specimens (n=14) (from baseline) showed four of these five miRNAs did not follow the same trend as the discovery phase or were less than 2-fold altered (Figure 2.12 A).

However, similar to the Discovery stage miR-758-3p was detected in the obese cohort and undetected in the MetS cohort, confirming the result of the discovery phase. Therefore, miR-758-3p was identified as a potential biomarker for MetS (Figure 2.12 B).

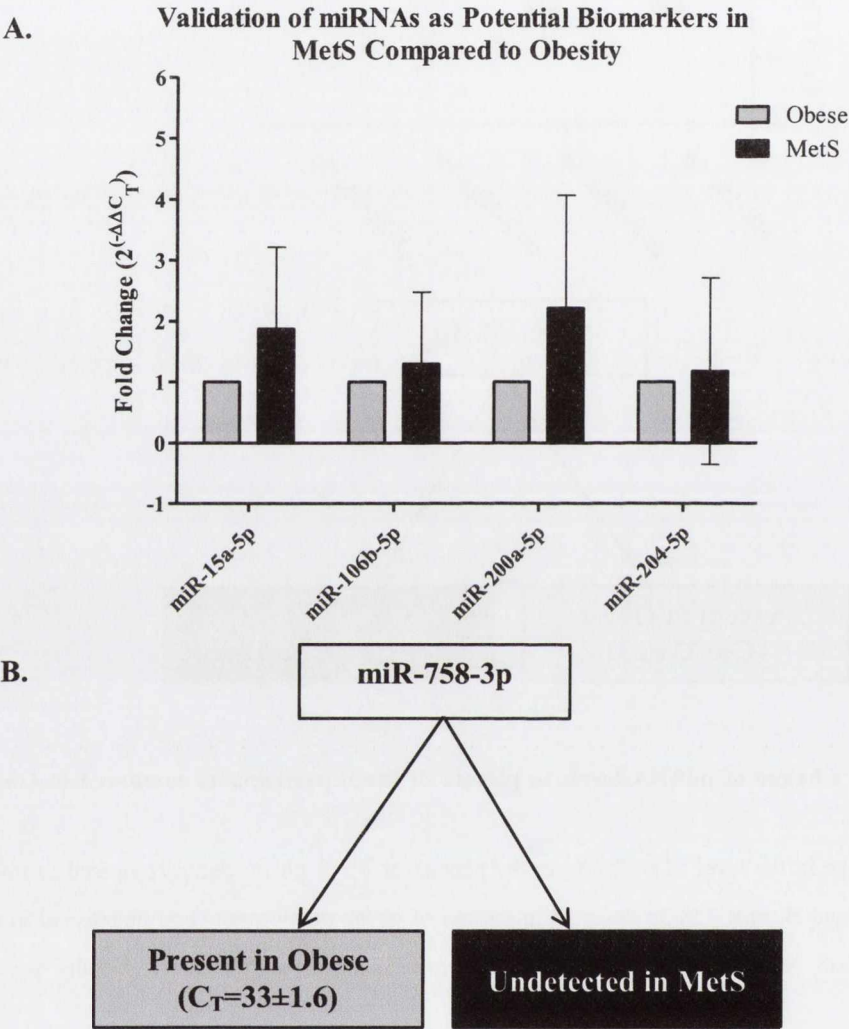


Figure 2.12: Validation of miRNAs as potential biomarkers for MetS
A. Four of the five miRNAs were not validated as suitable biomarkers for MetS. **B.** miR-758-3p was undetected in the MetS cohort but detected in the Obese cohort. Data represent mean $\pm$ SEM for each miRNA. Results are not significant.

2.3.5. miRNA Profiling Following the 12-week Dietary Intervention

Following visit 2 to the clinic each participant was assigned to one of 4 diets (high MC-SFA-Whey, high MC-SFA-casein, low MC-SFA-whey, low-MC-SFA-casein) for 12-weeks. At visit 7 (*i.e.* after the 12-week diets) to the clinic each participant had completed the 12-week dietary intervention. Specimens were procured (as described in Section 2.2.1) and miRNA analysis was performed.

Again a cut-off point of $35C_T$ was taken, where a $C_T \leq 35$ was considered present, while a $C_T > 35$ was considered absent. The detection of miRNAs in the plasma from obese and MetS participants at visit 7 baseline is shown in Figure 2.13. Of the 25 miRNAs analysed in this study, 17 were detected in the plasma from both obese and MetS participants, miR-29b-3p, miR-33a-3p, miR-130a-3p and miR-758-3p were only detected in plasma from obese participants. Four miRNAs (miR-33b-3p and miR-33b-5p, miR-200a-3p and miR-204-5p) were undetected in both cohorts (Figure 2.13).



Figure 2.13: Detection of miRNAs in plasma of Obese and MetS participants visit 7.

Venn diagram illustrating the distribution of miRNAs detected plasma from Obese and MetS participants

2.3.5.1. Changes in miRNA Levels within the MetS Cohort

At visit 7 baseline, any influence of the 12-week diets should be evident and 20 of the 25 miRNAs analysed were detected. Overall the 12-week diets resulted in a decrease in the levels of 10 miRNAs (-3.52 ± 0.83 -fold to -2.04 ± 1.09 -fold) (Figure 2.14) by more than 2-fold when comparing visit 7 to visit 2. However, none of the miRNAs were significantly affected by the diet. miR-758-

3p was excluded from this analysis as it was not detected in the plasma from the MetS cohort at visit 2 or visit 7.

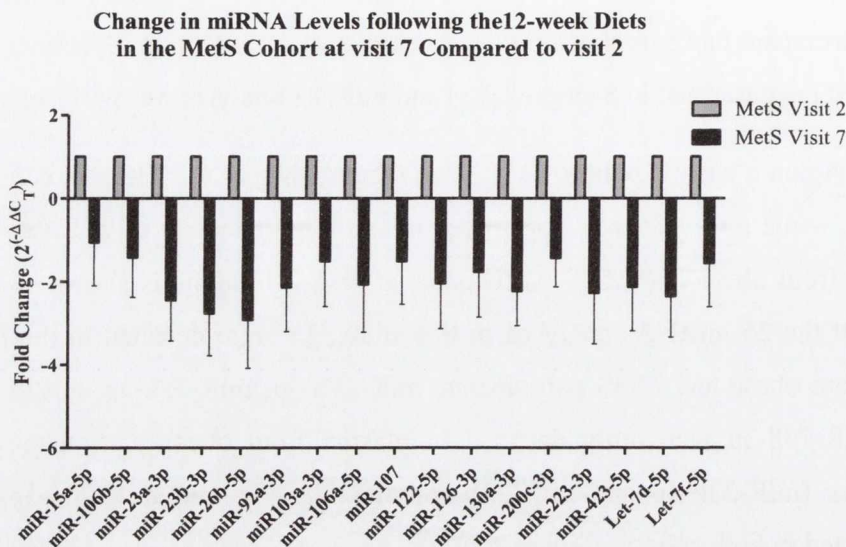


Figure 2.14: Change in miRNA levels for the MetS cohort at visit 7 compared to visit 2
Fold change in circulating miRNA levels in the MetS cohort at visit 7 compared to visit 2, showing that the 12-week diets did not significantly affect circulating miRNA levels. Data represent mean $\pm$ SEM for each miRNA. Results are not significant.

2.3.5.2. Changes in miRNA Levels within the Obese Cohort

Within the obese cohort, at visit 7 compared to visit 2, 22 of the 25 miRNAs analysed were detected. Overall the 12-week diets resulted in an increase in the levels of 13 miRNAs (2.15 ± 0.56 -fold to 4.07 ± 0.60 -fold) (Figure 2.15) by more than 2-fold when comparing visit 7 to visit 2. The levels of miR-758-3p (2.57 ± 1.1 -fold, $p=0.23$) were increased at visit 7 compared to visit 2. However, again, none of these changes were statistically significant indicating no statistical influence of the diet on circulating miRNAs.

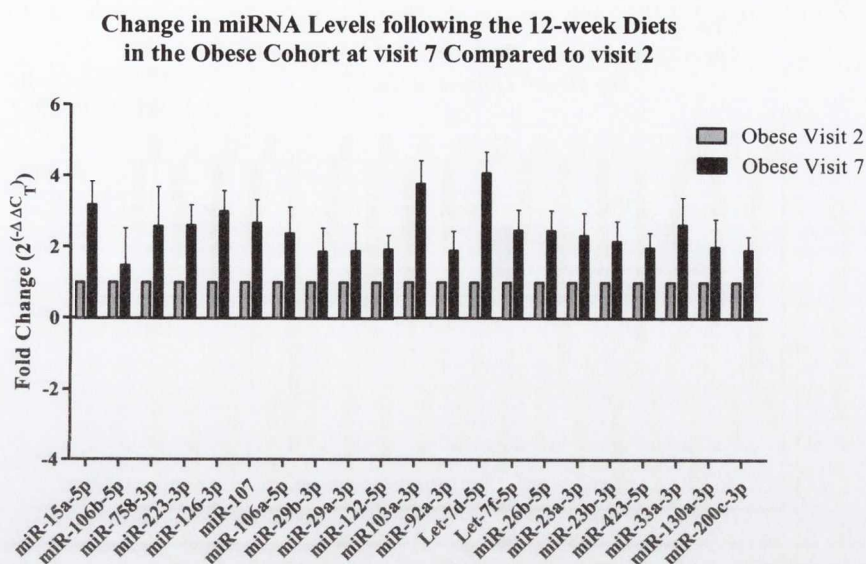


Figure 2.15: Change in miRNA levels for the Obese cohort at visit 7 compared to visit 2.

Fold change in circulating miRNA levels in the MetS cohort at visit 7 compared to visit 2, again, showing that the 12-week diets did not significantly affect circulating miRNA levels. Data represent mean $\pm$ SEM for each miRNA. Results are not significant.

2.3.5.3. Changes in miRNA Levels between the MetS and obese Cohorts

When comparing the obese and MetS cohorts at visit 7, the diets resulted in an overall decrease in the levels of the miRNAs in the MetS cohort compared to the obese cohort (Figure 2.16). Specifically, 21 of the 25 miRNAs analysed were decreased (-12.38 ± 0.82 -fold to -2.74 ± 0.77 -fold). 17 of these miRNA were significantly decreased (p range=0.002-0.05) *e.g.* miR-15a-5p (-9.44 ± 0.74 -fold, $p=0.01$) was substantially and significantly decreased.

Similar to visit 7, miR-758-3p was undetected in the MetS cohort and detected in the obese cohort ($C_T=33 \pm 1.1$) following the 12-week diets.

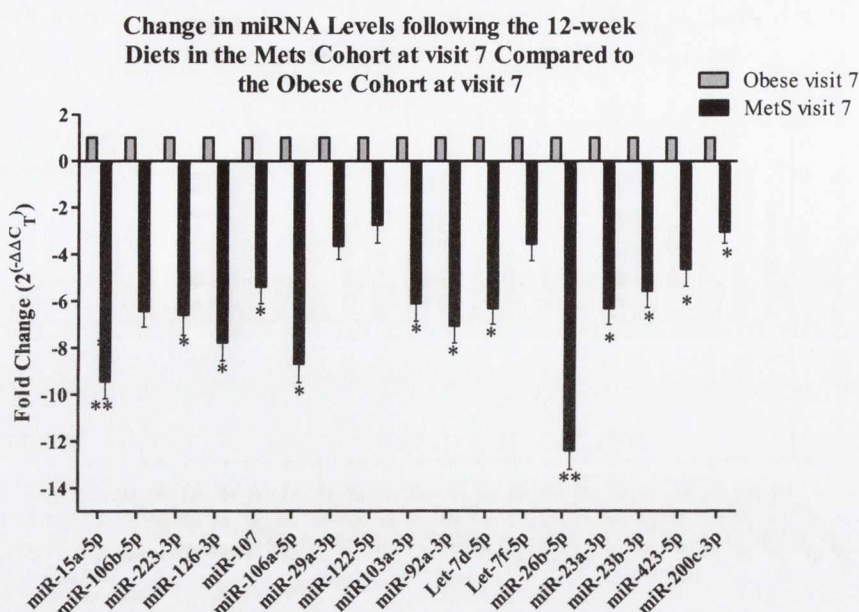


Figure 2.16: Change in miRNA levels in the MetS cohort compared to the Obese cohort–visit 7

Fold change in circulating miRNA levels in the MetS cohort at visit 7 compared to the obese cohort at visit 7. * $p < 0.05$, ** $p < 0.01$. Data represent mean $\pm$ SEM for each miRNA. Results are not significant.

2.3.5.4. Analysis of miRNA Levels for Each Individual 12-week Diet

2.3.5.4.1. Comparison of the Mets Cohort and Obese Cohort

Considering the diets individually for the MetS cohort at visit 7 compared to the obese cohort at visit 7, the high MC-SFA-whey diet (White A) resulted in an overall decrease in the levels of 22 miRNAs (-68.1 ± 2.24 -fold to -2.29 ± 0.88 -fold) (Appendix I, Section D, Figure D.1 A). miR-758-3p was detected in the plasma from the obese cohort ($C_T = 34 \pm 1.5$) but undetected in the MetS cohort. The high MC-SFA-casein diet (White B) resulted in a decrease in the levels of 20 miRNAs (-21.54 ± 3.04 -fold to -2.65 ± 2.39 -fold) (Appendix I, Section D, Figure D.1 B). miR-758-3p was again detected in the plasma from the obese cohort ($C_T = 34 \pm 1.6$) but undetected in the MetS cohort. The low MC-SFA-whey diet (Green A) resulted in a decrease in the levels of 5 miRNAs (-8.01 ± 1.52 -fold to -2.08 ± 0.82 -fold) and an increase in the levels of let-7f-5p (2.01 ± 0.79 -fold) (Appendix I, Section D, Figure D.1 C). miR-758-3p was detected in the plasma of the MetS cohort ($C_T = 33 \pm 0.6$) and obese cohort ($C_T = 33 \pm 1.4$) for this diet group. The low MC-SFA-casein diet (Green B) resulted in a decrease in the levels of 20

miRNAs (-112.92 ± 1.11 -fold to -3.46 ± 0.98 -fold) (Appendix I, Section D, Figure D.1 D). miR-758-3p was undetected in the plasma of the MetS cohort and detected in the plasma of the obese cohort ($C_T = 29.2 \pm 2.7$).

2.3.5.4.2. Comparison Within the MetS Cohort– Visit 7 to Visit 2

Considering the diets individually for the MetS cohort at visit 7 compared to visit 2. The high MC-SFA-whey diet (White A) resulted in an overall decrease in the levels of 22 miRNAs (-20.26 ± 0 -fold to -2.67 ± 0.88 -fold) (Appendix I, Section D, Figure D.2 A). miR-758-3p was undetected at both visit 2 and visit 7. The high MC-SFA-casein diet (White B) resulted in an overall decrease in the levels of 21 miRNAs (-28.50 ± 4.12 -fold to -2.39 ± 0.02 -fold) (Appendix I, Section D, Figure D.2 B). miR-758-3p was undetected at both visit 2 and visit 7. The low MC-SFA-whey diet (Green A) resulted in a decrease in the levels of 4 miRNAs (-13.83 ± 1.5 -fold to -2.08 ± 0.28 -fold) and an increase in the levels of 7 miRNAs (2.01 ± 0.79 -fold to 7.44 ± 0.65 -fold) (Appendix I, Section D, Figure D.2 C). miR-758-3p was detected at visit 7 ($C_T = 33.5 \pm 0.6$) but undetected at visit 2. Comparing visit 7 to visit 2 indicated miR-758-3p was increased substantially. The low MC-SFA-casein diet (Green B) resulted in an increase in the levels of 14 miRNAs (2.05 ± 1.3 -fold to 22.30 ± 1.99 -fold) (Appendix I, Section D, Figure D.2 D). miR-758-3p was undetected at visit 2 and visit 7.

2.3.5.4.3. Comparison Within the Obese Cohort– Visit 7 to Visit 2

Considering the diets individually for the obese cohort at visit 7 compared to visit 2, the high MC-SFA-whey diet (White A) resulted in an overall increase in the levels of 19 miRNAs (2.29 ± 0.78 -fold to 7.62 ± 0.70 -fold) (Appendix I, Section D, Figure D.3 A). miR-758-3p was detected at visit 2 ($C_T = 34 \pm 0.9$) but undetected at visit 7 ($C_T = 34 \pm 1.5$). The high MC-SFA-casein diet (White B) resulted in an overall decrease in the levels of 8 miRNAs (-3.66 ± 1.72 -fold to -2.16 ± 1.70 -fold) by more than 2-fold (Appendix I, Section D, Figure D.3 B). miR-758-3p was detected at visit 2 ($C_T = 33 \pm 1.4$) and visit 7 ($C_T = 34 \pm 1.6$). The low MC-SFA-whey diet (Green A) resulted in an overall increase in the levels of 21 miRNAs (3.29 ± 1.42 -fold to 108.35 ± 1.29 -fold) by more than 2-fold (Appendix I, Section D, Figure D.3 C). miR-758-3p was detected at visit 2 ($C_T = 33 \pm 1$) and at visit 7

($C_T=33\pm1.4$). Comparing the levels of miR-758-3p 3p in the plasma of the obese cohort at visit 7 to visit 2 in the obese cohort found miR-758-3p (3.30 ± 1.42 -fold, $p=0.32$) to be increased at visit 7. However, this increase was not significant. The low MC-SFA-casein diet (Green B) resulted in an overall decrease in the levels of 9 miRNAs (-3.23 ± 1.62 -fold to -2.06 ± 1.40 -fold) by more than 2-fold (Appendix I, Section D, Figure D.3 D). miR-758-3p was detected at visit 2 ($C_T=32\pm0.7$) and at visit 7 ($C_T=29\pm2.7$). Comparing the levels of miR-758-3p 3p in the plasma of the obese cohort at visit 7 to visit 2 in the obese cohort found miR-758-3p (13.25 ± 2.74 -fold, $p=0.29$) to be increased at visit 7.

2.3.5.5. Change in miRNA Levels Following High in Fat Meal

Following the high fat meal alterations in the levels of the 25 miRNAs analysed can be seen. Firstly, miR-758-3p is not detected in the MetS cohort or the obese cohort for any of the 4 diet groups pre- or post- the 12-week diets. This may be the result of cholesterol loading, as the diet is high in fat and miR-758-3p is responsive to cholesterol loading in mouse peritoneal macrophages and human THP-1 macrophages [164] (Appendix I, Section D, Figure D.1, Table D.2, Table D.3). The alterations of all miRNAs analysed, which are associated with the high fat meal can be found in the Appendix. Specifically, alterations of the miRNAs 30mins following the high fat meal pre- and post- 12-week diet (Appendix I, Section D, Figure D.1), alterations of the miRNAs 120mins following the high fat meal pre- and post- 12-week diet (Appendix I, Section D, Figure D.2) and alterations of the miRNAs 240mins following the high fat meal pre- and post- 12-week diet (Appendix I, Section D, Figure D).

2.3.6. *In vitro* analysis of miR-758-3p and its potential targets ABCA1 and ABCG2.

2.3.6.1. Use of miR-Walk for the Prediction of ABCA1 and ABCG2 as targets of miR-758-3p

miR-758-3p was identified as a miRNA with potential as a biomarker for MetS from a discovery phase (custom profiling) and a validation phase (RT-qPCR) as it was undetected in the MetS cohort but detected in the obese cohort and therefore has potential to distinguish MetS from obesity. Using publically-

available prediction software from the University of Heidelberg, miR-walk, miR-758-3p predicted targets were identified. Following a literature search for all predicted targets the cholesterol efflux pump ABCA1/CERP was chosen for further study as cholesterol- both HDL and LDL- are associated with both obesity and MetS (Figure 2.17). Another target, the breast cancer resistance drug efflux pump ABCG2/BCRP was also chosen for further study because obesity and MetS have both been epidemiologically linked to cancer and in particular TNBC (see Sections 1.1.3.3 and 1.3). Therefore, TNBC cell line variants –Hs578T and Hs578Ts(i)₈- were chosen for *in vitro* analysis of miR-758-3p.

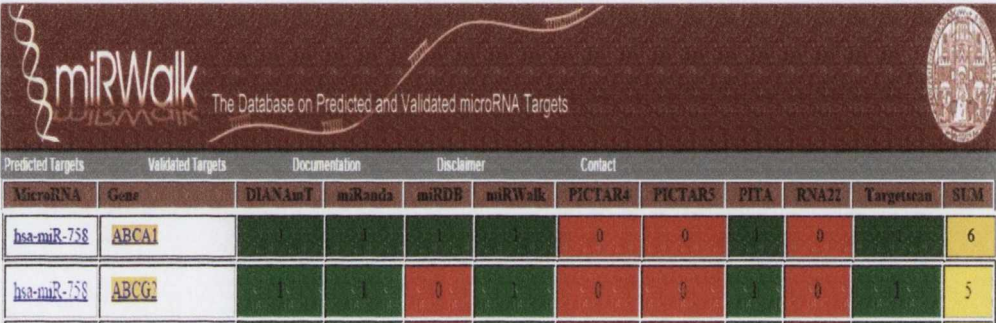


Figure 2.17: Identification of ABCA1/CERP and ABCG2/BCRP as potential targets of miR-758-3p using miR-walk.

2.3.6.2. Analysis of miR-758-3p in Breast Cancer Cell Lines

Numerous breast cancer cell lines were available, including the TNBC cell line Hs578T, its isogenic sub clone Hs578Ts(i)₈ and MDA-MB-231 cells and HER2-overexpressing breast cancer cell lines, HCC1954 and EFM192A cells. Pellets from all cell lines were collected in triplicate and RNA isolated using TriReagent™ as described in Sections 2.2.5.2.2 and 2.2.5.2.3. RT-qPCR was performed using RNA isolated from these cells for miR-758-3p, endogenous control RNAs miR-150-5p, miR-486-5p, U6 snRNA and UniSp6 spike in and again run in triplicate for each cell line and for each miRNA analysed. Analysis of the endogenous controls showed U6 snRNA as the most consistently expressed RNA in these cell lines. Therefore, the raw data was normalised to U6 snRNA (Figure 2.18).

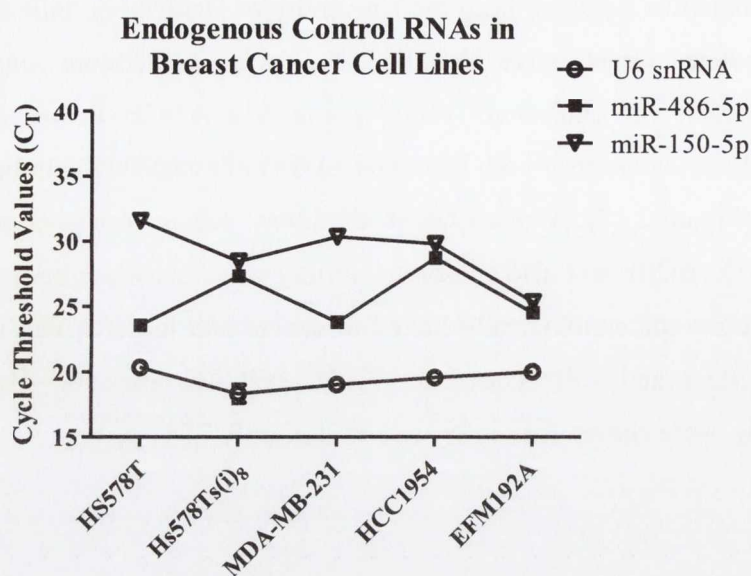


Figure 2.18: Expression of endogenous control RNAs in breast cancer cell lines

Endogenous control RNAs U6 snRNA, miR-486-5p and miR-150-5p were screened in TNBC cell lines Hs578T, Hs578Ts(i)₈, MDA-MB-231 and HER2+ breast cancer cell lines HCC1954 and EFM192A. Data represent mean $\pm$ SEM for 3 technical repeats and 3 biological repeats (n=9).

Analysis of the RT-qPCR C_T values of miR-758-3p in the Hs578T and Hs578Ts(i)₈ cell line variants and in MDA-MB-231, HCC1954 and EFM192A cell lines revealed miR-758-3p is highly expressed in Hs578T cells (mean C_T=25.4) and HCC1954 cells (mean C_T=26.8) while the HS578Ts(i)₈ cells (mean C_T=28.8), MDA-MB-231 cells (mean C_T=28.2) and EFM192A cells (mean C_T=29.7) have a lower expression (Figure 2.19 A). The fold difference in the levels of miR-758-3p in the isogenic subclones Hs578T and Hs578Ts(i)₈ (-11.99 $\pm$ 0.15-fold) was substantial and significant (p=0.001) (Figure 2.19 B).

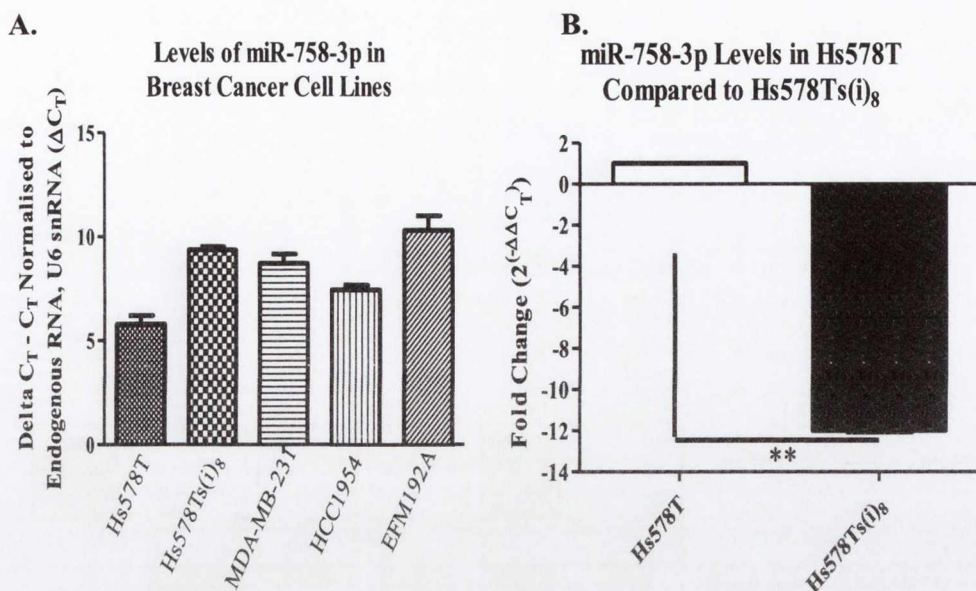


Figure 2.19: Expression of miR-758-3p in breast cancer cell lines selected for analysis
miR-758-3p levels in breast cancer cell lines as determined by RT-qPCR. **A.** Normalised C_T values for each cell line, **B.** Fold change of miR-758-3p in Hs578Ts(i)₈ compared to Hs578T. Data represent mean ± SEM for 3 biological repeats, where ** p<0.001.

As miR-walk predicted the targets of miR-758-3p as ABCA1/CERP and ABCG2/BCRP, the next step involved immunoblotting of the protein isolated from each cell pellet.

We hypothesised that both ABCA1 and ABCG2 would be increased in the Hs578Ts(i)₈ cells compared to the Hs578T cells as analysis of miR-758-3p in these cell line variants showed Hs578T cells to have higher levels than the Hs578Ts(i)₈ cells. This hypothesis held true for both proteins (Figure 2.20 A). MDA-MB-231, HCC1954 and EFM192A cells showed no detectable ABCA1, however ABCG2 was detected in these 3 cell lines (Figure 2.20 A). Quantitative analysis, by densitometry, showed ABCA1 and ABCG2 to be significantly increased in the Hs578Ts(i)₈ cells compared to the Hs578T cells by 4.63±0.7-fold (p=6x10⁻⁵) and 2.93±0.84-fold (p=0.006), respectively (Figure 2.20 B). β-actin was used as a loading control for all immunoblots.

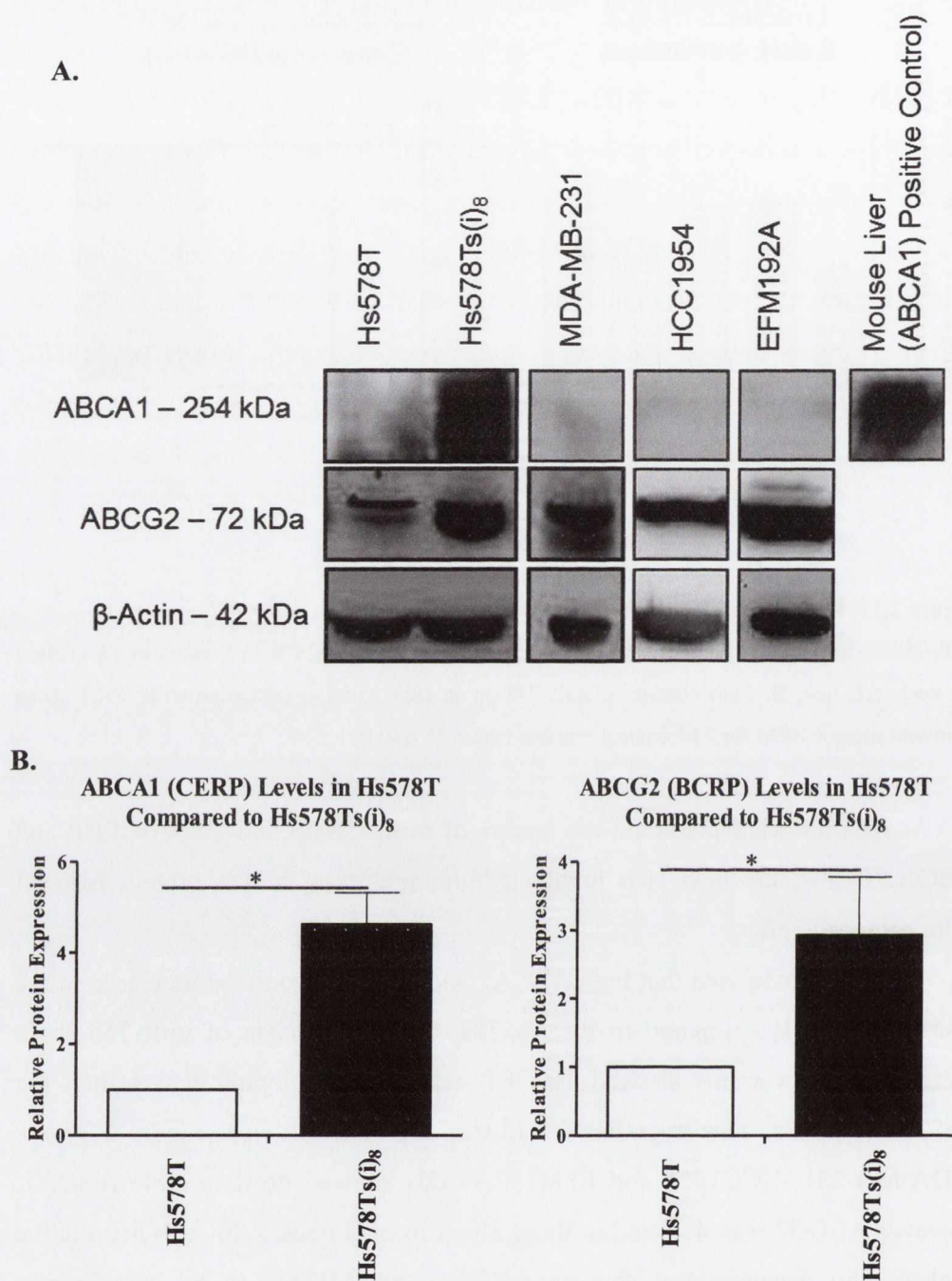
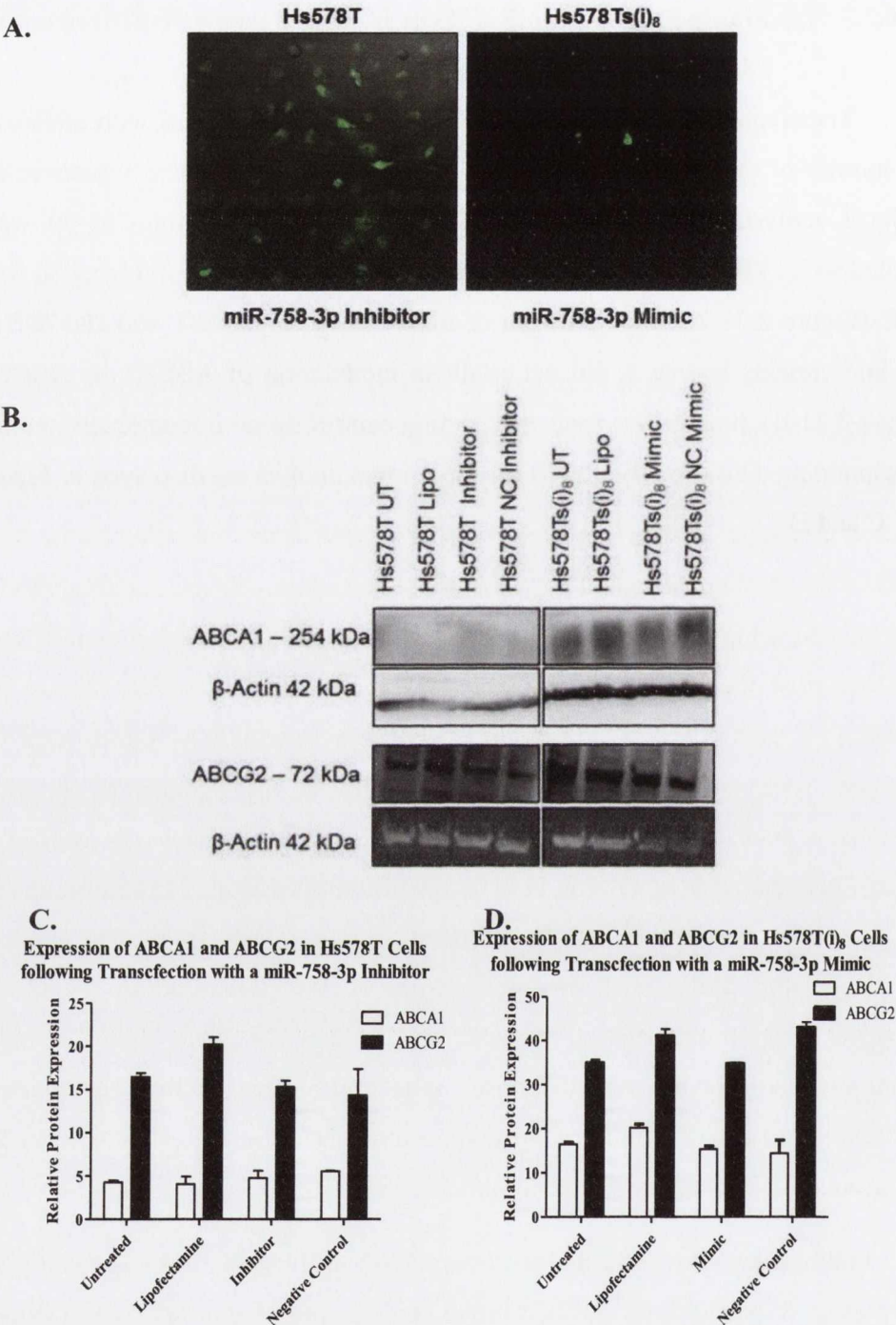


Figure 2.20: Analysis of ABCA1 and ABCG2 levels in breast cancer cell lines

A. Immunoblotting of ABCA1 and ABCG2 in HS578T, HS578Ts(i)₈, MDA-MB-231, HCC1954, EFM192A cell lines. **B.** Densitometry for the amount of each protein expressed in each cell line in triplicate and fold change for the Hs578T and Hs578Ts(i)₈ cell line variants. Data represent mean $\pm$ SEM for 3 biological repeats, where * $p < 0.05$.

2.3.6.3. Manipulation of miR-758-3p in Hs578T and Hs578Ts(i)₈ Cell Line Variants

Transfection of Hs578T and Hs578Ts(i)₈ cell line variants with inhibitors and mimics of miR-758-3p respectively were successful. This was visualised by confocal analysis for FITC as the inhibitor and mimic of miR-758-3p were labelled with FITC. Negative control inhibitors and mimics were not labelled with FITC (Figure 2.21 A). Manipulation of miR-758-3p in Hs578T and Hs578T(i)₈ cell line variants however, did not result in modulation of ABCA1 or ABCG2 (Figure 2.21 B). β -actin was used as a loading control for all immunoblots and the corresponding densitometry graphs for these immunoblots are displayed in Figure 2.21 C and D.



2.4. Discussion

Interest in extracellular diagnostic biomarkers for diseases is growing, as it offers potential for minimally-invasive procedures, such as procurement of blood specimens in the clinic (see Section 1.2). Previously our group reported on the presence of RNAs freely-circulating in serum/plasma from cancer patients [167] and not only did our group detect the presence of RNA but also the presence of miRNAs [168-170]. Biomarker use in both the clinic and as end-points for clinical trials has become commonplace. Biomarkers are not a new phenomenon, they have been used in clinical assessment for decades, in the form of basic biomarkers, *i.e.* pulse rate and blood pressure, but it is the use of more complex biomarkers, such as blood-based biomarkers, that is now emerging at the forefront of diagnostic research [171]. Biomarkers take many forms, such as proteins and nucleic acids (see Section 1.2). A small class of RNA, known as miRNAs, are becoming the focus of much research for biomarker discovery; by us and others [94, 168, 172-174]. miRNAs offer huge potential as biomarkers for diseases, such as MetS as they possess many of the features of an ideal biomarker, such as, they are stable in bio-fluids, their sequence is conserved among species, their expression may be specific to tissues and disease stage and their presence is easily assessed [175] (see Section 1.2.4). Traditional methods for diagnosing MetS, such as hypertension or WC, are used in the clinic. However, due to the lack of a uniform definition for MetS there are false positives and false negative diagnosis; it is for this reason that an objective (unbiased), quantifiable method for diagnosing MetS must be established in the clinic and miRNAs hold promise for this purpose.

In this study, we performed custom miRNA profiling on RNA isolated from the plasma of participants enrolled in the DairyHealth human intervention study in Aarhus University Hospital, Denmark. Participants were classified into two groups, MetS (n=26) and obese (n=26). These participants entered the clinic for blood procurement twice, on visit 2 and visit 7. At visit 2, participants were fasting and a blood specimen was procured (T_0), a meal test high in fat was then consumed and a blood specimen was procured 30mins (T_{30}), 120mins (T_{120}) and 240mins (T_{240}) following this meal test. Following blood procurement at visit 2, participants were randomised into one of four dietary groups: high MC-SFA-whey, high MC-SFA-casein, low MC-SFA-whey and low MC-SFA-casein. 12-

weeks post dietary intervention (visit 7), participants re-entered the clinic fasting and blood specimens were procured again. The primary purpose of the meal test was to determine the post-prandial response of this meal on these participants pre- and post- 12-week dietary intervention, in terms of oral glucose tolerance testing (OGTT), insulin levels, triglyceride levels and free fatty acid levels [163]. However, from our point of view the purpose of the meal test was to determine whether dietary intervention had an effect on circulating miRNA levels in the plasma of these participants and whether these miRNAs could be potential biomarkers for response to dietary intervention.

Prior to miRNA profiling of the RNA isolated from the plasma of the MetS and obese participants, successful RNA isolation was determined by assessing the quantity and quality of the isolated RNA. Also, the miRNA content of the total RNA isolated was determined using the Agilent small RNA kit. To assess the purity of the RNA isolated the 260/280 ratio was determined. For cellular samples the 260/280 ratio for a pure RNA sample is expected at approximately 2.0. However, for our RNA isolated from plasma specimens the average 260/280 for the obese and MetS specimens was 1.5 ± 0.03 and 1.5 ± 0.02 , respectively. These values although lower than the expected value of ~ 2.0 , are within the range for plasma specimens [176] as extracellular RNA does not contain ribosomal RNA (rRNA) as can be seen in the Agilent electropherograms (Figure 2.7). Preliminary results in our group have shown 260/280 ratios of 1.4-1.6 for RNA isolated from conditioned medium, the *in vitro* mimic of circulating RNA [177]. The quality of the RNA as determined by the 260/280 is within the acceptable range for plasma specimens. For the obese and MetS cohorts the yield of RNA was $4.9 \pm 0.3 \mu\text{g/ml}$ plasma and $4.6 \pm 0.3 \mu\text{g/ml}$ plasma, respectively, before the 12-week diets. For the obese and MetS cohorts, the yield of RNA was $5.1 \pm 0.3 \mu\text{g/ml}$ of plasma and $4.2 \pm 0.3 \mu\text{g/ml}$ of plasma, respectively, following the 12-week diets. The fold change for the yield of RNA from the plasma specimens pre- and post-12-week diets was not significantly different in the obese cohort (1.04 ± 0.03 -fold, $p=0.68$) or the MetS cohort (-1.12 ± 0.03 -fold, $p=0.33$).

qPCR provides the researcher with a numerical value, known as the cycle threshold (C_T). This number represents the number of cycles the molecule underwent in the qPCR analysis before being detected [178]. Therefore, the higher the C_T value, the lower the amount of the miRNA of interest and vice

versa. To normalise the miRNA data in order to calculate the relative fold changes between cohorts of specimens (Obese vs MetS), an endogenous control RNA is commonly used. However, in plasma specimens, RNAs that are suitable for this role are difficult to identify from one cohort to another and also within cohorts. Therefore, we performed a pre-screen of 192 miRNAs to determine the most consistently detected miRNAs in our cohort of specimens. From this analysis miR-128, miR-150-5p, miR-320a and miR-486-5p were selected as they were the most consistently detected. U6 snRNA is a commonly used endogenous control RNA for normalisation of cellular data, therefore we analysed it in our specimens too. U6 snRNA was also consistently detected in our specimens and so was chosen as an endogenous control RNA for profiling.

These five endogenous control RNAs were analysed along with twenty-five selected miRNAs. These miRNAs were selected from the pre-screen and from literature relating a miRNA to MetS (see Table 2.9). Analysis of the profiling data determined miR-150-5p and miR-486-5p as the endogenous control RNAs for this study, as they had the lowest variability and their detection was not significantly different in our specimens. Initial assessment of the miRNA levels in our specimens focused on their overall content. To analyse this, C_T values ≤ 35 were considered 'present', while all C_T values > 35 were regarded as 'not present'. This analysis showed that before any dietary intervention 18 of the miRNAs analysed were present in plasma from both the obese and MetS participants, 2 were present in the obese cohort only and 1 was detected in the MetS cohort only, while 4 miRNAs were undetected, *i.e.* miR-33a-3p, miR-33b-3p, miR-33b-5p and miR-200a-3p. Interestingly, these miRNAs were also undetected at visit 7. This was an interesting observation as miR-33a and miR-33b have both been reported as miRNAs associated with components of MetS and obesity [92, 127, 128, 134, 179]. Specifically, Davalos *et al* [134] showed miR-33 to regulate FA metabolism and insulin signalling; miR-33 over-expression in hepatic cells reduced FA synthesis and insulin signalling and inhibition of miR-33 resulted in increased FA synthesis and insulin signalling. They identified miR-33 binding sites in a number of FA metabolism and insulin signalling genes, such as IRS2. Rayner *et al* [179] found miR-33 regulates cholesterol efflux through a genome-wide screen of miRNAs involved in cholesterol content in mouse macrophages. miR-33 levels were effected by

cholesterol load or inhibition and miR-33 levels affected ABCA1 levels inversely. However, Gao *et al* [128] also found both of these miRNAs to be undetected in their cohort of hyperlipidemic individuals with and without CAD.

Prior to selection of miRNAs as potential biomarkers for MetS and validation analysis, the fold difference in miRNA levels in the obese cohort compared to the MetS cohort was analysed using the delta delta C_T method ($\Delta\Delta C_T$) [180]. This analysis identified three miRNAs (miR-15a-5p, miR-106b-5p, miR-200a-3p) that were more than 2-fold decreased but not significantly, miR-204-5p was more than 2-fold increased although not significantly and miR-758-3p which was detected in the plasma from the obese participants but undetected in the plasma from the MetS participants. These five miRNAs were, therefore, selected for validation in a separate sub-cohort of participants (n=14).

Attempts at validation of these miRNA findings was performed by individual RT-qPCR for each miRNA. miR-150-5p and miR-486-5p were again used as endogenous control RNAs and UniSP6 was used as a positive control and for RT efficiency. miR-758-3p was absent in the MetS cohort but detected in the obese cohort and was therefore concluded to be a potential biomarker for MetS independent of obesity. All other miRNAs were less than 2-fold altered and were not significantly changed and, therefore, did not validate as biomarkers for MetS.

Of particular interest, the result that miR-758-3p was undetected in the MetS cohort (profiling and validation cohorts) but detected in the obese cohort is a substantial finding as this would indicate the loss of miR-758-3p expression in progression from Obesity to full-blown MetS. The absence of miR-758-3p greatly improves the potential or relevance of this miRNA as a biomarker for MetS, as its absence could be tested in the clinic with greater efficacy than comparing the down-regulation of a biomarker to a set of standard controls.

The next step in the identification of a biomarker for MetS was the identification of its targets. Using a publically-available database, miR-Walk, ABCA1/CERP and ABCG2/BCRP were selected from the targets identified for further study. Numerous studies have reported on a link between miR-758-3p and metabolism, particularly in relation to cholesterol homeostasis. For example, miR-758-3p is involved in the post-transcriptional regulation of lipid metabolism genes, while one of its predicted targets -ABCA1- is involved in cellular

cholesterol efflux [127, 132]; this has been shown in human and mouse macrophages. It has also been shown in mice fed a high fat diet that miR-758-3p levels are decreased in macrophages, increasing the levels of ABCA1 and the efflux of cholesterol to ApoA1 (nascent HDL) [164]. Studies have shown that miR-758-3p targets the 3' UTR region of ABCA1 as does miR-33, suggesting miR-758-3p and miR-33 cooperate to regulate ABCA1. miR-106b (See Table 2.9) has also been shown to be involved in lipid metabolism and may function alongside miR-758-3p and miR-33. However, miR-33 has 3 binding sites in the 3' UTR of ABCA1 suggesting miR-33 regulates ABCA1 with higher efficacy than miR-758-3p [179]. Remarkably, miR-33 was not detected in our specimens (UniSP6 indicated assay efficiency), but this could be due to the effect obesity and MetS are having on miRNA levels. We therefore selected ABCA1 and also ABCG2/BCRP for subsequent study, as ABCA1 being a cholesterol efflux protein has a link to metabolic syndrome and also obesity. The breast cancer resistance protein ABCG2/BCRP was selected too, as MetS is associated with an increased risk of breast cancer (see Sections 1.1.3.3 and 1.3). Also, for this reason, we selected two TNBC cell lines (Hs578T and Hs578Ts(i)₈) to further investigate the role of miR-758-3p in regulating ABCA1 and ABCG2. Hs578Ts(i)₈ is an aggressive variant of the Hs578T cell line, derived following multiple extracellular matrix invasion steps. Remarkably, the TNBC cell line Hs578T had no detectable ABCA1, while its more aggressive subclone Hs578Ts(i)₈ had 4.63±0.7-fold ($p=6\times10^{-5}$) more ABCA1. Also, Hs578Ts(i)₈ cells expressed 32.93±0.84-fold ($p=0.006$) more ABCG2 than Hs578T cells. These results suggested that alterations in drug efflux pumps and lipid metabolism are implicated in the aggressive phenotype of TNBC.

An interesting step in the analysis of this cohort would have been to determine the levels of ABCA1 in the macrophages of these participants. As fresh blood specimens were not available, ABCA1 levels in the macrophages could not be determined. Furthermore, the involvement of participants in the study had ended and blood specimens could no longer be procured.

Following the 12-week dietary intervention, four of the 25 miRNAs analysed were again undetected (miR-33b-3p and miR-33b-5p, miR-200a-3p and miR-204-5p), miR-758-3p was only detected in the obese cohort, as was seen at visit 2, baseline. Interestingly, it was found that the 12-week diets were inducing

an increase in the levels of miRNAs in the obese cohort and a decrease in the levels of miRNAs in the MetS cohort. Comparison of the miRNA levels in the MetS cohort to the obese cohort following the 12-week diets showed 16 of the 21 miRNAs detected were significantly decreased. Again miR-758-3p was undetected in the MetS cohort following the 12-week diets and detected in the obese cohort. Analysis of the four diets individually indicated miR-758-3p was undetected in the MetS cohort for 3 diet groups *i.e.* high MC-SFA-whey, high MC-SFA-casein and low MC-SFA-casein. Interestingly, miR-758-3p was detected in the low MC-SFA-whey diet group. However, there were only 5 participants in this diet group. miR-758-3p was detected in all diet groups for the obese cohort.

Considering the results of WP2 (Section 1.4), they found the supplementation of whey, irrespective of MC-SFA (low/high) had the greatest effect on the participants in this study. The chylomicron (apoB48) post-prandial response was significantly ($p=0.025$) decreased following whey supplementation compared to the response following casein supplementation. It was concluded that further studies are required to elucidate any potential beneficial effects of whey-enriched dairy products on CVD risk [163]. Taking WP2 results into consideration when comparing the levels of the miRNAs in the MetS cohort pre- and post-12-week diets; miR-758-3p was substantially increased, but not significantly in the low MC-SFA-whey cohort. This may indicate the low-MC-SFA-whey diet has an impact on the levels of this miRNA, which may potentially indicate response to a dietary intervention low in MC-SFA with whey supplementation. Therefore, if miR-758-3p loss is associated with progression of obesity to MetS the increase of miR-758-3p following the low MC-SFA-whey dietary intervention may show there is potential for this treatment in individuals with MetS. Furthermore, miR-758-3p could be used for monitoring of individuals with MetS. From our point of view, the low MC-SFA and whey diet had the greatest effect on the levels of miR-758-3p in the MetS and obese cohorts following the 12-week diets. We can also conclude that whey appears to have potential as a dietary intervention in MetS and obesity, but agree with WP2 that further studies are required in a larger cohort with appropriate controls.

Our preliminary analysis of miR-758-3p levels in the plasma of obese participants and participants with MetS suggests a role for miR-758-3p as a

biomarker for the diagnosis of MetS, and may be useful to reduce the rate of false negatives and positives and ultimately help reduce the risk of T2DM, CVD and cancer. The potential of miR-758-3p as a biomarker for MetS and biomarker for response to dietary intervention with whey proteins now warrants further study in a larger cohort with appropriate healthy control specimens (appropriate healthy controls would be defined as healthy individuals of normal weight, with blood pressure and glucose within the normal ranges and normal triglyceride, HDL and LDL levels. Specimens for these control donors would be collected as part of the same study as the test specimens).

2.5. Conclusions

In conclusion, there is an urgent requirement for better diagnostic biomarkers for MetS. Here, we have identified miR-758-3p as a potential biomarker for MetS in a Danish population of males and females ranging in age from 20-80 years. Not only have we identified this as a potential diagnostic biomarker but also as a potential biomarker for response to dietary intervention, as whey-based dietary supplementation in the obese and MetS participants indicated a positive response in these participants following the 12-week intervention with whey. Targeting miR-758-3p may not be possible in the case of MetS as many miRNAs are involved in the regulation of ABCA1 and miR-758-3p is involved in regulating numerous other proteins as well but, for diagnosing MetS miR-758-3p may be beneficial.

**Chapter Three: Extracellular Vesicles and their
miRNA Content as Biomarkers in Individuals with
Clinical Obesity and Metabolic Syndrome**

3. Abstract

Introduction: Extracellular vesicles (EVs) are nano- and micron-sized vesicles that are emerging as hugely important biological factors in human development and disease. This is in view of their detection in a wide range of biological fluids such as serum, plasma, urine and saliva. Recently their presence has been extensively researched and found to have potential in a broad range of biological and diagnostic functions. As detailed in Chapters 1 and 2, more biomarkers for MetS are required. Having identified miR-758-3p as a plasma-based potential biomarker for MetS, the next step in the search was to assess EVs and their miRNA cargo as potential biomarkers for MetS.

Methods: EVs were isolated from 250µl of plasma derived from healthy donor control (n=14), obese (n=12) and MetS (n=13) participants by filtration and ultracentrifugation. Characterisation was performed to confirm that EVs had been purified. Characterisation included immunoblotting for CD81, PDC6I/Alix and Grp-94, transmission electron microscopy and particle tracking analysis. To assess the potential of circulating EV quantities as a biomarker for MetS, the quantity of isolated EVs was measured by determining total protein content as a surrogate, using the Bio-Rad BCA assay. EV-RNA was subsequently isolated using TriReagent™. Similarly, to assess the potential of EV-RNA as a biomarker for MetS, the RNA yield was measured using the NanoDrop 2000 spectrophotometer. The potential of these parameters as biomarkers for MetS was assessed by receiver operator characteristics curve (ROC) analysis. Global EV-miRNA profiling for 372 miRNAs (including 22 of the 25 miRNAs present on the custom miRNA profiling panels in Chapter 2), using Exiqon human miRNA profiling arrays, was performed to assess EV-miRNAs as potential biomarkers for MetS and their relevance compared to miRNAs isolated directly from plasma (termed plasma-miRNAs in Chapter 2). Validation of the miRNAs identified in the global profiling stage was also performed by individual RT-qPCR as described in Section 2.2.4.4.

Results: Successful isolation of EVs was confirmed via immunoblotting for CD81 and PDC6I/Alix, which were both detected in the control, obese and MetS specimens, while Grp-94, a negative control for EVs, was not detected in the EV populations isolated. Transmission electron microscopy and particle tracking

analysis confirmed vesicles of 30-130nm were isolated, indicating a population of exosomes/small EVs. Interestingly, the EV quantity as estimated by the Bio-Rad BCA assay was significantly increased in the MetS ($204.2 \pm 23 \mu\text{g/ml}$ plasma) cohort compared to the control ($171.8 \pm 25.9 \mu\text{g/ml}$ plasma) ($p=0.049$) and obese ($136.2 \pm 18.7 \mu\text{g/ml}$ plasma) ($p=0.004$) cohorts. However, the difference between the control cohort and obese cohort was not significant ($p=0.3$). ROC curve analysis showed EV quantities were sensitive to discriminate MetS from controls ($p=0.049$) and obesity ($p=0.003$). Analysis of EV-RNA yield showed EV-RNA was significantly ($p=0.049$) increased in the MetS cohort ($3.25 \pm 0.24 \mu\text{g/ml}$ plasma) compared to controls ($2.78 \pm 0.20 \mu\text{g/ml}$ plasma) but not significantly increased in the obese cohort ($3.54 \pm 0.31 \mu\text{g/ml}$ plasma) ($p=0.05$) compared to controls or in the obese cohort compared to the MetS cohort ($p=0.7$). ROC analysis indicated that the overall EV-RNA quantity increased in the MetS cohort compared to controls was not sensitive as a biomarker for MetS ($p=0.08$). Global miRNA profiling of 372 miRNAs for $n=7$ controls, $n=8$ obese and $n=8$ MetS participants identified 14 miRNAs, as potential biomarkers for MetS (miR-9-3p, miR-30e-5p, miR-31-5p, miR-100-5p, miR-133a, miR-133b, miR-134, miR-202-3p, miR-205-5p, miR-302c-5p, miR-370, miR-373-5p, miR-491-5p, miR-596) compared to healthy controls and obesity, when taking C_T values ≤ 35 as detected and >35 as undetected and a significance value of $p < 0.05$. Validation of three (miR-100-5p, miR-134 and miR-370) of these 14 miRNAs in $n=4$ controls, $n=4$ obese and $n=5$ MetS participants indicated miR-100-5p as a potential EV-miRNA biomarker for MetS. Individual RT-qPCR for miR-758-3p was performed for all EV specimens and showed miR-758-3p was not detected in the EVs from the healthy control, obese and MetS participants. Interestingly, individual RT-qPCR of miR-100-5p in the plasma (as not on custom plate used in Chapter 2) of obese and MetS participants, found miR-100-5p (3.4 ± 0.4 -fold, $p=0.02$) to be significantly increased in the plasma of the MetS cohort compared to the plasma of the obese cohort. Remarkably, predicted targets of miR-100-5p include ABCA1/CERP (See Section 2.3.6.1).

Conclusion: We have shown for the first time, that EVs quantities could be a useful minimally-invasive diagnostic tool for MetS, which could also discriminate MetS from obesity and healthy controls. It also suggests miR-100-5p as a potential EV-miRNA biomarker for MetS compared to healthy controls. Finally,

comparison of the EV-miRNA content and plasma-miRNA content showed both sources of miRNAs are quite different for biomarker discovery in MetS (*i.e.* miR-758-3p was detected in the plasma of the obese cohort but undetected in the EVs of the obese cohort and miRNA C_T values were lower in the plasma than in the EVs). Therefore, this study and the study described in Chapter 2 suggest it may not simply be plasma- or EV- miRNAs, but a combination of both that are most beneficial for MetS biomarker discovery and diagnosis. Further studies in larger cohorts, including appropriate healthy controls, are now warranted.

3.1. Introduction

Extracellular vesicles (EVs) are nano and micron-sized vesicles released by cells into the extracellular space [181] and are found freely circulating in most human biofluids, such as blood plasma, urine and saliva, which highlights their potential relevance *in vivo* [181]. EV is an umbrella term for exosomes (30-130nm), ectosomes/microvesicles (130nm-1 μ M), microparticles (100-1000nm), among other EV subsets (See Section 1.2.1). Over the last decade, EVs have grown in importance as they have been found to transfer their cargo via regulated processes to recipient cells, thus they are mediators of cell-to-cell communication, regulating a diverse range of biological processes; they are not just cellular debris [181, 182]. It has been shown that EVs are released by cells and some are physically internalised by recipient cells, in which they are apparently functional [183]. Cell-to-cell communication is pivotal in all multicellular organisms [184] in order to maintain tissue homeostasis and normal cellular functions [183] through the secretion of soluble factors or direct interaction. Added to this, EVs also seem to have an important role to play with both neighbouring cells and distant cells. EVs perform their cell-to-cell communication through the transfer of proteins, lipids and nucleic acids (See Section 1.2.1).

Not only do EVs have physiological functions, their pathophysiological roles in diseases such as cancer, infectious diseases, neurodegenerative disorders and metabolic diseases are emerging [181, 184]. EVs from adipose tissue have been shown to contribute to obesity- and MetS-related IR [181]. Specifically Kranendonk *et al* [185] showed EVs isolated from adipose tissue by ultracentrifugation from obese and MetS individuals (as determined by NCEP:ATPIII definition) interfere with insulin signalling in hepatocytes (HepG2 cells) with which they were co-cultured for 24 hours. Adipocyte fatty acid binding protein (FABP4), a protein involved in the pathogenesis of T2DM and cancer, has been detected in EVs isolated from adipocytes and shown to be partially secreted through an exosome-like vesicle [186]. Also, adiponectin- a protein whose expression is decreased in MetS (See Section 1.2.2)- was shown to be present, but decreased in levels in the EVs from serum of obese mice compared to non-obese mice [187]. Although EVs are involved in the pathogenesis of many diseases, the most researched and best understood is cancer. EVs seem to have many roles in tumorigenesis, including: inducing proliferation of cells, stimulating angiogenesis,

promoting matrix re-modelling, inducing metastasis and promoting immune escape. For example, members of our research group [188] have shown TNBC significantly ($p=0.013$) increased invasion of the recipient SKBR3 breast cancer cell line when compared to EVs derived from sera of healthy controls.

The identification of minimally-invasive biomarkers is an extremely challenging area of scientific research. Biomarkers for disease are required to be specific to the disease, sensitive enough to be detected [175] and should distinguish between individuals with a disease, such as MetS, from controls; therefore, allowing the early diagnosis of metabolic diseases. These biomarkers as detailed in Section 1.2 could also monitor progression of these diseases and predict an individuals' response to therapeutic intervention. Conventional methods [*i.e.* BMI, blood pressure, lifestyle (smoking status, alcohol consumption, activity level, diet)] [189]- although important- are insufficient [189].

EV analysis is minimally-invasive, as they are present in various bio-fluids (serum, plasma, urine) and contain biological molecules such as nucleic acids, proteins and lipids, that may reflect both the physiological and pathophysiological state of the cell of origin [190] (See Section 1.2). From available data, although limited, EVs seem to reflect the functional and dysfunctional state of both the releasing cells and tissue pathogenic pathways underlying metabolic diseases [189]. A critical step in identifying EVs as biomarkers for MetS would require the identification of correlations between EV signatures, disease and healthy states [189].

miRNA isolation from EVs offers a potential source of biomarkers for disease [191]. Cheng *et al* [191] isolated RNA from cell-free plasma and EVs purified from the same plasma specimen using ultracentrifugation. The results of this analysis demonstrated that EV-RNA content was 4.7 ± 2.1 ng ($67.9 \pm 1.7\%$ of total small RNA) and total plasma-RNA content was 2.2 ± 0.5 ng ($53.9 \pm 8\%$ of total small RNA), indicating EVs contain 47% more miRNAs than freely circulating RNA. However, the question still remains is the laborious task of isolating EVs and then their RNA content for subsequent miRNA profiling necessary, or is isolating directly from plasma, offering enough information about the disease state compared to the healthy state, therefore being a suitable biomarkers?

The aim of this Chapter was, therefore, to investigate EVs and their miRNA cargo as potential biomarkers for MetS. We also aimed to compare the miRNAs detected in EVs to those isolated directly from the same plasma specimens (here termed plasma-miRNAs) without EV enrichment. We began this investigation by isolating EVs from the plasma of healthy control, obese and MetS participants' plasma specimens by filtration and ultracentrifugation. An imperative step of EVs research is quality assessment of the isolated population. We therefore performed immunoblotting for EV and non-EV protein markers, transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA) to confirm successful EV isolation. Global miRNA profiling using Exiqon human miRNA panels for a total of 372 miRNAs (including 22 of the 25 miRNAs present on the custom miRNA panels from Chapter 2 [excluding miR-33a-3p, miR-33b-3p and miR-758-3p]) was then performed to identify potential EV-miRNA biomarkers for MetS.

3.2. Methods

3.2.1. Plasma Specimens

Plasma specimens from the healthy controls (n=14), obese (n=12) and MetS (n=13) participants were selected for EV isolation as they were to be used for functional assays with the TNBC cell line Hs578T. Seven healthy control plasma specimens from females aged 20-26 were obtained from Dr. Sharon O'Toole, Obstetrics and Gynaecology research group, Institute of Molecular Medicine, Trinity College Dublin, St. James' Hospital. Plasma specimens for the age group 42-72 we obtained from Asterand Biosciences. Finally, 3 specimens from individuals aged 68, 78 and 79 were obtained from Dr. Kathy Gately, Translational Oncology Research Group, Department of Clinical Medicine, Institute of Molecular Medicine, Trinity College Dublin, St. James' Hospital (Table 3.1). Asterand specimens were collected in EDTA blood collection tubes, centrifuged at 1300g for 10-15mins and plasma was aliquoted and stored at -80°C. All other specimens were collected in EDTA blood collection tubes, centrifuged at 1000g for 15mins and plasma was aliquoted and stored at -80°C until required.

Table 3.1: Plasma specimens from healthy control, Obese and MetS participants selected for EV isolation and further studies.

Healthy Control Specimens		Obese Specimens		MetS Specimens	
Specimen I.D.	Age	Specimen I.D.	Age	Specimen I.D.	Age
01-FI	28	074-AAT	42	014-TGJ	25
02-SH	42	009-KL	29	040-DHP	46
03-NI	26	013-JS	57	008-DCS	57
04-SA	21	038-EK	61	077-HK	27
65946	52	030-ABV	60	042-BJ	62
69866	72	088-SW	62	006-BH	67
69904	75	095-EPL	66	019-DB	64
69914	72	093-IH	78	098-KH	52
69916	65	048-SC	20	036-KAC	64
69906	65	039-CH	22	007-ST	55
69911	74	037-SDO	34	079-MBJ	65
CI-14-048	68	025-AM	29	045-AM	65
CI-14-043	78			082-INA	67
CI-14-061	79				

3.2.2. Isolation of Extracellular Vesicles from Plasma Specimens

EVs were isolated using filtration and ultracentrifugation (Figure 3.1). Specifically, 750µl or 250µl of plasma for functional studies or miRNA profiling, respectively, was diluted in 5ml of PBS. Using a syringe (Sigma-Aldrich; Cat. #: Z248029), a BD Precisionglide syringe needle (Sigma-Aldrich; Cat. #: 11804) and a 0.22µm Acrodisc 32mm syringe filter (VWR; Cat. #: 514-4131), the diluted plasma specimen was filtered into an 8.3ml Quickseal Polyallomer ultracentrifuge tube (Beckman Coulter; Cat. #: 34461), PBS was added until full, weighed (all 6 tubes were within 0.01g of each other) and sealed. The tubes were then ultracentrifuged (Beckman Coulter; Optima XPN) at 110,000g, 4°C, for 1 hour 15mins using a SW32.1Ti swinging bucket rotor. The supernatant was subsequently removed, leaving 1ml at the bottom of the tube, the pellet was re-suspended and transferred to a fresh 8.3ml ultracentrifuge tube, filled with PBS, weighed and sealed. The tubes were then centrifuged again. The supernatant was removed and 100µl of PBS was left at the bottom of the tube, the pellet re-suspended and 100µl of PBS added. The EVs in 200µl of PBS were transferred to a low protein-binding Eppendorf tube and stored at -80°C until required (Figure 3.1).

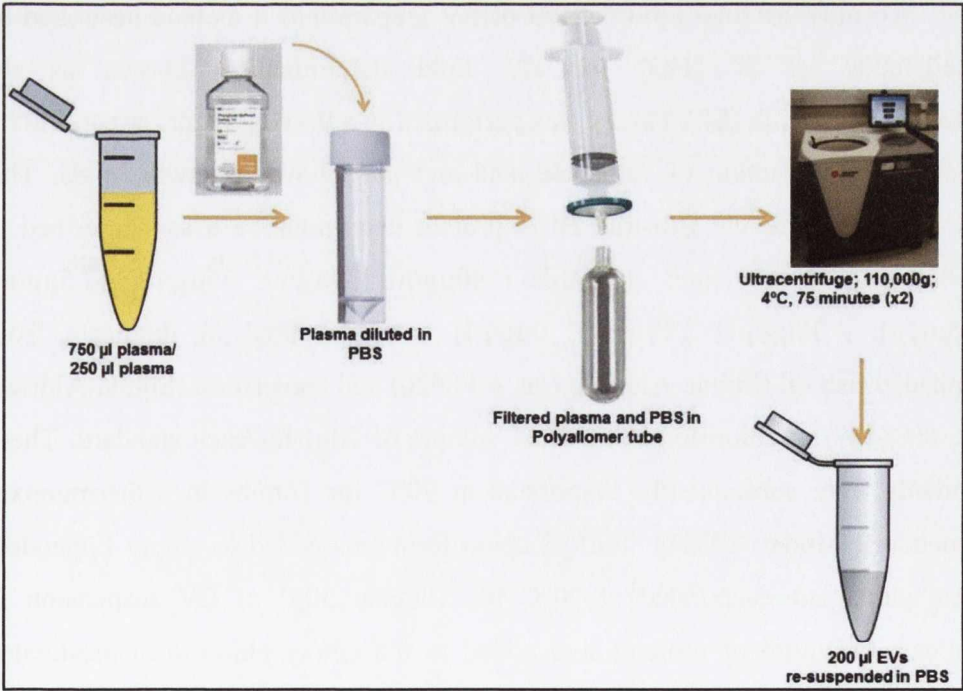


Figure 3.1: Schematic illustration for EV isolation; filtration and ultracentrifugation used to isolate EVs from plasma specimens.

3.2.3. Characterisation of Plasma EVs

The International Society for Extracellular Vesicles has set out the minimal experimental requirements for determination of an EV population [192]. These requirements include: immunoblotting, transmission electron microscopy (TEM) and Nanoparticle tracking (NTA).

3.2.3.1. Total Protein Determination of Plasma EV Preparations

Total protein concentration of EV preparations was determined using the Bio-Rad protein assay dye (Bio-Rad, Cat. #: 5000006) reagent. BSA standards were prepared in ddH₂O (200mg/ml, 100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml, 3.125mg/ml, 0mg/ml). Re-suspended EVs were diluted 1:10 in ddH₂O and the protein assay was performed as in Section 2.2.5.2.5.

3.2.3.2. Total Lipid Determination of Plasma EV Preparations

[Total lipid determination of EV preparations was performed in a fume hood. All steps involving heating were performed with open Eppendorf tubes].

To measure total lipid content of EV preparations a method described by Osteikoetxea *et al* [193] for EV lipid determination known as the sulfophosphovanilin (SPV) assay was performed in a 96-well plate format. This is a colorimetric reaction of sulphuric acid and phosphovanilin with lipids. This assay is similar to the Bio-Rad BCA protein determination assay described in Section 3.2.3.1, but lipid standards (140µg/µl, 70µg/µl, 35µg/µl, 17.5µg/µl, 8.75µg/µl, 4.38µg/µl, 2.19µg/µl, 0µg/µl) were prepared in duplicate from menhaden fish oil (Sigma-Aldrich; Cat. #:F8020) and cholesterol (Sigma-Aldrich; Cat. #:C8667) in chloroform to a final volume of 70µl for each standard. These standards were subsequently evaporated at 90°C for 10mins in a thermomixer (Eppendorf; Model: 22331). 70µl of chloroform was added to empty Eppendorf tubes and again evaporated at 90°C for 10mins. 50µl of EV suspension in duplicate (15µg/ml of protein) was added to the empty chloroform pre-treated Eppendorf tubes and 50µl of PBS was added to the lipid standards. The pre-treatment with equal volumes of chloroform, and PBS in the case of EV suspensions, ensured the same background colour development in all Eppendorf

tubes. 250µl of 96% sulphuric acid (Fluka; Cat. #: 84721) was added to each Eppendorf and incubated at 90°C for 20mins. Subsequently, 220µl of each sample and standards in sulphuric acid were transferred to the wells of a 96-well plate and the plate was allowed to cool to room temperature for 30mins. Finally, 110µl of 0.2mg/ml vanillin (Sigma-Aldrich; Cat. #:V1104) in 17% phosphoric acid (Sigma-Aldrich; Cat. #: P6560) was added to each well and incubated at room temperature for 10mins. The 96-well plate was read at 540nm using the FlouStar Optima microplate reader (BMG Labtech) (Figure 3.2). Total lipid concentrations were determined in Excel using regression analysis applied to absorbance readings and R² values. The lipid concentrations were extrapolated from the standard curve of menhaden fish oil.

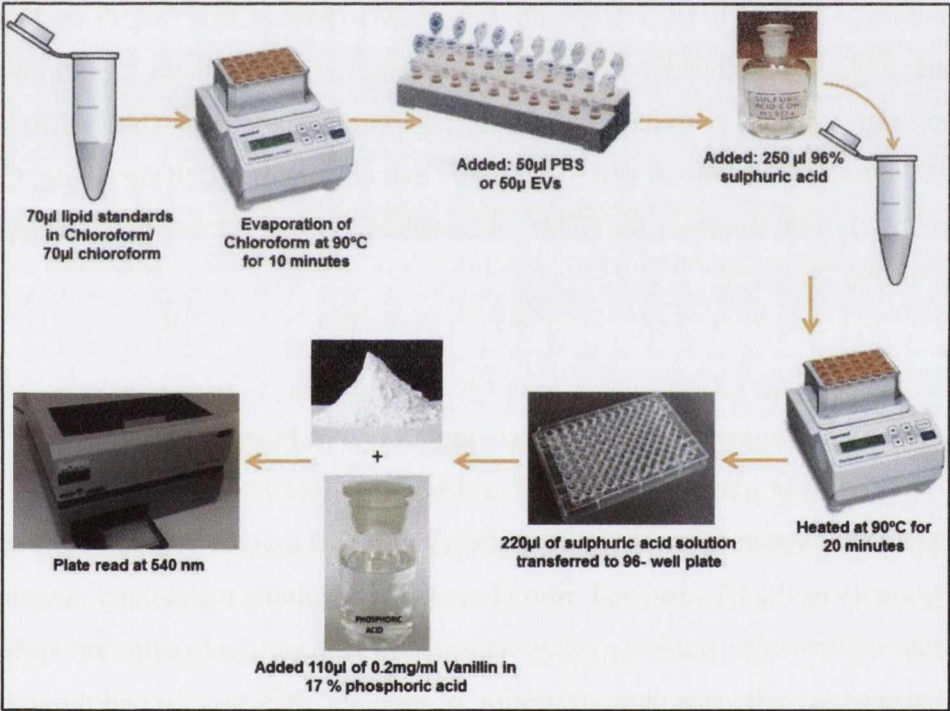


Figure 3.2: Schematic illustration of the total lipid determination of plasma EVs

3.2.3.3. Immunoblotting for EV Markers

Total protein extraction from EVs was performed as described in Section 2.2.5.2.5. Specifically, following EV isolation all PBS supernatant was removed and EVs were lysed in 30µl of Cell Extraction Buffer (Invitrogen, Cat. #: FNN0011) supplemented with 1X protease inhibitor cocktail (Roche, Cat. #: 05892970001) as opposed to being re-suspended in 200µl PBS.

Total EV protein concentration was determined using the Bio-Rad protein assay dye reagent as described in Section 3.2.3.1. However, protein from each EV sample was diluted 1:5 in ddH₂O and BSA standards were prepared for a narrower range of protein concentrations (100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml, 3.125µg/ml, 1.563µg/ml). Loading buffer was added to 15µg of each sample to a final concentration of 1x and samples were heated to 95°C for 10mins (See Section 2.2.5.2.5).

Immunoblotting was performed as described in Section 2.2.5.2.5. However, 15µg total EV proteins were resolved on NuPage® Novex® 10% Bis-Tris precast gels for protein SDS-PAGE electrophoresis (Biosciences, Cat. #: NP0323). Membranes were incubated with primary antibodies– Grp-94 (Cell Signalling, Cat. #: 2104P, 1:1000 dilution), CD81 (Santa Cruz, Cat. #: sc-23962 Clone: 5A6, 1:200 dilution), PDC6I/Alix (Abcam, Cat. #: Ab76608, 1:500) for 24 hours and secondary antibodies- anti-Rabbit (Cell Signalling, Cat. #: 7074s, 1:1000 dilution) for Grp-94 and PDC6I/Alix and anti-mouse (Cell signalling, Cat. #: 7076s, 1:1000 dilution) for CD81. All antibodies were diluted in 3% BSA in PBS/0.01% Tween 20.

3.2.3.4. Nanosight Particle Tracking (NTA) of Plasma EVs

NTA was performed on EVs isolated from all plasma specimens. The NTA NS500 system (NanoSight, Amesbury, UK) was used to determine the size and quantity of the EVs isolated from plasma. This evolving technology measures the rate of Brownian motion of the nanovesicles using a light scattering system that enables accurate size characterisation of the EVs. PBS was filtered through a 0.2µm filter and was run as a negative control. EV samples, diluted 1:60 in filtered PBS, were run at 24°C and had a viscosity of 0.909cP. As the sample was being analysed, six 60sec videos were taken. During this analysis the software identified, tracked and measured the Brownian motion of each vesicle in a sample from frame to frame of each video. Videos were analysed using NTA software to determine the size and quantity of the EVs in the sample.

3.2.3.5. Transmission Electron Microscopy (TEM) of Plasma EVs

[TEM imaging of EVs was performed in the Centre for Microscopy and Analysis in Trinity College Dublin, with the help of Neal Leddy, Chief Technical Officer]

Following EV isolation, a 10µl aliquot from the 200µl EV suspension was placed onto Parafilm (Sigma-Aldrich; Cat. #: P7793). A formvar carbon coated nickel grid was placed on top of the EV aliquot, ensuring that the coated side of the grid faced the drop. The grid was then incubated at room temperature for 30-60mins, washed by placing 3x30µl drops of PBS onto the Parafilm and placed on each drop for 5mins, using absorbent paper between each drop to remove excess PBS. A drop of paraformaldehyde (2%) was placed on the Parafilm and the grid was placed on top to fix the sample for 10mins. The washing step was repeated (3x30µl drops of PBS onto the Parafilm and the grid was placed on each drop for 5mins, using absorbent paper between each drop to remove excess PBS). The grid was contrasted in 2% uranyl acetate (BDH, Cat. #: 30550) and images taken using a JEOL JEM-2100 transmission electron microscope at 100kV.

3.2.4. RNA Isolation from Plasma EVs and Quantification of RNA

To the 200µl re-suspended EVs, 50µl of PBS was added. To this, 750µl of TriReagent™ was added, mixed by inversion and incubated at room temperature for 10mins. RNA isolation and quantification was completed as in Section 2.2.2.

3.2.5. Global miRNA Profiling of EV-RNA– Discovery Phase

Global miRNA profiling was performed using Exiqon microRNA ready-to-use PCR, human panel I, V3.M panels which were selected as the platform for miRNA profiling (Exiqon, Cat no.: 203613). A total of 370 miRNAs, miR-150-5p and miR-486-5p endogenous control RNAs, UniSP6 internal reverse transcription (RT) positive control, H₂O negative control, UniSp3IPC (plate control) SNORD38B, SNORD49A, Cel-miR-39-3p, UniSp2, UniSp4, UniSp5 and U6snRNA (endogenous control RNA) were analysed. 22 of the 25 miRNAs placed on the custom miRNA panels in Chapter 2 were placed on the global miRNA panels, miR-33a-3p, miR-33b-3p and miR-758-3p were not on the global

miRNA profiling panels. As miR-758-3p was of interest this was analysed by individual RT-qPCR (See Section 3.3.5).

The input volume of RNA for these plasma specimens was determined as 1.6µl in Section 2.3.3.1. Therefore, this volume was used in order to compare plasma-miRNAs in Chapter 2 to EV-miRNAs in this chapter. Twenty-three plasma EV-RNA specimens were selected randomly for this discovery phase (Table 3.2).

Table 3.2: Plasma specimens selected for Global miRNA profiling of EV-RNA– Discovery Phase

Control (n=7)	Obese (n=8)	MetS (n=8)
01-FH	074-AAT	014-TGJ
02-SH	013-JS	040-DHP
04-SA	038-EK	008-DCS
65946	030-ABV	077-HK
69866	095-EPL	042-BJ
69904	093-IH	006-BH
69916	037-SDO	007-ST
	037-SDO	079-MBJ

3.2.5.1. RT-qPCR Reaction for Discovery Cohort

The RT reaction was performed according to Exiqon’s miCURY LNA™ Universal RT miRNA PCR instruction manual v5.2 March 2013 for Serum/Plasma and other biofluids and Human miRNome PCR Panels. cDNA synthesis was performed as in Section 2.2.4.2.1 for a 40µl reaction, therefore 6.4µl of RNA was added to the RT reaction. qPCR was performed according to manufacturers’ protocol. The cDNA was diluted 50X and the PCR was set up as described in Table 3.3. qPCR was performed as per Section 2.2.4.2.2.

Table 3.3: Set up of Exiqon Exilent SYBR Green Master Mix for qPCR reaction

Reagent	RT-qPCR Reaction Volume (µl)
SYBR Green PCR Master Mix– 2X	2000
Nuclease-free Water (part of kit)	1960
cDNA	40

3.2.5.2. Analysis of Global miRNA Profiling Data- Discovery Cohort

Raw data from the Applied Biosystems ABI7900 HT sequence detection system was analysed as per Section 2.2.4.2.3. However, only miR-486-5p (as miR-150-5p was not consistently expressed in these EVs [Figure 3.10]) was used to normalise each C_T value, resulting in the ΔC_T value and hierarchical clustering (unsupervised) was performed in D-Chip using the normalised C_T values.

3.2.5.3. Statistical Analysis

Statistical Analysis of the data to generate p-values was performed as described in Section 2.2.4.3.3.

3.2.6. Individual RT-qPCR – Validation Cohort

Three miRNAs were identified in the discovery stage of the study from the global miRNA profiling as possible EV-miRNA biomarkers for MetS at baseline (before any dietary intervention). RT-qPCR was performed to validate these miRNAs in a sub-cohort of specimens, n=13 specimens (Table 3.4). Primers for four miRNAs, *i.e.* miR-100-5p (Exiqon, Cat. #: 205689), miR-134-3p (Exiqon, Cat. #: 205989), miR-370 (Exiqon, Cat. #: 204011) and miR-758-3p were chosen for validation. miR-150-5p and miR-486-5p endogenous control RNAs and UniSp6 internal control RNA were also used and validation analysis was performed as per Section 2.2.4.4.

Table 3.4: Specimens Selected for Global miRNA Profiling– Validation Phase

Control (n=4)	Obese (n=4)	MetS (n=5)
03-NI	099-KL	019-DB
69906	025-AM	045-AM
69911	088-SW	082-INA
69914	048-SC	098-KH
		036-KAC

3.2.6.1. RT-qPCR for Validation Cohort

cDNA was synthesised as per Section 3.2.5.1. cDNA was diluted 40X in DEPC-treated H₂O (Ambion, Cat. #: AM9916), and the cDNA was amplified as described in Section 3.2.5.1.

3.2.6.2. Data Analysis

Data analysis was performed as described in 3.2.5.2. Specifically, all plates were set to the same threshold, so to normalise interpolate differences and miR-486-5p (as miR-150-5p levels were inconsistent in these EVs) was used to normalise each C_T value, resulting in the ΔC_T value. Fold changes were determined using the delta delta C_T ($\Delta\Delta C_T$) method.

3.2.6.3. Statistical Analysis

Statistical analysis was performed as per section 3.2.5.3. However, we performed one-way *ANOVA* analysis with post-hoc Tukey analysis in order to compare the three cohorts for significant difference and to then determine the significance in the difference between each cohort separately.

3.2.7. Functional Assessment of Plasma EVs with Hs578T Cell Line Variant

3.2.7.1. Cell Culture

Hs578T cells were cultured as described in Section 2.2.5.2.1. For functional assays Hs578T cells were treated with EVs isolated from the plasma of healthy control, obese and MetS participants. FBS was depleted (dFBS) of EVs as FBS naturally contains EVs. FBS EVs may alter the results of functional studies where the effect of the EVs of interest may be masked. Specifically, FBS was aliquoted into 28ml conical ultracentrifuge tubes (Beckman Coulter; cat. #: 358651) and ultracentrifuged at 110,000g, 4°C for 16 hours. dFBS was aliquoted and stored at -20°C.

3.2.7.2. Proliferation Assay for Hs578T Cells after Treatment with Plasma EVs

Hs578T cells were treated with EVs isolated from the plasma from healthy controls, obese and MetS participants. Specifically, Hs578T cells were seeded at 2×10^3 cells/100µl/well in a 96-well plate in DMEM supplemented with 10% FBS (Gibco, Cat. #: 10270106) 2mM L-Glutamine (Sigma-Aldrich; Cat. #: I0516) and 10µg/ml insulin (Sigma-Aldrich; Cat. #: I9278). Cells were allowed to attach overnight and the following morning medium was removed and replaced with 10µg of EVs/200µl/well in DMEM supplemented with 2mm L-glutamine, 10% dFBS, and 10µg/ml insulin in triplicate. EVs were incubated with cells at 37°C and 5% CO₂ for 72 hours.

After 72 hours of growth in the presence of EVs, cell proliferation was measured using the acid phosphatase method. Briefly, culture medium was removed from wells and plates were washed with 100µl PBS (x2 times) (Sigma-Aldrich, Cat. #: D8537) per well. The plate was then blotted on tissue paper to ensure all PBS was removed. Phosphatase substrate (10 mM p-nitrophenol phosphate [VWR; Cat. #: 27963.101]) was freshly prepared in 1M sodium acetate buffer (Table 3.5) and 100µl was added to each well. Plates were covered in tin-foil and placed in the incubator at 37°C and 5% CO₂, for 1.5 hours. The reaction was stopped by adding 50µl 1M NaOH to each well. Absorbance was then read at 405nm using a FlouStar Optima microplate reader.

Table 3.5: Protocol for Preparation of 1M sodium Acetate Buffer

Reagent	Quantity/Volume
Sodium Acetate (Sigma-Aldrich; cat. #: S2889)	4.1g
TritonX-100 (Sigma-Aldrich; #: T8787)	500µl
dH ₂ O	500ml
pH adjusted to 5.5	

3.2.7.3. Anoikis Assay for Hs578T Cells Treated with Plasma EVs

24-well plates were coated in 200µl of poly(2-hydroxyethyl methacrylate) [poly-HEMA] (Sigma-Aldrich, cat, #: P3932) solution. Poly-HEMA was prepared by dissolving 12mg/ml overnight in 100ml 95% ethanol. Plates were allowed to

air-dry in a laminar flow overnight. This coating process was repeated a second time. Control wells were coated in 95% ethanol, in the same way. Hs578T cells were seeded at 2×10^4 cells/500 μ l/well. At the same time as seeding, 10 μ g EVs/well were added and plates were incubated at 37°C and 5% CO₂ for 72 hours in DMEM supplemented with with 2mm L-glutamine, 10% dFBS, and 10 μ g/ml insulin in triplicate.

72 hours following seeding the Hs578T cells with EVs, 50 μ l of alamar blue (Invitrogen; cat. #: DAL1100) solution was added to each well. Plates were covered in tin-foil and placed at 37°C and 5% CO₂ for 3.5 hours. After this incubation time absorbance was read at 570nm using a FluoStar Optima microplate reader.

3.3. Results

3.3.1. EVs were successfully isolated from Healthy Control, Obese and MetS Participants' Plasma Specimens

The first step in the analysis of EVs was to establish whether EVs had been successfully isolated. To assess this, immunoblot analysis was performed to determine the presence of two proteins accepted as markers of EVs and a negative control protein marker. Specifically, proteins analysed were:

1. PDC6I/Alix (96-100 kDa): a cytoplasmic protein which sorts cargo into multivesicular bodies for processing into intraluminal vesicles.
2. CD81 (22-26 kDa): a transmembrane protein/tetraspanin involved in the maturation and trafficking of CD19 from the endoplasmic reticulum to the Golgi and to the cell membrane.
3. Grp-94 (94 kDa): an intracellular protein that acts as a negative control for EVs. It is undetected in EVs/exosomes and associated with compartments other than cell membrane or endosomes.

TEM and NTA were performed to determine whether the isolated vesicles were within the expected size-range (30-130nm) for EVs. Total protein content was assessed for EVs isolated from control, obese and MetS participants' plasma specimens by Bio-Rad BCA assay to establish whether the quantities present in each cohort were similar or significantly different.

Immunoblot analysis for the presence of PDC6I/Alix, CD81 and Grp-94 (Figure 3.3 A), TEM analysis (Figure 3.3 B) and NTA (Figure 3.3 C) confirmed successful isolation of EVs. Analysis of EV quantities isolated from control ($171.8 \pm 25.9 \mu\text{g/ml}$ plasma), obese ($136.2 \pm 18.7 \mu\text{g/ml}$ plasma) and MetS ($204.2 \pm 23 \mu\text{g/ml}$ plasma) participants' plasma specimens showed a significant difference in the levels of EVs ($p=0.03$) between the three cohorts (one-way ANOVA). However, EV quantities were not significantly ($p=0.3$) different between the control and obese cohorts. Post-hoc Tukey analysis showed a significant difference in the levels of EVs isolated from the MetS and control cohorts ($p=0.049$) and the MetS and obese cohorts ($p=0.004$) (Figure 3.3 D). Interestingly, ROC curve analysis also indicated that EVs derived from MetS

participants are sensitive to discriminate MetS participants from healthy controls ($p=0.049$) and MetS participants from obese participants ($p=0.0033$) (Table 3.6).

TEM analysis indicated the vesicles isolated from plasma specimens were nano-sized EVs ($\sim 100\text{nm}$ in diameter) with the characteristic round shape of EVs (Figure 3.3 B). NTA also indicated EVs of $\sim 100\text{nm}$ were successfully isolated from plasma specimens. Specifically, there was no significant difference in the size of the EVs isolated from the 3 cohorts ($p=0.65$) (Figure 3.3 C). A method for characterising EVs involving lipid concentration determined EVs were also successfully isolated from all specimens with no significant difference ($p=0.23$) in the type of vesicle isolated from the plasma from healthy control (0.330 ± 0.1 protein-lipid ratio), obese (0.225 ± 0.8 protein-lipid ratio) and MetS (0.132 ± 0.4 protein-lipid ratio) participants (Figure 3.3 E).

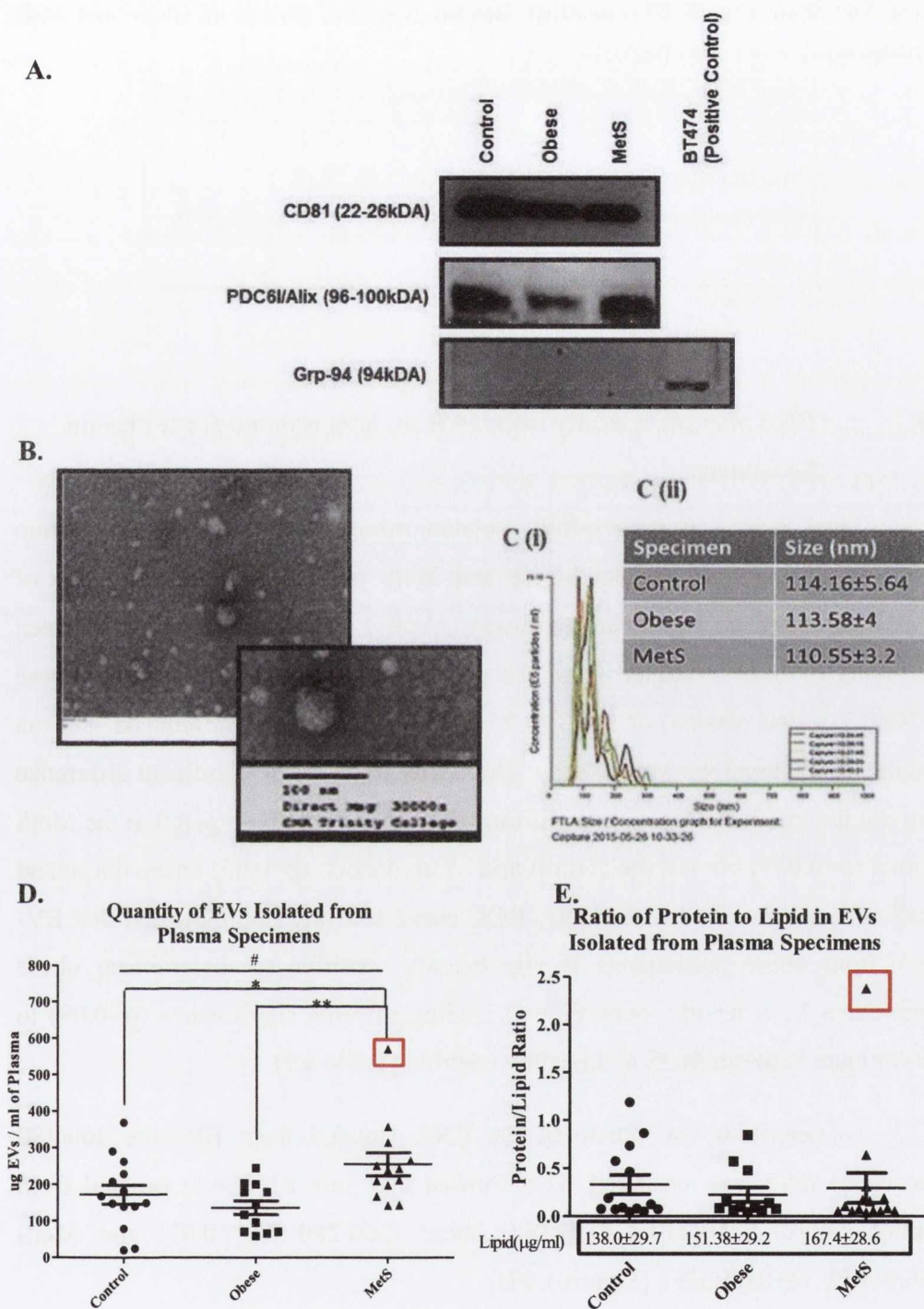


Figure 3.3: Validation of EVs isolated from plasma of control Obese and MetS

A. Immunoblot analysis of two proteins detected in EVs and one protein not detected in EVs. **B.** TEM analysis of the isolated EVs. **C.** (i) Representation of nanosight particle tracking (ii) average size of EVs from each cohort as determined by NTA. **D.** Quantification of EVs as determined by the Bio-Rad BCA assay. **E.** Characterisation of EVs by the lipid sulfophosphovanilin [SPV] assay. Outlier values (highlighted in red box) as determined by Grubbs test were included in the figure but excluded from statistics. # $p < 0.05$ one-way ANOVA, where * $p < 0.05$, ** $p < 0.01$ post-hoc Tukey

Table 3.6: Sensitivity of EV quantities isolated from the plasma of Obese and MetS participants as biomarkers for MetS

ROC Analysis	MetS vs Control	MetS vs Obese
AUC	0.73	0.85
Standard Error	0.099	0.08
95% Confidence Interval	0.5303 to 0.9203	0.6948 to 0.9976
p-value	0.049	0.0033

AUC: area under the curve

3.3.2. RNA was successfully isolated from EVs isolated from Plasma Specimens

Total RNA was successfully isolated from 250µl of EVs isolated from 250µl of plasma from control, obese and MetS participants. Quantification of RNA determined no significant (one-way *ANOVA*, $p=0.09$) differences between the levels of RNA obtained from the control ($2.78\pm0.20\mu\text{g/ml}$ plasma), obese ($3.54\pm0.31\mu\text{g/ml}$ plasma) or MetS ($3.25\pm0.24\mu\text{g/ml}$ plasma) participants' plasma specimens (Figure 3.4 A) at baseline. There was, however, a significant difference between the yield of RNA obtained from the control cohort compared to the MetS cohorts ($p=0.049$) but not the control and obese cohorts ($p=0.05$) or the obese and MetS cohorts ($p=0.7$). Interestingly, ROC curve analysis also indicated that EV-RNA from obese participants is significantly sensitive to discriminate obese participants from healthy controls and tending towards significance ($p=0.08$) to discriminate between MetS and healthy controls (Table 3.7).

To determine the purity of the RNA isolated from EVs the 260/280 absorbance ratio was measured. This showed that pure RNA was isolated from healthy control (260/280= 1.61 ± 0.01), obese (260/280= 1.61 ± 0.02) and MetS (260/280= 1.54 ± 0.03) EVs (Figure 3.4 B)

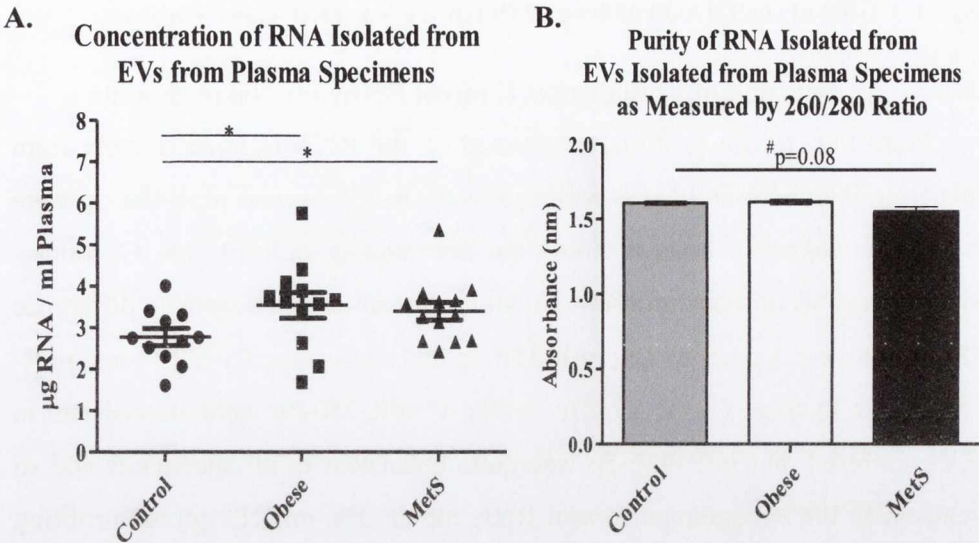


Figure 3.4: Quantification and purity of RNA isolated from plasma EVs.
A. Quantification of RNA isolated from plasma EVs as determined by the NanoDrop. B. Purity of RNA isolated from plasma EVs as determined by 260/280 values. Data for B represent the mean $\pm$ SEM for the each cohort, where # one-way ANOVA, * $p < 0.05$.

Table 3.7: Sensitivity of EV-RNA yield to discriminate between cohorts

ROC Analysis	Control vs Obese	Control vs MetS
AUC	0.75	0.71
Standard Error	0.1	0.1
95% Confidence Interval	0.5362 to 0.9638	0.501 to 0.9249
p-value	0.04	0.08

AUC: area under the curve

3.3.3. Global miRNA Profiling of Plasma EVs- Discovery Cohort

3.3.3.1. Selection of Endogenous Control RNAs for Normalisation

From the custom profiling performed on the RNA isolated directly from plasma from the obese and MetS participants, miR-150-5p and miR-486-5p were selected as endogenous control RNAs for normalising miRNA raw C_T values. Following analysis of these miRNAs for all specimens after interpolate difference (UniSP3IPC) were accounted for, miR-150-5p had an average C_T =29.16 and miR-486-5p had an average C_T =31.19. The levels of miR-150-5p were inconsistent in the EVs analysed, but miR-486-5p was quite consistent in all specimens and so was chosen as the endogenous control RNA for the EV-miRNA global profiling study (Figure 3.5).

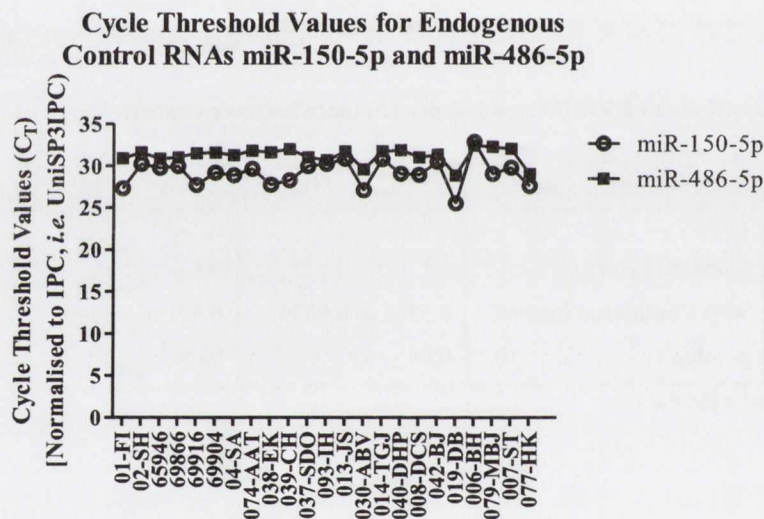
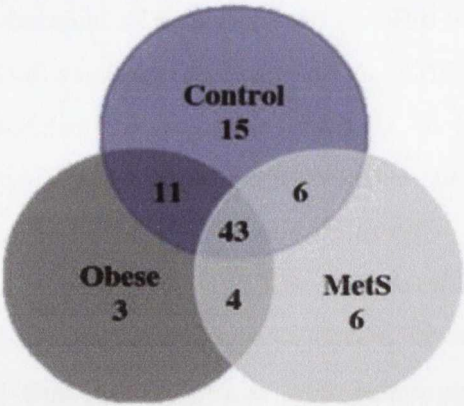


Figure 3.5: Endogenous control RNA levels in EVs from control, Obese and MetS specimens
Levels of miR-150-5p and miR-486-5p following interpolate difference normalisation using UniSp3IPC for endogenous control RNA selection.

3.3.3.2. Global miRNA Profiling of EVs isolated from Plasma identified 14 miRNAs as Potential Biomarkers for MetS

A cut-off point of $35C_T$ was taken, where miRNAs detected $\leq 35C_T$ were considered as 'present' where as those with a C_T value $>35C_T$ were considered 'undetected'. miRNA profiling for n=372 miRNAs (including 22 of the 25 miRNAs present on the custom profiling panels from Chapter 2) for the contents of EVs isolated from the plasma of healthy control, obese and MetS participants detected 43 (including endogenous control RNAs, miR-150-5p and miR-486-5p) miRNAs in all three cohorts. A further 15 miRNAs were detected in the control cohort only, 3 miRNAs were detected in the obese cohort only, 6 miRNAs were detected in the MetS cohort only, 11 miRNAs in the control and obese cohorts, 6 miRNAs in the control and MetS cohorts and 4 miRNAs in the obese and MetS cohorts (Figure 3.6 A and B; Appendix I, Section E, Table E.1). 372 miRNAs were analysed in this global miRNA profiling study, therefore, 23.7% of the 372 miRNAs (n=88) were detected in the EVs of control, obese and MetS participants.

A.



B.

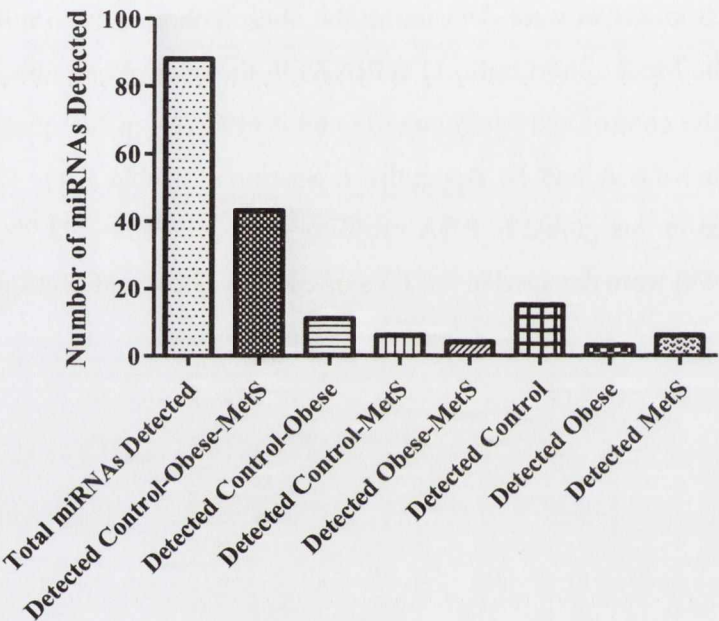


Figure 3.6: Detection of miRNAs in EVs taking C_T values of ≤ 35 as present.
A. Venn diagram illustrating the distribution of miRNAs detected in Control, Obese and MetS participants' EVs isolated from plasma specimens. **B.** Graphical representation of the number of miRNAs detected in control, obese and MetS EVs.

Hierarchical Clustering was performed for the control, obese and MetS cohorts using the normalised C_T (ΔC_T) for each miRNA detected during the global profiling analysis. Clustering of all 86 miRNAs (88 detected, analysis performed on 86 as miR-150-5p and miR-486-5p endogenous control RNAs were removed after normalising) was observed for the obese and MetS cohorts separate from the control cohort (Figure 3.7).

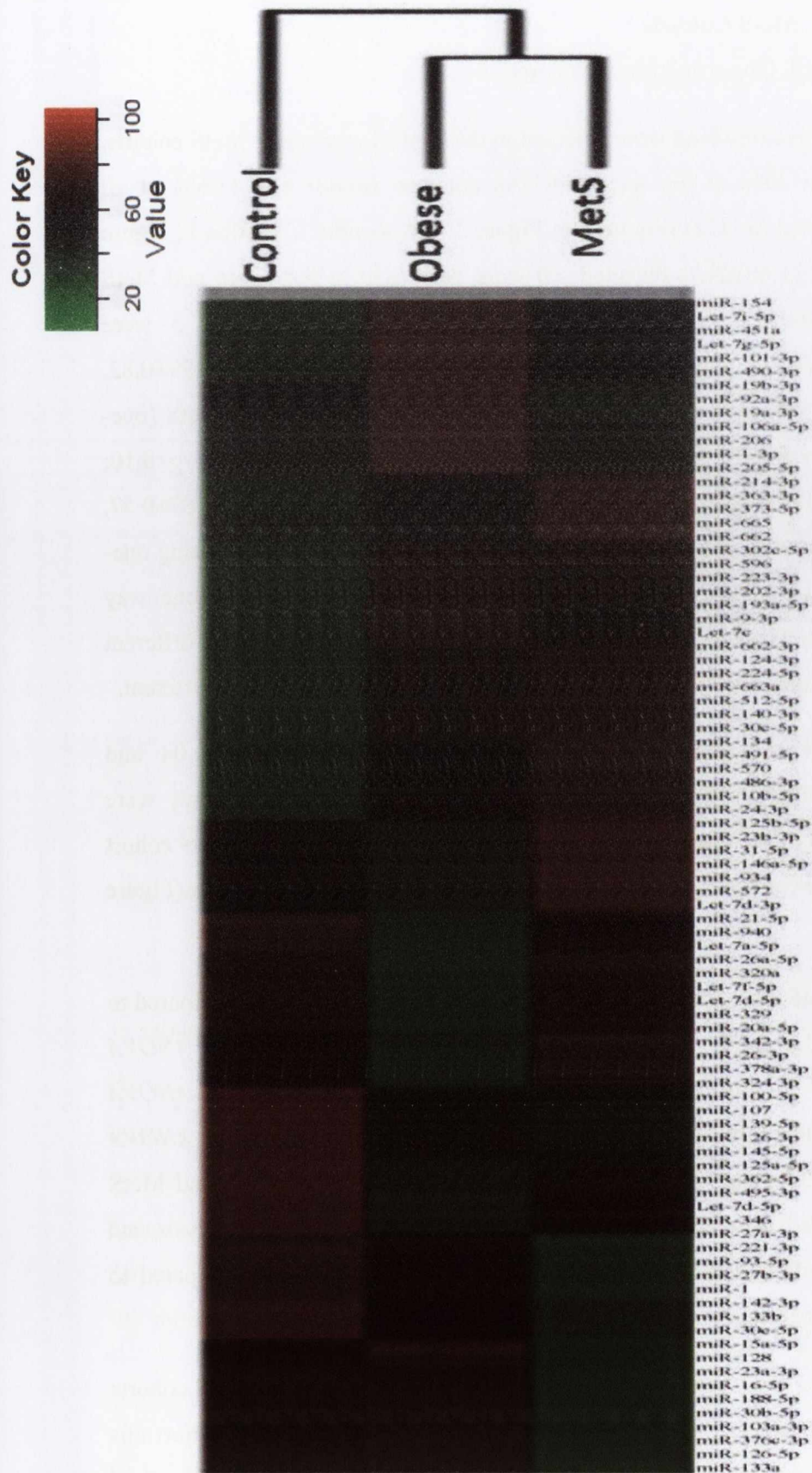


Figure 3.7: Hierarchical clustering of miRNAs detected in EVs isolated from the plasma from control, Obese and MetS participants.

Heatmap generated from the normalised C_T values. Values in red have a high C_T value and are lowly detected. However, values in green have lower C_T values and are highly detected. Values in red have a high rank value and, therefore, a low C_T value and are highly detected. However, values in green are lower rank values and, therefore, are higher C_T values and have a lower detection.

3.3.3.2.1. Global Profiling of EV-miRNAs in the Control, Obese and MetS Cohorts

Detected Control, Obese and MetS Cohorts

Forty-three miRNAs were detected in the control, obese and MetS cohorts, this equated to 49% of the total miRNAs detected (n=88) and 11.6% of all miRNAs analysed (n=372) (Figure 3.6; Figure 3.8; Appendix I, Section F, Figure F.1). Of these 43 miRNAs detected, 10 were decreased in the obese and MetS cohorts compared to controls by >2-fold. Of these 10 miRNAs 3 were significantly altered. Specifically, miR-134 (obese vs control $[-8.99 \pm 0.82, p=0.10]$; MetS vs control $[-14.34 \pm 0.57, p=0.03]$; control vs obese vs MetS [one-way *ANOVA* $p=0.04$]), miR-184 ($-266.22 \pm 2.44, p=0.04$; $-14.07 \pm 2.05, p=0.10$; one-way *ANOVA* $p=0.089$) and miR-491-5p ($-3.15 \pm 0.75, p=0.17$; $-7.87 \pm 0.57, p=0.01$; one-way *ANOVA* $p=0.04$). Significance testing was performed using one-way *ANOVA* with post-hoc Tukey analysis. Significance values for one way *ANOVA* indicates at least 2 of the 3 cohorts analysed are significantly different and post-hoc Tukey analysis determines which cohorts are significantly different.

miR-134 and miR-491-5p were significantly decreased ($p=0.04$ and $p=0.04$, respectively) when compared by one-way *ANOVA* analysis; they were also significantly decreased ($p=0.03$ and $p=0.01$, respectively) in the MetS cohort compared to the control cohort when analysed by post-hoc Tukey analysis (Figure 3.8).

Three miRNAs were increased in the obese and MetS cohorts compared to controls, miR-126 ($2.93 \pm 0.52, p=0.06$; $3.33 \pm 0.56, p=0.05$; one-way *ANOVA* $p=0.10$), let-7b-5p ($3.69 \pm 0.48, p=0.03$; $2.20 \pm 1.11, p=0.45$; one-way *ANOVA* $p=0.35$) and miR-133b ($1.82 \pm 0.41, p=0.22$; $3.31 \pm 0.37, p=0.02$; one-way *ANOVA* $p=0.04$). miR-133b was significantly increased ($p=0.04$) in the obese and MetS cohorts compared to controls when analysed by one-way *ANOVA* analysis and miR-133b was significantly ($p=0.02$) increased in the MetS cohort compared to controls when analysed by post-hoc Tukey analysis (Figure 3.8).

miR-133a was significantly altered in the control, obese and MetS cohorts ($p=0.049$) when analysed by one-way *ANOVA*. However, in the obese cohort this was <2-fold decreased compared to controls ($-1.17 \pm 0.42, p=0.73$) and increased by >2-fold in the MetS cohort compared to controls ($3.03 \pm 0.64, p=0.10$); neither

were significantly changed when compared by post-hoc Tukey analysis (Figure 3.8).

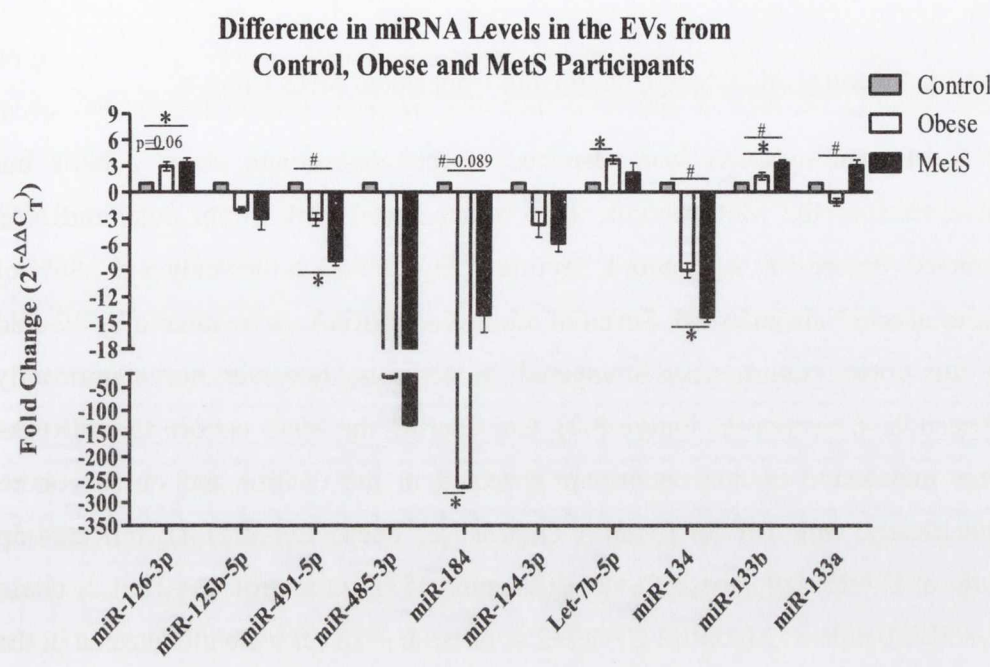


Figure 3.8: Change in EV-miRNA levels in the Obese and MetS cohorts compared to controls.

Fold change for each miRNA in the obese cohort compared to controls and the MetS cohort compared to controls. Data represent the mean $\pm$ SEM for each miRNA, where* Tukey post-hoc $p<0.05$, # one-way ANOVA $p<0.05$.

Detected Control Cohort, Undetected Obese and MetS Cohort

Fifteen miRNAs were detected in the control cohort and undetected in the obese and MetS cohorts. This equated to 17% of the total miRNAs detected in the study ($n=88$) and only 4.0% of the total miRNAs analysed ($n=372$) (Figure 3.6). Analysis of the C_T values for the control, obese and MetS cohorts indicated 6 miRNAs were detected in the control cohort with a C_T value <30 , *i.e.* miR-9-3p (control $C_T=27\pm1.6$), miR-202-3p (control $C_T=28\pm3$), miR-205-5p (control $C_T=24\pm1.1$), miR-302c-5p (control $C_T=22.7\pm1.8$), miR-370 (control $C_T=28\pm2.7$) and miR-596 (control $C_T=29\pm1.6$).

Nine miRNAs were detected in the control cohort with a C_T value >30 and ≤ 35 , *i.e.* miR-19a-5p (control $C_T=34\pm1.3$), miR-25-3p (control $C_T=35\pm1.1$), miR-140-3p (control $C_T=32\pm1.7$), miR-146a-5p (control $C_T=34\pm0.9$), miR-193a-5p

(control $C_T=34\pm0.7$), miR-214-3p (control $C_T=35\pm0.8$), miR-363-3p (control $C_T=32\pm1$), miR-512-5p (control $C_T=31\pm1.9$) and let-7i-5p (control $C_T=33\pm0.6$).

Detected Control and Obese Cohorts, but Undetected MetS Cohort

Eleven miRNAs were detected in the control and obese cohorts but undetected in the MetS cohort. This equated to 12.5% of the total miRNAs detected (Figure 3.6; Appendix I, Section F, Figure F.2) in the study and 2.96% of the total miRNAs analysed. Seven of the eleven miRNAs were altered by >2-fold in the obese cohort when compared to controls, however not significantly (Appendix I, Section F, Figure F.2). Considering the MetS cohort, the miRNAs were undetected in this cohort but detected in the control and obese cohort. Specifically, miR-10b-5p (control $C_T=26\pm1.6$, obese $C_T=30\pm1.4$), miR-224-5p (control $C_T=25\pm0.9$, obese $C_T=27\pm2.5$), miR-373-5p (control $C_T=27\pm1.2$, obese $C_T=30\pm2$), miR-572 (control $C_T=29\pm2.4$, obese $C_T=28\pm2$) were all detected in the control and obese cohorts with C_T values ≤ 30 . miR-30c-5p (control $C_T=33\pm0.9$, obese $C_T=32\pm0.6$), miR-31-5p (control $C_T=34\pm0.9$, obese $C_T=33\pm0.5$), , miR-320a (control $C_T=32\pm0.9$, obese $C_T=31\pm0.5$), , miR-652 (control $C_T=33.8\pm0.6$, obese $C_T=34\pm0.6$), miR-662 (control $C_T=27\pm2.5$, obese $C_T=31.6\pm1.4$), let-7f-5p (control $C_T=33\pm0.6$, obese $C_T=33\pm0.7$) and let-7c-5p (control $C_T=32\pm0.7$, obese $C_T=32\pm0.7$) were all detected in the control and obese cohorts with C_T values >30 and ≤ 35 .

Detected Control and MetS Cohorts, but Undetected Obese Cohort

Six miRNAs were detected in the control and MetS cohorts but undetected in the obese cohorts. This equated to 6.8% of the total miRNAs detected ($n=88$) in the study and 1.6% of the total miRNAs analysed in the study ($n=372$) (Figure 3.6;). Of these 6 miRNAs none were significantly altered in the MetS cohort compared to controls (Appendix I, Section F, Figure F.3). For the obese cohort these 6 miRNAs were not detected *i.e.* miR-20a-5p (control $C_T=32.1\pm1.0$, MetS $C_T=31\pm0.6$), miR-342-3p (control $C_T=33.5\pm1.0$, MetS $C_T=33\pm1.3$), let-7g-5p (control $C_T=28.9\pm2.9$, MetS $C_T=31\pm1.3$), miR-101-3p (control $C_T=33\pm1.6$, MetS $C_T=33\pm0.8$), miR-30b-5p (control $C_T=34\pm1.3$, MetS $C_T=33\pm1.0$), miR-106a-5p (control $C_T=31.2\pm0.5$, MetS $C_T=32\pm1.1$).

Detected Obese Cohort, but Undetected Control and MetS Cohorts

Three miRNAs were detected in the obese cohort and undetected in the control and MetS cohorts. This equates to 3.4% of the total miRNAs detected (Figure 3.6) in the study (n=88) and 0.81% of the total miRNAs analysed in the study. miR-27a-3p (obese $C_T=33\pm0.4$), miR-125a-5p (obese $C_T=33\pm1.1$) and miR-107 (obese $C_T=34\pm0.9$) were all increased in the obese cohort compared to the control cohort. For obese and MetS comparisons See Section 3.3.3.2.2.

Detected MetS Cohort, but Undetected Control and Obese Cohorts

Six miRNAs were detected in the MetS cohort and undetected in the control and obese cohorts. This equated to 6.8% of the total miRNAs detected (n=88) in the study (Figure 3.6) and 1.6% of the total miRNAs analysed in the study (n=372) (Figure 3.6). Comparing these miRNAs in the MetS cohort to the control cohort found all six miRNAs to be increased in the MetS cohort [miR-1 (MetS $C_T=33\pm0.9$), miR-15a-5p (MetS $C_T=32\pm0.5$), miR-19b-3p (MetS $C_T=32\pm1.1$), miR-93-5p (MetS $C_T=33\pm0.9$), miR-103a-3p (MetS $C_T=32\pm1.1$) and miR-139-5p (MetS $C_T=33\pm0.9$). For obese and MetS comparisons See Section 3.3.3.2.2.

3.3.3.2.2. Global Profiling of EV-miRNAs in the Obese and MetS Cohorts.

Seventy-three miRNAs were detected in either both the obese and MetS cohorts or the obese and MetS cohorts individually. This equated to 83% of the total miRNAs detected in the study (control specimens were excluded for this part of the analysis) (Figure 3.6, Figure 3.9; Appendix I, Section F, Figure F.4). 43 of these 73 miRNAs were detected in the control, obese and MetS cohorts, therefore they were analysed by comparison to controls in Section 3.3.3.2.1. Of the remaining 30 miRNAs 3 were detected in the obese cohort, 6 in the MetS cohort, 4 in the obese and MetS cohorts, 6 miRNAs in the control and MetS cohorts (analysed by comparison to controls in Section 3.3.3.2.1) and 11 miRNAs in the control and obese cohorts (analysed by comparison to controls in Section 3.3.3.2.1).

Analysis of miRNAs detected in the obese and MetS cohorts but undetected in the controls identified four miRNAs. This equated to 4.55% of the total miRNAs detected in the study (n=88) (Figure 3.6) and 1% of the total miRNAs analysed in the study (n=372). Specifically, miR-30e-5p (obese $C_T=34\pm0.8$, MetS $C_T=32\pm1.2$) and miR-100-5p (obese $C_T=30\pm0.4$, MetS $C_T=30\pm0.3$), let-7d-3p (obese $C_T=33\pm0.6$, MetS $C_T=34\pm0.6$) and miR-145-5p (obese $C_T=34\pm1$, MetS $C_T=33\pm1$).

Analysis of the miRNAs detected in both the obese and MetS cohorts identified miR-133a (3.55 ± 0.64 , $p=0.03$) (Figure 3.9) as the only miRNA that was significantly increased and altered in the MetS cohort compared to the obese cohort. Another 8 miRNAs were altered by >2-fold, 4 of which were increased but not significantly and 4 of which were decreased, again not significantly.

Three miRNAs were detected in the obese cohort but undetected in the MetS cohort, *i.e.* miR-27a-3p (obese $C_T=33\pm0.4$) miR-107 (obese $C_T=3\pm0.9$) and miR-125a-5p (obese $C_T=34\pm1.1$).

Six miRNAs were detected in the MetS cohort but undetected in the obese cohort *i.e.* miR-1 (MetS $C_T=33\pm0.9$), miR-9-3p (MetS $C_T=31\pm2.6$), miR-15a-5p (MetS $C_T=32\pm0.5$), miR-19b-3p (MetS $C_T=32.1\pm1$), miR-103a-3p (MetS $C_T=32\pm1.1$) and miR-139-5p (MetS $C_T=33\pm0.9$).

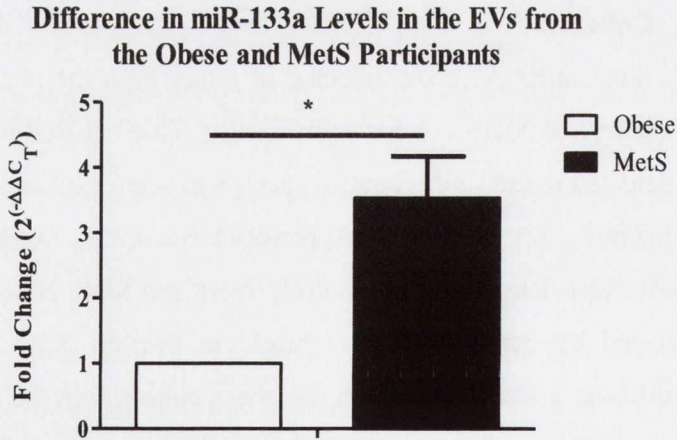


Figure 3.9: Change in EV-miRNA miR-133a levels in the MetS cohorts compared to the Obese cohort

Fold change for miR-133a in the MetS cohort compared to obese cohort. Data represent the mean $\pm$ SEM, where fold change was not significant. * $p<0.05$.

3.3.4. Validation of miRNAs of Interest as Potential EV-miRNA Biomarkers

Fourteen miRNAs were identified as of particular interest following global miRNA profiling as they were substantially altered, in the obese and MetS cohorts compared to controls, or in the MetS cohort compared to the obese cohort, or undetected in one or more cohorts as described in Table 3.8. Three of the 14 miRNAs identified during the global profiling stage were selected for validation. Specifically, miR-100-5p (detected obese and MetS, undetected controls), miR-134 (detected controls, obese and MetS) and miR-370 (detected controls only).

These three miRNAs were selected as miR-100-5p was undetected in controls but detected in obese and MetS participants and was highly expressed in both cohorts with C_T values for the obese cohort of 30 ± 0.4 and MetS cohort of 30 ± 0.3 . miR-134 was detected in the control, obese and MetS cohorts and it was substantially and significantly decreased ($p=0.04$) in control, obese and MetS cohorts when analysed by one-way *ANOVA* and significantly decreased ($p=0.03$) in the MetS cohort compared to controls when analysed by post-hoc Tukey. Of note, our group has shown miR-134 to be significantly decreased in the cells (8.9×10^{-11}) and EVs (3×10^{-9}) from the aggressive Hs578Ts(i)₈ TNBC cell line variant compared to the Hs578T cell line variant. Not only was miR-134 significantly decreased in the Hs578Ts(i)₈ cells but transfection of these cells with miR-134 significantly ($p<0.001$) reduced the migration of these cells and treatment of Hs578Ts(i)₈ cells with EVs isolated from Hs578Ts(i)₈ cells over-expressing miR-134 significantly ($p<0.01$) reduced the migration and invasion of the recipient cells [194]. Interestingly, this was not only seen in the TNBC cell lines but also in breast tumour EVs compared to healthy control EVs. This data had been mined from publically available datasets through Gene Expression Omnibus. Finally, miR-370 was selected as it was only detected in the control specimens and therefore, undetected in the obese and MetS cohorts. Again the C_T value (control $C_T=28 \pm 2.7$) for miR-370 indicated a high level of miR-370 in the controls.

Table 3.8: miRNAs of interest following global miRNA profiling

Detection of miRNAs in EVs from Each Cohort	miRNAs of Interest
Detected control, obese and MetS participants	miR-134 (p=0.03, control vs MetS) miR-491-5p (p=0.01, control vs MetS)
Detected controls, Obese and MetS	miR-133a (p=0.03, Obese vs MetS) miR-133b (p=0.02, control vs MetS)
Detected controls, undetected Obese and MetS	miR-9-3p (control $C_T=27\pm1.6$) miR-202-3p (control $C_T=28\pm3$) miR-205-5p (control $C_T=24\pm1.1$) miR-302c-5p (control $C_T=23\pm1.8$) miR-370 (control $C_T=28\pm2.7$) miR-596 (control $C_T=29\pm1.6$)
Detected Obese and MetS, undetected controls	miR-30e-5p (obese $C_T=34\pm0.8$, MetS $C_T=32\pm1.2$) miR-100-5p (obese $C_T=30\pm0.4$, MetS $C_T=30\pm0.3$)
Detected controls and Obese, undetected MetS	miR-31-5p (control $C_T=34\pm0.9$, obese $C_T=33\pm0.5$) miR-373-5p (control $C_T=27\pm1.2$, obese $C_T=30\pm2$)

3.3.4.1. Selection of Endogenous Control RNAs for Validations

Individual RT-qPCR for miR-100-5p, miR-134 and miR-370 was performed with endogenous control RNAs miR-150-5p and miR-486-5p. Analysis of endogenous control RNAs showed quite a consistent level for each miRNA in the specimens selected for validation. miR-150-5p had an average C_T of 32.37, miR-486-5p had an average C_T of 34.74 and the average C_T for both miRNAs was 33.55 (Figure 3.10). In order to be consistent with analysis of EV-miRNAs, the C_T value of 34.74 for miR-486-5p was selected for normalising the data.

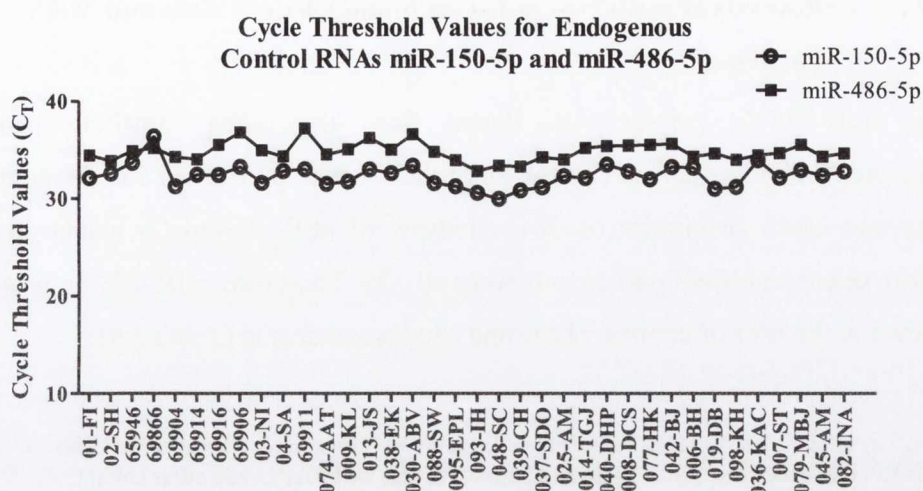


Figure 3.10: Endogenous control RNAs for normalising EV-miRNA validation data
miR-150-5p and miR-486-5p were analysed by RT-qPCR in all specimens. Data represents mean $\pm$ SEM of 3 technical repeats.

3.3.4.2. Validation of miR-100-5p, miR-134 and miR-370 in a Sub-Cohort of Participants

Individual RT-qPCR for miR-100-5p, miR-134 and miR-370 showed miR-370 and miR-134 were undetected in the control, obese and MetS sub-cohorts (n=13) and miR-100-5p (control $C_T=33\pm1$, obese $C_T=33\pm0.6$, MetS $C_T=34\pm1$) was detected in all 3 cohorts. The trend observed for miR-100-5p (1.90 ± 0.22 , $p=0.62$; 1.69 ± 0.29 , $p=0.63$) is similar in the validation phase as the global profiling phase (Figure 3.11).

Attempt to Validate miR-100-5p in a Separate Sub-cohort of 13 Specimens

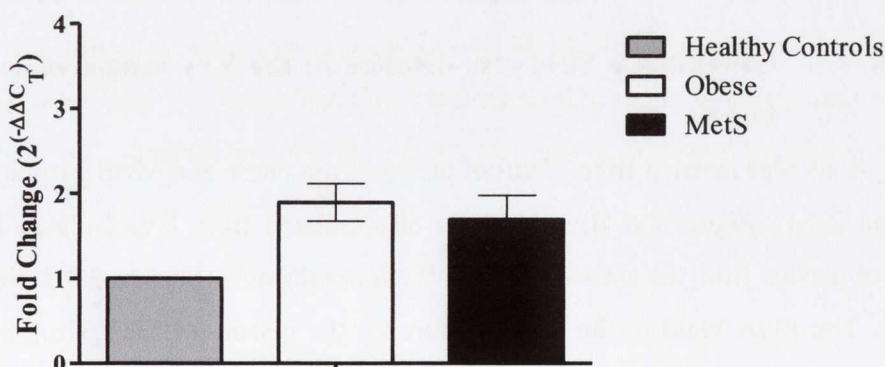


Figure 3.11: Levels of miR-100-5p in the validation cohort
Validation of miR-100-5p in a validation cohort of 13 control, obese and MetS participants. Data represent the mean $\pm$ SEM for miR-100-5p, where results were not significant.

3.3.5. Analysis of miR-758-3p Levels in the EVs of Obese and MetS Participants

miR-758-3p primer was absent from the global profiling panels. Therefore, individual RT-qPCR for this miRNA was performed for the control, obese and MetS specimens (n=36). Analysis of miR-758-3p C_T values in the control, obese and MetS participants were all >35. Therefore, miR-758-3p was not detected in the EVs of control, obese and MetS participants (Table 3.9).

Table 3.9: C_T values for miR-758-3p in Control, Obese and MetS validation cohort

Control	C _T	Obese	C _T	MetS	C _T
01-FH	37	074-AAT	38	014-TGJ	39
02-SH	37	009-KL	37	040-DHP	39
65946	39	013-JS	38	008-DCS	3
69866	40	038-EK	38	077-HK	40
69904	38	030-ABV	38	042-BJ	39
69914	40	088-SW	38	006-BH	38
69916	40	095-EPL	40	019-DB	37
69906	38	093-IH	38	098-KH	37
03-NI	36	048-SC	35	036-KAC	39
04-SA	39	039-CH	35	007-ST	38
69911	40	037-SDO	38	079-MBJ	36
		025-AM	37	045-AM	38
				082-INA	36

3.3.6. Comparison of the Analysis of Plasma and EV RNA and miRNAs

3.3.6.1. Lower RNA Yield was detected in the EVs compared to the Plasma

RNA was isolated from 250µl of plasma from obese and MetS participants (Section 2.3.1., Figure 2.6 B). RNA was also isolated from EVs isolated from 250µl of plasma from the same obese and MetS participants (Section 3.3.2, Figure 3.4 B). The RNA yield in the obese cohort for the plasma (4.53±0.31mg/ml of plasma) compared to the obese cohort for the EVs (3.68±0.32mg/ml of plasma) was not significantly different (p=0.11). However, the RNA yield in the MetS cohort for the plasma (4.90±0.42mg/ml plasma) compared to the MetS cohort for

the EVs ($3.09 \pm 0.20 \text{ mg/ml}$ plasma) was significantly different ($p=0.0058$) (Figure 3.12).

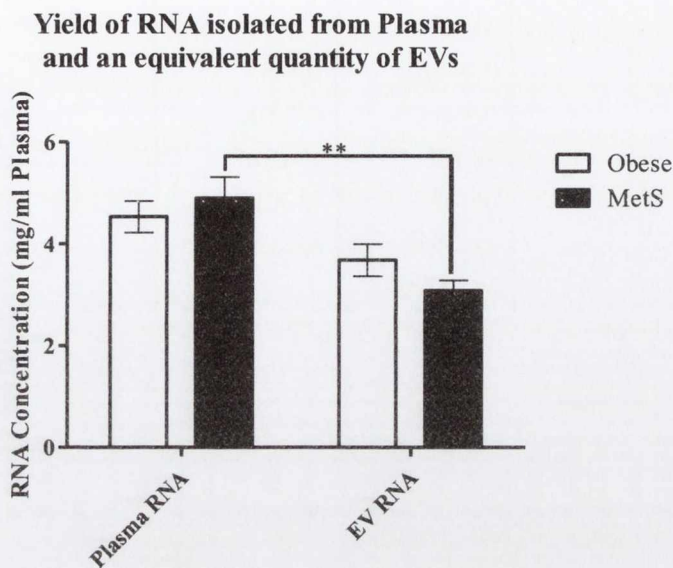


Figure 3.12: Comparison of the yield of RNA in the plasma and EVs of participants with clinical Obesity and MetS.

Data represent the mean $\pm$ SEM for each cohort, where $**p<0.01$.

3.3.6.2. Plasma and EVs from Obese and MetS Participants Contain Different miRNAs and Different miRNA Levels

Female participants from the obese and MetS cohorts were selected for EV-miRNA profiling. Therefore, re-analysing the miRNA data for the same cohort of female participants in the custom miRNA profiling described in Chapter 2 for RNA isolated directly from plasma (Table 3.10), determined 5 of the 25 miRNAs analysed were detected in the female obese cohort, and 14 of the 25 miRNAs analysed were detected in the female obese and MetS cohorts (Figure 3.13). miR-33a-3p, miR-33b-3p, miR-33b-5p, miR-200a-3p, miR-200c-3p and miR-204-5p were undetected in both cohorts and miR-29a-3p, miR-29b-3p, miR-106b-5p, miR-130a-3p and miR-758-3p were undetected in the MetS cohort. Thus, analysis of the difference in the miRNA levels in the plasma and EVs of the same participants was performed.

Table 3.10: List of female participants selected for custom miRNA profiling (plasma)

Obese	MetS
009-KL	014-TGJ
13-JS	007-ST
038-JK	042-BJ
074-AAT	077-HK
98-KH	40-DHP
093-IH	45-AB
037-SDO	008-DCS
030-ABV	

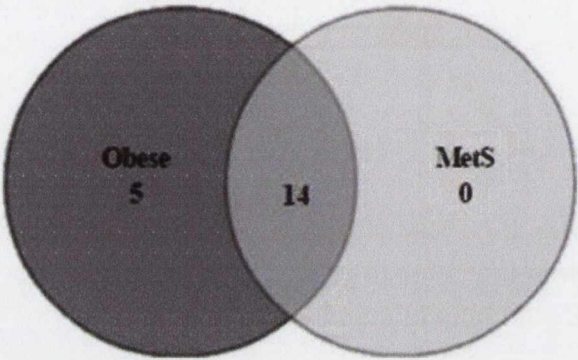


Figure 3.13: Detection of miRNAs in the plasma of Obese and MetS participants

Of the 25 miRNAs analysed in the plasma of obese and MetS participants, 22 of these miRNAs were present on the global miRNA profiling panels used to analyse the miRNA content of EVs isolated from the plasma of the same obese and MetS participants. Specifically, miR-33a-3p, miR-33b-3p and miR-758-3p were not present on the global miRNA profiling panels. Of the 22 miRNAs present on the global miRNA profiling panel, 11 miRNAs were detected in the EVs. Two miRNAs (miR-107 and let-7f-5p) were detected in the obese cohort, 3 miRNAs (miR-15a-5p, miR-103a-3p and miR-106a-5p) were detected in the MetS cohort and 6 miRNAs were detected in both the obese and MetS cohorts.

As there were 11 miRNAs detected in both the plasma and EVs, these miRNAs were further analysed. Analysis of each miRNA in the plasma and EVs for the MetS cohort and the obese cohort found 11 miRNAs to be decreased (higher C_T values) in both the obese and MetS cohorts in the EVs compared to the

plasma (Table 3.11). Analysis of this difference identified miR-223-3p (p=0.009), miR-130a-3p (p=0.04), miR-92a-5p (p=0.01), let-7f-5p (p=0.02), miR-23a-3p (p=0.003) and miR-15a-5p (p=0.01) to be significantly decreased in the EVs of the obese cohort compared to the plasma of the obese cohort. Statistical analysis of the MetS cohort identified miR-223-3p (p=0.02), miR-107 (p=0.03), miR-92a-5p (p=0.001), let-7f-5p (p=0.002), miR-23a-3p (p=0.007) and miR-23b-3p (p=0.01) to be significantly decreased in the EVs compared to the plasma.

Table 3.11: Difference in C_T values obtained for the plasma compared to the EVs in the Obese and MetS cohorts.

	Obese– Plasma	Obese– EVs	Difference C _T	MetS– Plasma	MetS– EVs	Difference C _T
miR-223-3p	23.83	29.99	6.17	25.24	29.93	4.69
miR-126-3p	26.31	28.79	2.48	28.03	28.60	0.56
miR-107	29.52	34.03	4.51	30.25	33.91	3.66
miR-106a-5p	27.30	33.46	6.16	28.64	31.67	3.03
miR-103a-3p	28.27	33.28	5.02	28.73	32.03	3.30
miR-92a-5p	25.25	31.69	6.44	26.77	30.45	3.68
let-7d-5p	30.92	31.82	0.91	30.28	32.52	2.24
let-7f-5p	27.82	33.38	5.54	28.15	33.56	5.40
miR-23a-3p	23.96	29.18	5.22	25.42	28.43	3
miR-23b-3p	26.03	29.71	3.67	27.35	30.99	3.64
miR-15a-5p	28.29	36.76	8.47	30.40	32.26	1.86

Interestingly, comparison of the fold changes of the MetS cohort compared to the obese cohort in the plasma and EVs found that 9 miRNAs showed the opposite fold change for the MetS cohort compared to the obese as detailed in Figure 3.14. Of note, miR-15a-5p was significantly increased in the plasma (22.62±0.52, p=0.01) but decreased in the EVs (-4.32±1.84, p=0.39) (Figure 3.14).

Comparison of miRNA Levels in the Plasma and EVs from MetS Participants Compared to Obese Participants

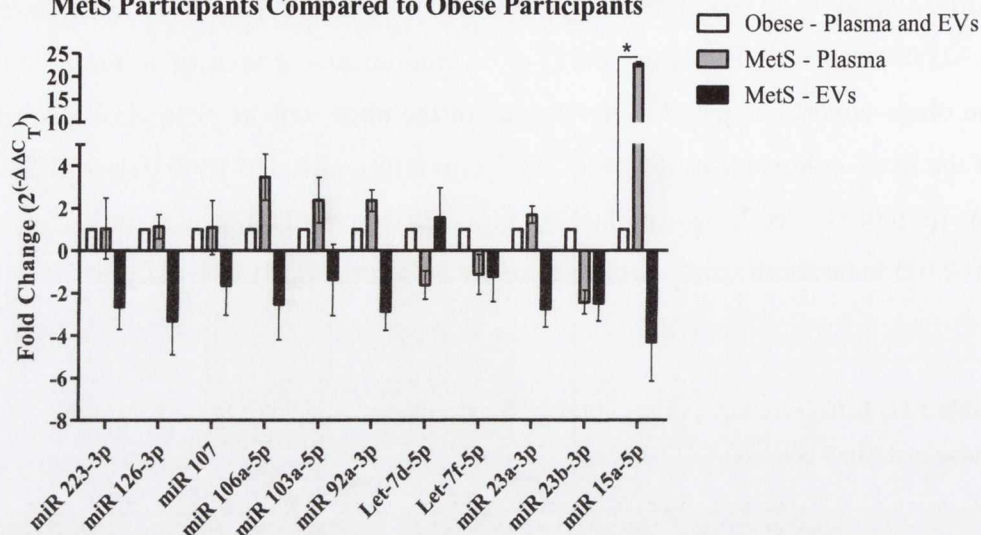


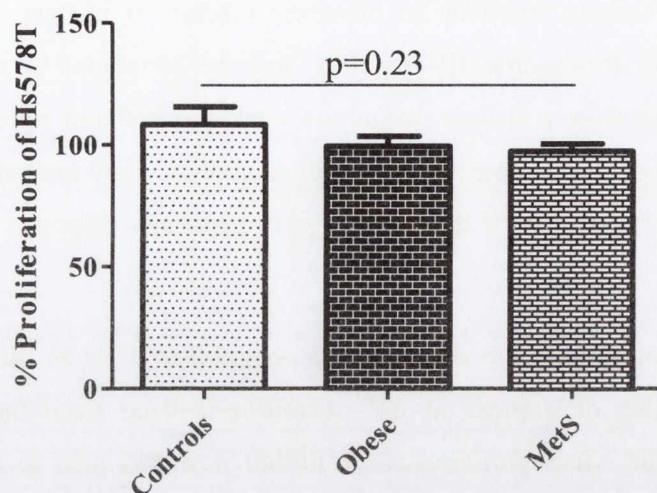
Figure 3.14: Comparison of miRNA levels in the plasma and EVs from Obese and MetS participants

Data represent the mean $\pm$ SEM, where $*p < 0.05$.

3.3.7. EVs from Obese and MetS Participants have no Functional Effects on Hs578T Breast Cancer Cells

Treatment of Hs578T cells with EVs from obese and MetS participants did not increase the growth rate (as would be expected) of this cell line variant in comparison to Hs578T cells treated with healthy control EVs (Figure 3.15 A). In fact, there was no difference in the growth rate following treatment with any EV. Also, treatment of the Hs578T cell line variant with EVs from obese and MetS participants did not induce the cells to resist *anoikis* compared to treatment of the cells with control EVs (Figure 3.15 B)

A. Treatment of Hs578T cells with 10ug/well EVs from Control, Obese and MetS Participants



B. Resisance of Hs578T Cells treated with 10μg/well EVs from Control, Obese and MetS Participants to *Anoikis*

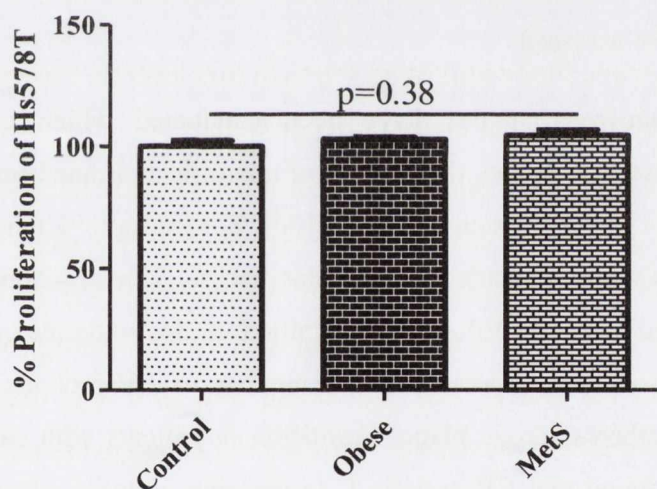


Figure 3.15: Functional assessment of the effects of EVs co-cultured with Hs578T cells
A. proliferation assay and **B.** *Anoikis* assay. Data is expressed as the mean $\pm$ SEM of 3 independent experiments, where results were not significant.

3.4. Discussion

Cell-derived vesicles known as EVs are gaining more and more attention as a novel and unique approach to detecting disease. It has recently become apparent that disease-specific EV profiles may be beneficial to distinguish a healthy phenotype from a disease phenotype, such as MetS and cancer. Not only may EV profiles differentiate between these phenotypes, they may also be useful to monitor the progression of the disease and assess any response to treatment [195].

This chapter sought to discover the potential of EVs as biomarkers for MetS by analysing differences in EV quantities isolated from the plasma of control, obese and MetS participants. A further aim was also to discover the potential of EV-miRNAs as biomarkers for MetS and to determine the differences in the miRNA profile of EV-miRNAs in healthy control, obese and MetS participants compared to the plasma-miRNA profile (Chapter 2). The potential of obese and MetS EVs to induce alterations in the functionality of a TNBC cell line (Hs578T) was also assessed.

While numerous studies have been conducted which examine EV quantities in obese participants the method of quantification has been specific to an obesity related disorder, such as CVD [196]. For example, Kranendonk *et al* [196] isolated EVs from patients with vascular disease, 51% of whom manifested MetS as described by the NCEP:ATPIII definition. In this study the quantification of EVs was based on the presence of a number of EV-proteins specifically associated with atherosclerotic plaque formation in patients with cardiovascular problems. They found that EV-cystatin C (a cysteine protease, present in obese subjects and associated with CVD) was significantly higher ($p=0.01$) in MetS participants ($9.97\text{pg}/\mu\text{g}$) compared to non-MetS participants ($9.84\text{pg}/\mu\text{g}$). In the results presented in this chapter, the quantity of EVs was assessed using the Bio-Rad BCA assay. This determined that the difference in the quantities of EVs in the control ($171.8\pm 25.9\mu\text{g}/\text{ml}$ plasma), obese ($136.2\pm 18.7\mu\text{g}/\text{ml}$ plasma) and MetS ($204.2\pm 23\mu\text{g}/\text{ml}$ plasma) cohorts were significantly different (one-way *ANOVA* $p=0.01$). Interestingly, the quantity of EVs in the control and MetS cohorts was significantly different ($p=0.05$) and the quantity of EVs in the obese and MetS cohort was also significantly different ($p=0.004$) when analysed by

post-hoc Tukey analysis. This result is pertinent to this study as our goal is to differentiate obesity from MetS, in view of the obstacle clinician's face when diagnosing MetS, as obesity is central to its definition (See Section 1.1.2.1) [155]. Also, our results are consistent with the findings of Agouni *et al* [89, 90] (See Section 1.2.1) who found in two separate studies EVs to be increased in MetS compared to healthy controls when analysed by flow cytometry following labelling of the EVs with antibodies against surface antigens.

The first step in the analysis of EVs isolated from our study cohorts was to determine whether the population isolated was in fact composed of EVs. Recently, the Society for Extracellular Vesicles [192] published a joint statement regarding the minimal set of EV experimental characteristics required in order to claim an EV population (See Section 3.3.1). Firstly, immunoblotting was performed to detect a minimum of 3 EV-specific proteins, of which we chose CD81 (a transmembrane or lipid bound extracellular protein), PDC6I/Alix (a cytoplasmic protein) and Grp-94 (an intracellular protein – negative control). Secondly, EV populations were characterised by 2 methods, *i.e.* TEM and NTA. Recently, an article described the characterisation of EVs based on lipid concentration [193]; this study showed that protein-lipid ratios were different for different types of EVs (exosomes, microvesicles and apoptotic bodies). As the focus of our study is obesity and MetS, the lipid concentration of the EVs from each cohort was determined, and the results showed that the protein/lipid ratios of vesicles isolated from the healthy control (0.330 ± 0.1 protein-lipid ratio), obese (0.225 ± 0.8 protein-lipid ratio) and MetS (0.132 ± 0.4 protein-lipid ratio) participants' plasma specimens were the same ($p=0.23$). These results taken together indicate a single EV population of EVs was isolated from each cohort by the filtration and ultracentrifugation technique.

One of the main aims of this chapter was to determine whether EVs, EV-RNA and EV-miRNAs have potential as biomarkers for MetS and how these compare to plasma-RNA and plasma-miRNA biomarkers also for MetS. As is stated above, there was a significant ($p=0.004$) difference in the quantities of EVs isolated from the plasma of obese and MetS participants. Quantification of RNA derived from control, obese and MetS participants EVs found a significant difference in the yield of RNA derived from the EVs of the control cohort compared to the MetS cohort ($p=0.049$) but not the obese cohort compared to the

control cohort ($p=0.05$) or the obese cohort compared to the MetS cohort ($p=0.7$). To analyse whether EV quantity and EV-RNA yield are sensitive enough to discriminate healthy controls from obesity and MetS, and obesity from MetS, we performed Receiver Operator Characteristic Curve (ROC) analysis to determine the area under the curve (AUC). ROC analysis is commonly used to assess the diagnostic ability of a test to discriminate between healthy controls and disease populations; we have also used it to discriminate between two disease states. ROC analysis determines an AUC value, which is indicative of the tests overall diagnostic ability. ROC analysis determined the quantity of EVs in the MetS cohort is sensitive to discriminate MetS from healthy controls ($p=0.049$) and MetS from obesity ($p=0.003$). The EV-RNA yield was not sensitive to discriminate the MetS cohort from the control cohort but was tending towards significance ($p=0.08$) or MetS from obesity ($p=0.48$). To the best of our knowledge, a comprehensive study of the plasma EV-RNA yield in obese and MetS participants compared to healthy controls and indeed EV-RNA yield in obese participants compared to MetS has not been performed. However, results obtained in this laboratory have shown increased ($p<0.001$) EV-RNA in the sera of breast cancer patients (TNBC, ER+, HER2+) compared to healthy control sera [197].

In Chapter 2 RNA was isolated from 250 μ l of plasma, therefore EVs were purified from 250 μ l of plasma and RNA was subsequently isolated from the total volume of EVs (which were re-suspended in 250 μ l PBS). The RNA yield was therefore comparable in the plasma and EVs. Comparison of the RNA yield in the obese cohort found no significant difference ($p=0.11$) in the plasma (4.53 ± 0.31 mg/ml of plasma) compared to the EVs (3.68 ± 0.32 mg/ml of plasma); however, comparison of the RNA yield in the MetS cohort found a significant difference ($p=0.006$) in the yield of RNA in the plasma (4.90 ± 0.42 mg/ml plasma) compared to the EVs (3.09 ± 0.20 mg/ml plasma). This indicates that the progression from obesity to MetS results in a reduction in EV-RNA quantity.

In recent years, miRNAs have shown great potential as biomarkers for the diagnosis and prognosis of diseases including metabolic disorders and cancer [198] (See Section 1.2.4). Such expression profiling, using clinical specimens has primarily focused on investigating the miRNA expression in either peripheral blood [199] or in cell-free serum [157] or plasma [200, 201]. However, this approach has limitations, including degradation of freely circulating miRNAs by

ribonucleases and lack of standardised protocols for expression analysis [191]. To investigate the potential of EV-miRNAs as biomarkers for MetS we performed global miRNA profiling on RNA isolated from EVs which had been purified from the plasma of control, obese and MetS participants. Analysis of the levels of 372 miRNAs (including 22 of the 25 miRNAs present on the custom panels in Chapter 2) in the EVs of these cohorts identified 14 miRNAs of interest (miR-9-3p, miR-30e-5p, miR-31-5p, miR-100-5p, miR-134, miR-133a, miR-133b, miR-202-3p, miR-205-5p, miR-302c-5p, miR-370, miR-373-5p, miR-491-5p, miR-596). Four of these miRNAs were detected in the control, obese and MetS cohorts (miR-133a, miR-133b, miR-134, miR-491-5p), 6 were detected in controls only (miR-9-3p, miR-202-3p, miR-205-5p, miR-302c-5p, miR-370, miR-596), 2 were detected in the obese and MetS cohorts (miR-30e-5p, miR-100-5p) and 2 were detected in the control and obese cohorts (miR-31-5p, miR-373-5p). From these 14 miRNAs, miR-100-5p, miR-134 and miR-370 were selected for validation in a separate sub-cohort of control, obese and MetS participants (n=13). Upon validation miR-100-5p- although not significantly altered- showed the same trend in the validation cohort as in the profiling cohort, *i.e.* the levels were increased in the obese and MetS cohorts compared to controls. As miR-100-5p has not been placed on the custom profiling panel for the plasma in Chapter 2, miR-100-5p, miR-134 and miR-370 were analysed in the plasma of a selection of obese and MetS participants. miR-100-5p (3.42 ± 0.40 , $p=0.02$) was significantly increased in the MetS cohort compared to the obese cohort (Appendix I, Section F, Figure F.5).

Analysis of the miRNA levels freely circulating in the plasma and in the EVs from the same obese and MetS participants identified 11 miRNAs that were detected in both the plasma and EVs (miR-15a-5p, miR-23a-3p, miR-23b-3p, 92a-3p, miR-103a-3p, miR-106a-5p, miR-107, miR-126, miR-223-3p, let-7d-5p, let-7f-5p). Analysis of the C_T values for each of these miRNAs in the plasma and EVs showed all miRNAs to be decreased in the EVs compared to the plasma, with some significantly decreased. Therefore, a useful biomarker for MetS should not be analysed simply in plasma or EVs but in a combination of both.

Interestingly, analysis of the fold change in the plasma and EV for the MetS cohort compared to the obese cohort found 9 of these miRNAs showed the opposite trend in the plasma and EVs, while miR-23b-3p and let-7d-5p showed the same trend when comparing fold-change for the MetS cohort and the obese

cohort in the plasma and EVs. Also of interest, miR-15a-5p was detected in both the plasma and EVs. This miRNA was selected as a potential plasma-miRNA biomarker for validation; however, it did not validate in the plasma. In the plasma study miR-15a-5p (-2.8 ± 1.41 -fold, $p=0.4$) was detected in both the obese and MetS cohorts and was decreased by >2 -fold, however in the EVs miR-15a-5p (22.63 ± 0.52 -fold, $p=0.01$) was only detected in the MetS and was significantly increased.

Remarkably, analysis of miR-100-5p predicted targets using miR-walk identified ABCA1 as a predicted target of this miRNA. It was also predicted to target VLDL and Cytochrome P450 26B1-a monooxygenase that catalyses many reactions including drug metabolism and lipid and cholesterol synthesis. miR-100-5p and miR-758-3p are both predicted to target cisplatin resistance-related protein. This is interesting as obesity and MetS lead to an increased risk of TNBC and as cisplatin is used in the treatment of TNBC any perturbations of factors regulating the expression of cisplatin resistance-related protein 9 could lead to resistance to cisplatin treatment.

Unfortunately, treatment of Hs578T cells with EVs from healthy control, obese and MetS individuals did not result in an increased proliferation of the Hs578T cells treated with EVs from the obese and MetS cohorts compared to controls. Similarly, treatment of the Hs578T cells with EVs from the obese and MetS individuals did not increase the ability of the cells to resist *anoikis* compared to controls. These results show that unlike EVs isolated from the conditioned media of breast cancer cell lines, which increase the proliferation, migration and invasion of breast cancer cell lines [188], EVs derived from Obese and MetS individuals are not aggressive enough to cause such effects.

3.5. Conclusion

The question of the necessity of EVs and their cargo as potential biomarkers for disease has had much scrutiny due to the complexity of EV isolations and the ongoing examination of EV definitions and markers for identification. Analysis of EV quantity in numerous diseases has shown an increase in the EV quantity in disease compared to healthy controls. In this study, we have shown an increased quantity of EVs in MetS compared to obesity, and also that this increase was sensitive enough as a diagnostic tool for MetS. To the best of our knowledge this is the first study illustrating EVs as biomarkers for metabolic disorders and also the first study differentiating 2 metabolic disorders that are highly related. The potential of plasma- and EV-miRNAs as biomarkers for MetS was also studied in this chapter and Chapter 2. This chapter identified miR-100-5p as a potential EV-miRNA biomarker for MetS compared to controls. Interestingly, analysis of miR-100-5p in a selection of plasma specimens identified miR-100-5p as also a potential plasma-miRNA biomarker also. However, this similarity in miRNA levels in the plasma and EVs was not seen for nine other miRNAs that were detected in both the plasma and EVs, in fact the trend seen for both the plasma and EVs was opposite. In conclusion, this study shows for the first time that EVs themselves, EV-miRNAs and plasma-miRNAs are minimally-invasive biomarkers that can discriminate MetS from obesity and healthy controls. As the EV-miRNA content and the plasma- miRNA content are quite different, biomarker discovery may not simply be plasma- or EV-based but a combination of both. Further studies in larger cohorts, including appropriate healthy controls, and co-analysing each of these, are now warranted.

Chapter Four: Investigation of the Bioenergetics of Two Triple Negative Breast Cancer Cell Line Variants

4. Abstract

Introduction: Triple negative breast cancer (TNBC) accounts for 15-20% of all breast cancers and for a disproportionate number of breast cancer deaths. The poor outcome associated with TNBC is due to its innate aggressiveness and is augmented by the lack of targeted treatments available for this cancer type. Chemotherapeutic agents such as anthracyclines, taxanes and platinum compounds are used to treat patients with TNBC; however, the response rate to these treatments are very low, but TNBC patients often do better on platinum drugs than other breast cancer subtypes. Obesity is a known risk factor for many types of cancer, including breast cancer, and many epidemiological studies have linked TNBC risk to obesity. Obesity and metabolic syndrome (MetS) are both disorders of metabolism and since there is an association between these and TNBC, the metabolic phenotype of two TNBC cell line variants was investigated, in order to determine how alterations in the metabolism of these cell line variants contributes to the aggressiveness of TNBC.

Methods: The metabolic phenotypes of two TNBC cell line variants, namely Hs578T and Hs578Ts(i)₈, were investigated using the Seahorse extracellular flux assay to determine mitochondrial oxidative phosphorylation and glycolytic basal rates. Contributions of different steps of the metabolic pathway to the metabolism of these cell line variants were further elucidated by the addition of inhibitors or uncouplers of components of the electron transport chain or glycolysis. The mitochondrial biomass of both cell line variants was assessed by the citrate synthase assay and immunoblotting for voltage dependent anion channel 1 (VDAC1) protein. Potential alterations in mitochondrial membrane potential were investigated by staining of the cells with 10-N-Nonyl Acridine Orange (NAO) followed by flow cytometry. Short-term treatment of the aggressive Hs578Ts(i)₈ TNBC cells with the pyruvate dehydrogenase kinase inhibitor Dichloroacetate (DCA), the endocrine cytokine interleukin 6 (IL-6) receptor inhibitor Tolicizumab and both cell line variants with the neuropeptide Neuromedin U (NmU) were also performed to evaluate their effects on cell metabolism as NmU and IL-6 have been implicated in obesity, MetS and cancer. Assessment of NmU and IL-6 in both cell line variants and in the plasma of healthy control, obese and MetS participants was performed by ELISA.

Results: Seahorse extracellular flux analysis was used to compare oxygen consumption rates (OCR) and glycolytic flux in Hs578T and Hs578Ts(i)₈ cell line variants. We demonstrated substantial oxidative phosphorylation, as measured by basal OCR in Hs578T cells (292.25 ± 2.30 pMoles/min/mg protein) with significantly ($p=7.64 \times 10^{-6}$) lower basal OCR in the Hs578Ts(i)₈ cells (140 ± 6.830 pMoles/min/mg protein). This decrease in OCR was not due to a reduction in mitochondria themselves, since VDAC1 protein levels and the citrate synthase assay determined that the biomass of the mitochondria in Hs578Ts(i)₈ cells was equivalent to the biomass of Hs578T cells (74.34 ± 6.71 versus 66.93 ± 2.28 nmol/min/mg protein). Supporting the notion of reduced mitochondrial function was the indirect measurement of mitochondrial membrane potential using NAO staining which indicated a 2.0 ± 0.9 -fold significantly decreased ($p=0.0006$) accumulation of NAO in the Hs578Ts(i)₈ cells when compared to the Hs578T cells. This indicated that the mitochondria of Hs578Ts(i)₈ cells were dysfunctional and/or lacking substrate (*e.g.* succinate) supply to the mitochondria for oxidative phosphorylation. Simultaneously to OCR measuring, the Seahorse Extracellular Flux Analyser evaluated the glycolytic activity of the cells and it was found that while Hs578T cells were performing basal glycolysis (27.75 ± 2.18 mpH/min/mg protein), Hs578Ts(i)₈ cells showed a significantly ($p=0.005$) higher degree of glycolytic flux (39 ± 1.47 mpH/min/mg protein).

Subsequent analysis of intracellular levels of NmU in Hs578T and Hs578Ts(i)₈ cell line variants found NmU to be decreased (1.5 ± 0.02 -fold, $p=0.02$) in the more aggressive Hs578Ts(i)₈ cells. Treatment of Hs578T cells with exogenous NmU-25 peptide resulted in a significant decrease in the basal oxidative phosphorylation by 1.4 ± 0.7 -fold ($p=0.0003$) and the maximal oxidative phosphorylation by 1.7 ± 0.5 -fold ($p=7.44 \times 10^{-5}$). However, a concomitant increase in glycolysis was not observed. In fact, the basal glycolytic rate was significantly ($p=0.002$) decreased following NmU treatment. NmU-25 peptide treatment had no apparent effect on the mitochondrial oxidative phosphorylation of Hs578Ts(i)₈ cells, but did significantly ($p=1.3 \times 10^{-6}$) inhibit glycolysis.

Investigation of the levels of IL-6 secreted by the Hs578T and Hs578Ts(i)₈ cells found the Hs578Ts(i)₈ cells to secrete significantly more (2.6 ± 0.15 -fold, $p=0.0002$) IL-6 than the Hs578T cells. Treatment of Hs578Ts(i)₈ cells with an

inhibitor of the IL-6 receptor, Tocilizumab again significantly decreased their basal (1.5 ± 0.9 -fold, $p=0.0007$) and maximal (1.5 ± 0.8 -fold, $p=0.0002$) mitochondrial oxidative phosphorylation. Similarly, basal glycolysis was also significantly (1.8 ± 0.8 -fold, $p=0.0003$) decreased in Hs578Ts(i)₈ cells following Tocilizumab treatment.

Analysis of NmU in the plasma of female healthy controls and participants with obesity and MetS showed NmU to be significantly decreased ($p=0.02$) in the obese cohort (0.89 ± 0.05 ng/ml) compared to the control cohort (1.16 ± 0.1 ng/ml). The MetS cohort was decreased (0.93 ± 0.06 ng/ml) compared to the control cohort and was tending towards significance ($p=0.06$) using an ELISA from one supplier, this was not observed when using an ELISA from another supplier, therefore further analysis is required. Analysis of the levels of IL-6 in the plasma of the female participants in the obese and MetS cohorts determined IL-6 was increased in the MetS cohort (2.30 ± 0.5 pg/ml) compared to the obese cohort (1.59 ± 0.2 pg/ml) by 1.44 ± 0.48 -fold ($p=0.19$); however, this was not significant.

Conclusion: TNBC has been epidemiologically linked to obesity and here we have shown that TNBC cell line variants present alterations in their metabolic phenotype. In our model, progression of TNBC to a more aggressive phenotype results in the inhibition of normal aerobic mitochondrial oxidative phosphorylation and a switch to aerobic glycolysis. The decrease in mitochondrial oxidative phosphorylation may be due to mitochondrial dysfunction in the aggressive cell line and this may have potential as a biomarker for TNBC, however this would need further study in additional TNBC cell lines and TNBC tissue specimens. NmU and IL-6 levels were shown to have the same trend in plasma specimens and cell lines, *i.e.* NmU was decreased and IL-6 was increased in the plasma from obese and MetS participants compared to controls, although not significantly, and significantly in the aggressive TNBC cell line variant compared to the parental TNBC cell line variant. We hypothesised that these metabolic factors may link obesity and MetS to TNBC, however upon treatment of the cells with exogenous NmU-25 and Tocilizumab there was a further decline in the mitochondrial oxidative phosphorylation and glycolysis of the cells. The metabolic switch taking place in Hs578T cells as they progress to aggressive TNBC holds great promise as the mechanism of increased aggressiveness in these cells and as a potential target for therapy.

4.1. Introduction

Metabolic Syndrome (MetS) and obesity are complications which arise from many factors including alterations of an individuals' metabolism. Physiological metabolism requires an energy homeostasis; this regulates energy expenditure and consumption [202], which is essential for health [52] (See Section 1.3). Obesity and MetS arise due to many factors including sedentary lifestyle [203, 204]. Humans, over the last number of decades have increased energy intake, in the form of calories, while at the same time reducing their energy expenditure [52]. Over the course of 1 day of inactivity there may be negative effects on cellular processes in tissues regulating MetS risk factors, such as plasma triglycerides and HDL cholesterol [205]. Obesity is fundamental to MetS [155] and both increase the risk of cancer at multiple sites. This significant association between obesity and cancer was detected over a decade ago. It has been estimated that the overall risk of death from cancer is 1.5- to 1.6-fold higher for men and women with a BMI >40 Kg/m².

Epidemiological data has closely linked obesity and MetS to breast cancer, the most frequently occurring cancer in women, *i.e.* women have a 12% life-time risk of developing this disease. There has been a specific link shown between MetS, its components and breast cancer [206]. Data relating to post-menopausal women shows a positive association between obesity, breast cancer risk and reduced survival in women already diagnosed with breast cancer [207]. In a meta-analysis of 9 studies of breast cancer, obesity and MetS, MetS was associated with a 52% increased risk of breast cancer ($p<0.001$) in post-menopausal women. There was also a significantly increased risk of breast cancer in post-menopausal women with hyperglycaemia ($p=0.005$), high blood pressure ($p=0.03$) and low HDL cholesterol ($p=0.008$) indicating MetS components are also independent risk factors for breast cancer [208].

Overall survival and disease-free survival were significantly shorter in premenopausal obese women with TNBC compared to non-obese women with TNBC [206]. Interestingly, the impaired glucose tolerance seen in MetS correlates with disease progression in individuals treated with paclitaxel, 5-fluorouracil, epirubicin and cyclophosphamide [209]. MetS-related insulin resistance (IR) (See Section 1.1.3.3.2) and sub-acute inflammation (See Section 1.2.2 [IL-6]) offer

potential explanations for the mechanistic link between breast cancer and MetS [206]. However, this data is inconsistent. For TNBC, specifically, a meta-analysis evaluating the association between TNBC and obesity found women with a BMI of 30 or greater had a significantly (95% CI: 1.03-1.40) increased probability of presenting with TNBC. Also, classification of women based on menopausal status showed a significant association between BMI and TNBC in pre-menopausal women (95% CI: 1.23-1.65) but not post-menopausal women (95% CI: 0.79-1.24). Results suggest pre-menopausal women with a BMI equal to or greater than 30 have a 42% increased risk of developing TNBC than both over (25>BMI<30)- and normal-(18.5>BMI<25) weight individuals [210].

Obesity triggers systemic and metabolic alterations that can influence tumorigenesis [52]. It also results in the production of hormones, including adipokines and cytokines from adipose tissue. Increased production of the cytokine IL-6 is typically seen in obese individuals [211], as adipose tissue macrophages have altered properties [52] and their numbers are increased [211]. IL-6 also induces insulin resistance (IR). Treatment of leptin knockout mice with a neutralising IL-6 antibody reversed the effects of IL-6 on IR [212]. Interestingly, the receptors for the adipokine adiponectin produced by adipocytes are expressed on many cancer cells, including breast cancer cells (*i.e.* MCF-7, T47D, SKBR3, and MDA-MB-231) [52, 213]. Data points to adiponectin as an inhibitor of tumorigenesis, due to its ability to activate AMPK and inhibit mTORC1 and other pro-tumorigenic signalling molecules [214]. However, as is stated in Section 1.2.2, adiponectin is decreased in obesity and MetS. Leptin, another adipokine, which is increased in obesity and MetS (See Section 1.2.2) has pro-tumorigenic effects via its growth factor-like activity, resulting in angiogenesis [52]. Metabolism is fundamental to obesity and MetS, however, the involvement of metabolism in MetS and obesity is not specific to these disorders, it is also a fundamental part of cancer biology, even being termed a hallmark of cancer [145]. Cancer cell metabolism has recently been re-energised in the field of cancer research (See Section 1.3).

In 1924 Otto Warburg put forward what is now termed the ‘Warburg Effect’. The premise of the Warburg Effect is that cancer cells switch from using their mitochondria to produce ATP energy through oxidative phosphorylation to using glycolysis for ATP production. However, not only do cancer cells exhibit

altered glucose metabolism they also exhibit increased fatty acid synthesis and increased rates of glutamine metabolism [215]. It should also be noted however that this switch is not performed by all cancer cells, some cancer cells have normal mitochondrial oxidative phosphorylation, while others have altered or non-existent mitochondrial oxidative phosphorylation [216]. Oxidative phosphorylation is the breakdown of glucose to pyruvate which then enters the Krebs cycle in the mitochondria to produce ATP energy when oxygen is available (Figure 1.6 A). Glycolysis, on the other hand, is a much less efficient method that produces 18 times less ATP than oxidative phosphorylation and lactate under either aerobic and anaerobic conditions [146] (Figure 1.6 B). Under much scrutiny is the question of why, even in oxygen-rich conditions, cancer cells choose glycolysis, a much less efficient energy source, rather than oxidative phosphorylation. Vander Heiden *et al* [146] have proposed that the metabolism of cancer cells is altered to increase the uptake and incorporation of nutrients so as to increase growth rates. Normal cells must receive a signal from growth factors in order to uptake nutrients to divide; cancer cells have overcome this need through genetic mutations that functionally alter receptor-initiated signalling pathways, another hallmark of cancer [145]. Therefore, cancer cells show a constant uptake of nutrients independently of growth factors. Other proposed explanations for Warburg's observations are damage to the mitochondria or adaptation to hypoxia; however, it has been shown in many cancer cells that mitochondria are not impaired and also cancer cells have been shown to switch to glycolysis before the onset of hypoxia. The evolving data suggests a system that is dynamically regulated and programmed to fit the requirements of the cell for proliferation [146].

In this study, we proposed to investigate the metabolic bioenergetics of two TNBC cell line variants, as TNBC is associated with obesity and thus, MetS. Our model consisted of Hs578T cells and its isogenic more aggressive subclone Hs578Ts(i)₈ cells. We proposed to assess the metabolic phenotype of these cells using the Seahorse Extracellular Flux Analyser. Having determined there to be a switch in the metabolism of these cells we aimed to determine the switch conferring the altered metabolic phenotype of these cells and finally we aimed to reverse the switch by treating the cells with NmU-25 peptide and the IL-6

receptor (IL-6R) inhibitor Tocilizumab as both NmU and IL-6 have roles in energy, feeding, metabolism and cancer.

4.2. Methods

4.2.1. Cell Culture

Hs578T and Hs578Ts(i)₈ cells were cultured as described in Section 2.2.5.2.1.

4.2.2. Measurement of the Mitochondrial Function by the Seahorse Extracellular Flux Analyser

The metabolic rate of Hs578T and Hs578Ts(i)₈ cell line variants was analysed using the Seahorse XF^c24 Flux Analyser (XF24-3, Seahorse Bioscience). Assays were performed according to manufacturers' instructions. Specifically, on Day 1, cells were seeded at 1.8×10^4 cells/150µl/well in a 24-well XF microplate (Biosciences, Cat. #: 100777-004) and incubated for 2 hours to allow attachment of cells. After 2 hours 150µl of complete medium (See Section 2.2.5.2.1, *i.e.* DMEM supplemented with 10% FBS, 2mM L-Glutamine and 10µg/ml insulin) was added (seeded in 150µl to ensure cells attach to bottom of well and not to sides of well) and cells were incubated overnight at 37°C/5%CO₂. Also on Day 1, 500µl of calibrant solution (Seahorse Bioscience, Cat. #: 100840-000) was dispensed into each well of a 24-well XF24 utility plate (Seahorse Bioscience, Cat. #: 100880-001) with sensor cartridge and incubated overnight at 37°C in a non-CO₂ incubator (Medical Supply Company; Hybaid Maxi) (Figure 4.1 Day 1).

On Day 2, 10mM D-(+)-glucose (concentrations near 10mM are pre-diabetic levels. Concentrations above this are analogous to a diabetic condition within the cell culture system. This is important as the same effects on cells and molecules are seen *in vivo* and *in vitro*. The consequence to growing cells under conditions that are essentially diabetic is that cells and cell products are modified by the processes of glycation and glycooxidation) was prepared in XF assay medium (Seahorse Biosciences, Cat. #: 102365-100) and the wells of the 24-well plate containing the cells were washed with 500µl/well of the XF assay medium supplemented with 10mM D-(+)-glucose. Subsequently, 525µl of the XF assay medium supplemented with 10mM D-(+)-glucose was added to each well. Cells were incubated for 40mins at 37°C in a non-CO₂ incubator (Figure 4.1 Day 2).

Inhibitors and uncoupler were prepared (oligomycin A 1µg/ml [Sigma, Cat. #: O4876], Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP)

300µM [Sigma, Cat. #: C2920], rotenone 1µM [Sigma, Cat. No.:R88758], antimycin A 1µM [Sigma, Cat #: A8674] and 2-deoxy-D-glucose 30mM [Sigma, Cat #: D8375]) in XF assay medium supplemented with 10mM D-(+)-glucose and 55µl of each inhibitor was loaded into appropriate ports on the sensor cartridge (Figure 4.1 Day 2).

The utility plate and sensor cartridge loaded with inhibitors and uncoupler was placed in the Seahorse instrument and a calibration program was run. Following calibration, the utility plate was removed from the instrument and replaced with the pre-incubated cell plate (the sensor cartridge remained in the instrument) and the program was run.

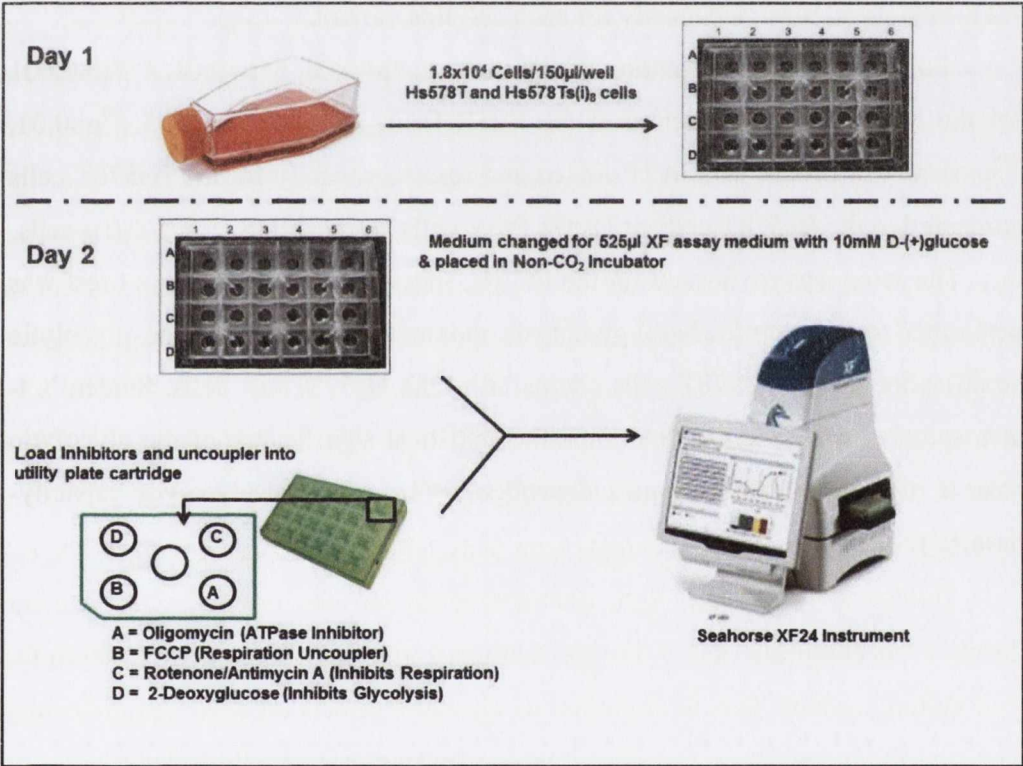


Figure 4.1: Schematic representation of Seahorse Extracellular Flux analysis of Hs578T and Hs578Ts(i)₈ cell line variants

After the run was complete, the 24-well XF plate was recovered and the cells collected for protein isolation. 30µl of cell lysis buffer (Invitrogen, Cat. #: FNN0011) supplemented with 1X protease inhibitor cocktail (Roche, Cat. #: 05892970001) was added to each well and protein lysis and quantification was performed as described in Section 2.2.5.2.5. OCR and ECAR readings were normalised using the protein concentrations.

4.2.2.1. Data and Statistical Analysis

The OCR and ECAR were plotted as illustrated in Figure 2 A and B, respectively. Statistical analysis for the basal OCR, Proton leak (FCCP addition-non-mitochondrial respiration), ATP-linked (basal oxidative phosphorylation-after oligomycin A added), maximal respiration and reserve capacity (maximal respiration-basal respiration) was performed using the Student's t-test to compare the basal OCR, maximal OCR and non-mitochondrial respiration of the Hs578T cells to the Hs578Ts(i)₈ cells. The Student's t-test was also performed to determine the statistical significance of the proton leak of each cell line variant independently (*i.e.* readings for the Hs578T cells after FCCP addition compared to Hs578T readings for non-mitochondrial respiration) the same was done for the ATP-linked and reserve capacity for each cell line variant.

For graphical representation of significance * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for the Hs578T cells compared to the Hs578Ts(i)₈ cells and # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ for proton leak, ATP-linked and reserve capacity for the Hs578T cells compared to the Hs578T cells or Hs578Ts(i)₈ cells compared to Hs578Ts(i)₈ cells.

The same was performed for the ECAR, specifically, the Student's t-test was performed to compare the basal glycolysis, maximal glycolysis and non-glycolytic acidification of the Hs578T cells compared to the Hs578Ts(i)₈ cells. Student's t-test was also performed to determine the statistical significance of the glycolytic reserve of each cell line variant independently (*i.e.* maximal glycolytic capacity-basal glycolysis).

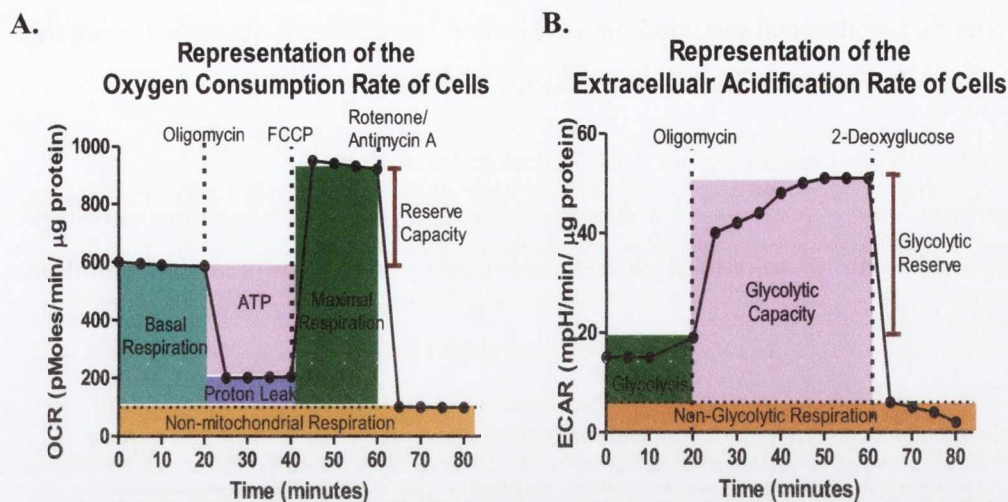


Figure 4.2: Representation graphs of the OCR and ECAR analysis of data

Figure to explain the output from the seahorse extracellular flux analyzer mito stress test.

A. The OCR explains the data analysis of the OCR illustrating the oxygen consumed for ATP-linked and unlinked respiration, the oxygen consumption not linked to respiration, the proton leak representing the remaining respiration not linked to ATP production (sign of mitochondrial damage) and the maximum oxygen the cells can consume for respiration and **B.** the ECAR explains the basal glycolysis before addition of oligomycin A, the glycolytic capacity of the cells following inhibition of ATPase synthase and depletion of oxygen consumption and the glycolytic reserve, indicating the difference in glycolytic capacity compared to basal glycolysis.

4.2.3. Citrate Synthase Assay

Hs578T and Hs578Ts(i)₈ cell line variants were seeded in T25 flasks at 1×10^5 cells/5mls complete medium. Cells were incubated for 24 hours at 37°C/5% CO₂. Cells were collected by scraping and centrifuged at 900rpm for 5mins, washed in 1ml of PBS and centrifuged at 900rpm for 5mins. The cell pellets were re-suspended in 60 μ l of PBS and placed in liquid nitrogen until frozen, then thawed and the freeze-thaw cycle was repeated 3 times to lyse the cells.

Reagents for the assay were prepared in 1ml cuvettes (Sigma-Aldrich, Cat. #: C5416) as described in Table 4.1. Prior to use, samples were incubated on ice. For the analysis, a Libra S12 spectrophotometer (Biochrom) and flatbed recorder (Kipp and Zonen) were used. The spectrophotometer was set at 412nm, 30°C and the recorder was set at 10mm/min chart speed and 0.2 voltage. Tris buffer (0.2M, pH 8.1, Sigma-Aldrich, Cat. #: T1503) was used to blank the spectrophotometer between readings. Each sample was read for 2mins to establish baseline activity, 50 μ l of Oxaloacetate (0.5mM; Sigma, Cat. #: O4126) was then added to start the

enzymatic reaction and recorded for 2mins. Cell Lysates were quantified using the Bio-Rad BCA assay as described in Section 2.2.5.2.5.

Citrate synthase enzyme activity was calculated as follows:

$$(\text{nmolmin}^{-1} \text{ mg}^{-1} \text{ protein}) = (\Delta \text{ Absorbance/min} \times 1,000 \times 1 [\text{volume cuvette}]) / ((13.6\text{mM}^{-1} \text{ cm}^{-1} \times \text{volume of sample used in ml}) \times (\text{sample protein concentration in mg ml}^{-1})).$$

Table 4.1: Concentration and volume of reagents and sample added to each 1ml cuvette

Reagent	Supplier	Cat. No.	Final Concentration	Volume (µl)
Tris Buffer	Sigma	T1503	0.2M, pH 8.1	800
DTNB	Sigma	D8130	0.1mM	100
Acetyl CoA	Sigma	A2181	0.3mM	30
Triton X-100	Sigma	T8787	0.1% in ddH ₂ O	10
Cell lysate				10

4.2.4. Flow Cytometric Analysis of 10-N-Nonyl Acridine Orange (NAO) Staining

[Performed with the help of Dr. Vanesa Martinez]

Hs578T and Hs578Ts(i)₈ cell line variants were seeded at 1x10⁵ cells/2ml/well of a 6-well plate and were allowed to attach overnight. Cells were trypsinised, washed in PBS, re-suspended and incubated in 1ml of 10µM NAO (Molecular Probes, Cat. #: A1372) in 5% FCS in PBS for 10mins in the dark. Unstained cells were incubated in 5% FCS in PBS for 10mins. Cells were subsequently centrifuged at 900rpm for 5mins at room temperature, washed in 2.5ml PBS (x2), re-suspended in 200µl PBS and transferred to Falcon round-bottom polystyrene tubes (Thermo Scientific; cat #: 352054). Cell staining for NAO was measured using a FACSCanto II flow cytometer (BD Biosciences) and analysed using BD FACSDiva software.

4.2.5. Cell Pellet Protein Extraction

Hs578T and Hs578Ts(i)₈ cell line variants were seed at 2×10^6 cells/10ml of complete medium in T75 flasks. Once at confluency cell pellets were collected, lysed and quantified as in Section 2.2.5.2.5.

4.2.5.1. Immunoblotting for Voltage Dependent Anion Channel 1 (VDAC1) Protein

Immunoblotting for VDAC1 was performed as described in Section 2.2.5.2.5 using 50µg of protein for each cell line variant in triplicate and separated using NuPage® Novex® 4-12% Bis-Tris for protein SDS-PAGE electrophoresis pre-cast gels (Biosciences, Cat. #: NP0323). Antibodies were diluted as follows VDAC1 (Abcam, Cat. #: Ab15890, 1:1000 dilution) and its secondary antibody anti-rabbit (Cell Signalling, Cat. #: 7074s, 1:1000 dilution). β-actin (Sigma, Cat. #: A1978, 1:1000 dilution) was used as a loading control. Densitometry on the bands of protein was performed using NIH ImageJ software and normalised to loading control, β-actin.

4.2.6. Analysis of the Effects of Dichloroacetate (DCA) on Hs578T and Hs578Ts(i)₈ Cell Line Variants

4.2.6.1. Cytotoxicity Assay to Determine the IC₅₀ of DCA

On Day 1, Hs578T and Hs578Ts(i)₈ cell line variants were seeded at 2×10^4 cells/500µl/well in a 24-well plate. On Day 2, cells were treated with 0mM, 5mM, 10mM or 20mM DCA and incubated for 24 hours. Cytotoxicity assays were then taken down as described in Section 3.2.7.2.

4.2.6.2. Seahorse Extracellular Flux Assay with DCA

Hs578Ts(i)₈ cells were seeded at 1×10^4 cells/150µl/well in an XF24-well plate. After a 2 hour incubation, 150µl complete medium was added (to ensure cell attachment to bottom of well) and incubated overnight to allow attachment. DCA (5mM- as determined as the non-toxic concentration of DCA for these cells in Section 4.2.6.1) was added to the appropriate wells and incubated for a further

24 hours (Figure 4.3). The Seahorse Extracellular Flux assay was then performed as per Section 4.2.2 (Figure 4.1, Day 2).

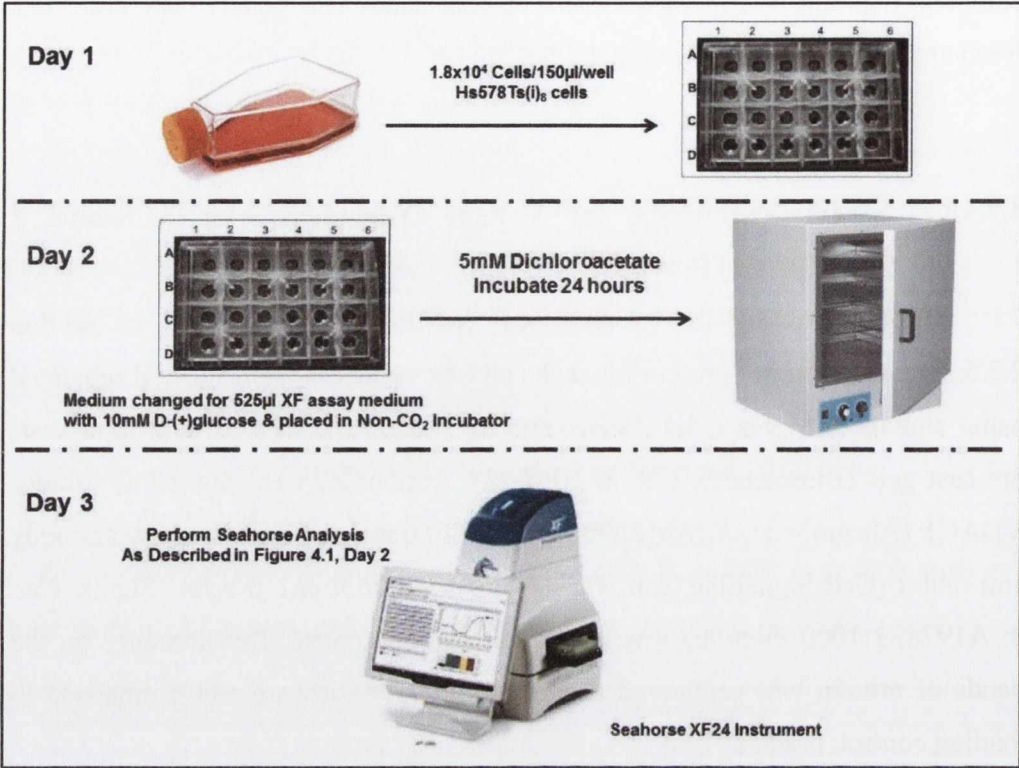


Figure 4.3: Schematic representation of Seahorse Extracellular Flux analysis of Hs578T and Hs578Ts(i)₈ cell line variants including Dichloroacetate treatment.

4.2.7. Neuromedin U (NmU)

4.2.7.1. NmU ELISA

Intracellular NmU was quantified in Hs578T and Hs578Ts(i)₈ cell line variants by ELISA (Peninsula Laboratories, Cat. #: S-1253.0001) which was performed according to the manufacturers' instructions using 30 μ g of total cellular protein diluted in PBS to bring total volume to 50 μ l. Cellular NmU quantification was repeated using another ELISA (Cloud-clone; Cat. #: CEB025Hu) which was performed according to the manufacturers' instructions using 30 μ g of total cellular protein diluted in PBS to bring total volume to 50 μ l as the Peninsula Laboratories ELISA was unreliable.

4.2.7.2. NmU Receptor 1 and 2 Immunoblotting

Immunoblotting for NmU R1 and NmU R2 was performed as described in Section 4.2.5.1. Protein levels were assessed using NmU R1 (C-12) anti-body (Santa-Cruz; Cat. No.: sc-47241; 1 in 200 dilution), its secondary anti-goat anti-body (Sigma, Cat. #: A5420; 1 in 1000 dilution), NmU R2 (P-22) antibody (Santa-Cruz; Cat. No.: sc-133832; 1 in 1000 dilution) and its secondary anti-rabbit anti-body (Cell Signalling, Cat. #: 7074s; 1:1000 dilution). β -actin was again used as a loading control. Densitometry on the bands of protein was performed using NIH ImageJ software and normalised to loading control, β -actin.

4.2.7.3. Proliferation Assay with NmU-25 Peptide

Hs578T and Hs578Ts(i)₈ cell line variants were seeded at 2×10^4 cells/500 μ l/well in a 24-well plate and allowed to attach overnight. Cells were then treated with 0 μ M, 0.5 μ M, 1 μ M, 2 μ M, 5 μ M or 10 μ M NmU-25 peptide and incubated for 72 hours. Proliferation assays were then taken down as described in Section 4.2.6.1.

4.2.7.4. Seahorse Extracellular Flux Assay with NmU-25 Peptide

Hs578T and Hs578Ts(i)₈ cells were seeded at 2×10^3 cells/150 μ l/well in an XF24-well plate. After a 2 hour incubation, 150 μ l media was added and incubated overnight to allow attachment. NmU-25 peptide (5 μ M- as determined in Section 4.2.6.1) was added to the appropriate wells and incubated for a further 72 hours (Figure 4.4). The seahorse extracellular flux assay was then performed as described in Section 4.2.2 (Figure 4.1, Day 2).

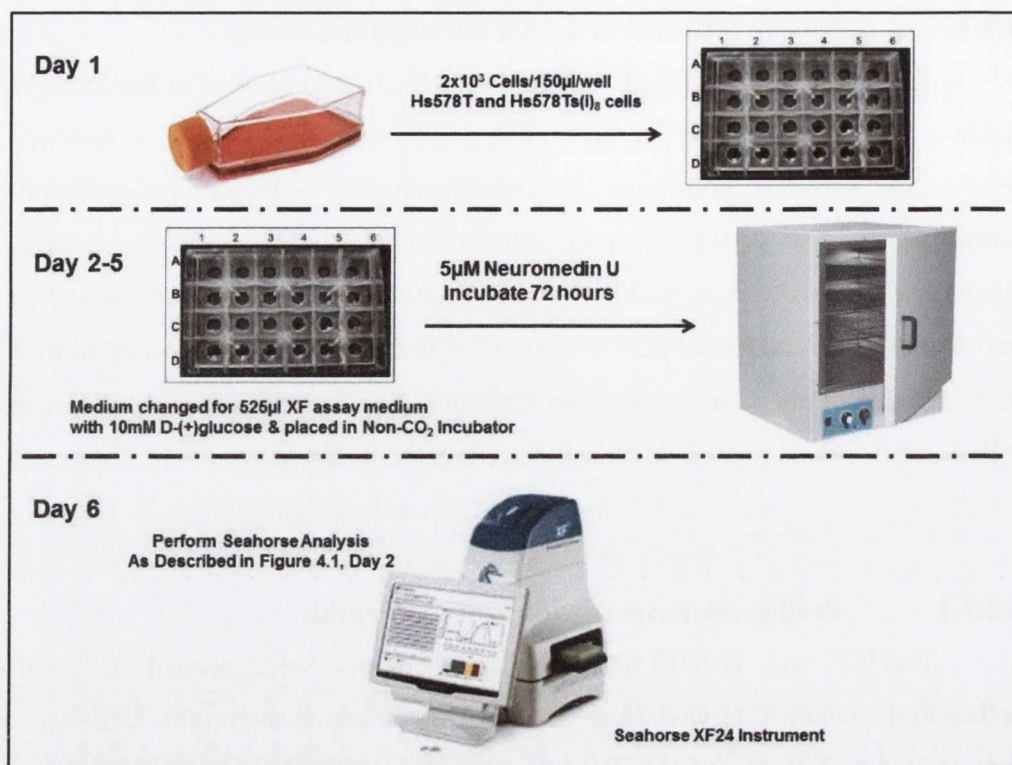


Figure 4.4: Schematic representation of Seahorse Extracellular Flux analysis of Hs578T and Hs578Ts(i)₈ cell line variants including Neuromedin U treatment

4.2.7.4.1. Data and Statistical Analysis

The OCR and ECAR were plotted as illustrated in

A and

B, respectively. Statistical analysis for the basal OCR, Proton leak (FCCP addition- non-mitochondrial respiration), ATP-linked (basal oxidative phosphorylation-after oligomycin A added), maximal respiration and reserve capacity (maximal respiration-basal respiration) was performed using the Student's t-test to compare the basal OCR, maximal OCR and non-mitochondrial respiration of the Hs578T-UT cells to the Hs578T-NmU-25 peptide treated cells and Hs578Ts(i)₈-UT cells to the Hs578Ts(i)₈-NmU-25 peptide treated cells. The Student's t-test was also performed to determine the statistical significance of the proton leak (readings after FCCP addition-readings after non-mitochondrial respiration) of:

Hs578T-UT cells compared to the Hs578T-UT,

Hs578T-NmU-25 treated cells compared to the Hs578Ts-NmU-25 treated cells,

Hs578Ts(i)₈-UT cells compared to the Hs578Ts(i)₈-UT cells,
Hs578Ts(i)₈-NmU-25 treated cells compared to the Hs578Ts(i)₈-NmU-25 treated cells.

The same was done for the ATP-linked and reserve capacity for each cell line variant. For graphical representation of significance * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for the Hs578T-UT cells compared to the Hs578T-NmU-25 treated or Hs578Ts(i)₈-UT cells compared to Hs578Ts(i)₈-NmU treated cells and # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ for proton leak, ATP-linked and reserve capacity for the Hs578T-UT cells compared to the Hs578T-UT, Hs578T-NmU-25 treated cells compared to the Hs578T-NmU-25 treated cells, Hs578Ts(i)₈-UT cells compared to the Hs578Ts(i)₈-UT cells and Hs578Ts(i)₈-NmU-25 treated cells compared to the Hs578Ts(i)₈-NmU-25 treated cells.

The same was performed for the ECAR, specifically, the Student's t-test was performed to compare the basal glycolysis, maximal glycolysis and non-glycolytic acidification of the Hs578T-UT cells compared to the Hs578T-NmU-25 peptide or Hs578Ts(i)₈-UT compared to Hs578Ts(i)₈-NmU-25 treated cells. Student's t-test was also performed to determine the statistical significance of the glycolytic reserve (*i.e.* Maximal glycolytic capacity-basal glycolysis) of:

Hs578T-UT compared to Hs578T-UT

Hs578T-NmU-25 treated compared to Hs578T-NmU-25 treated

Hs578Ts(i)₈-UT compared to Hs578Ts(i)₈-UT

Hs578Ts(i)₈-NmU-25 treated compared to Hs578Ts(i)₈-NmU-25 treated.

4.2.7.6. Mitochondrial Biomass of Hs578T and Hs578Ts(i)₈ Cell Line Variants following NmU-25 Peptide Treatment

The biomass of Hs578T and Hs578Ts(i)₈ cell line variants following NmU-25 peptide treatment was analysed by the citrate synthase assay and by immunoblotting for VDAC1. Cells were seeded at 1×10^5 cells in T25 flasks, allowed to attach overnight, treated with 5 μ M NmU-25 peptide and incubated for a further 72 hours. The citrate synthase assay was performed as described in Section 4.2.3.

Cells were seeded at 2×10^4 cells/well in a 6-well plate, incubated overnight, treated with 5 μ M NmU-25 peptide and incubated for a further 72

hours. An immunoblot for VDAC1 was then performed as described in Section 4.2.5.1. β -actin was used as a loading control. Densitometry on the bands of protein was performed using NIH ImageJ software and normalised to loading control, β -actin

4.2.7.7. Flow Cytometry Analysis of NAO Staining after NmU-25 Peptide Treatment

[Performed with the help of Dr. Vanesa Martinez]

Hs578T and Hs578Ts(i)₈ cell line variants were seeded at 1×10^5 cells/2ml/well in a 6-well plate and were allowed to attach overnight. Complete growth medium was then replaced with fresh complete growth medium containing 5 μ M NmU-25 peptide and incubated for a further 72 hours. Flow cytometry was then performed as described in Section 4.2.4.

4.2.8. Interleukin 6 (IL-6)

4.2.8.1. IL-6 ELISA

Hs578T and Hs578Ts(i)₈ cell line variants were seeded at 1×10^5 cells/500 μ l/well in a 24-well plate and incubated overnight. The next day complete medium was changed for serum-free medium and incubated for a further 72 hours. The serum-free conditioned medium (CM) was then collected and an IL-6 ELISA (Peprotech, Cat. #:900-M16) was performed on the CM according to the manufacturer's instructions with some modifications. Specifically, on Day 1, 100 μ l of capture antibody (1 μ g/ml) in PBS was placed in each well of a 96-well Nunc plate (ThermoFisher, Cat. #: NC-442404). On Day 2, the wells of the 96 well-plate were washed with 300 μ l of wash buffer (0.05% Tween 20 in PBS) 4 times and 100 μ l of 10% FCS in PBS was added to each well to block for 1 hour. The wells were washed again (x4) and 100 μ l of detection antibody (0.5 μ g/ml) in 10% FCS in PBS was added and incubated for 2 hours. The wells were washed again (x4) and 100 μ l of Avidin-HRP (1:2000 dilution) in 10% FCS in PBS was added and incubated for 30mins. The wells were washed again (x4) and 100 μ l of 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was added and incubated for 30mins. The plate was read at 405nm at 5min

intervals during this 30min incubation until the top standard reached 1.4 units. IL-6 concentrations were normalised to the cell protein concentrations by lysing of cells and determination of protein concentration by Bio-Rad BCA assay as described in Section 2.2.5.2.5.

4.2.8.2. Proliferation Assay to Determine the IC₅₀ of Tocilizumab

On Day 1, Hs578T and Hs578Ts(i)₈ cell line variants were seeded at 2×10^4 cells/500µl/well in a 24-well plate. On Day 2, cells were treated with 0µg/ml, 5µg/ml, 10µg/ml or 20µg/ml Tocilizumab (obtained from St. James Hospital Pharmacy) and incubated for a further 72 hours in serum-free medium. Proliferation assays were then taken down as described in Section 4.2.6.1.

4.2.8.3. Seahorse Extracellular Flux Assay with Tocilizumab

Hs578Ts(i)₈ cells were seeded at 2×10^3 cells/150µl/well in an XF24-well plate. After a 2 hour incubation, 150µl medium was added and incubated overnight to allow attachment. Tocilizumab (20µg/ml- as determined in Section 4.2.8.2) was added to the appropriate wells and incubated for a further 72 hours (Figure 4.5). The seahorse extracellular flux assay was then performed as described in Section 4.2.2 (Figure 4.1, Day 2).

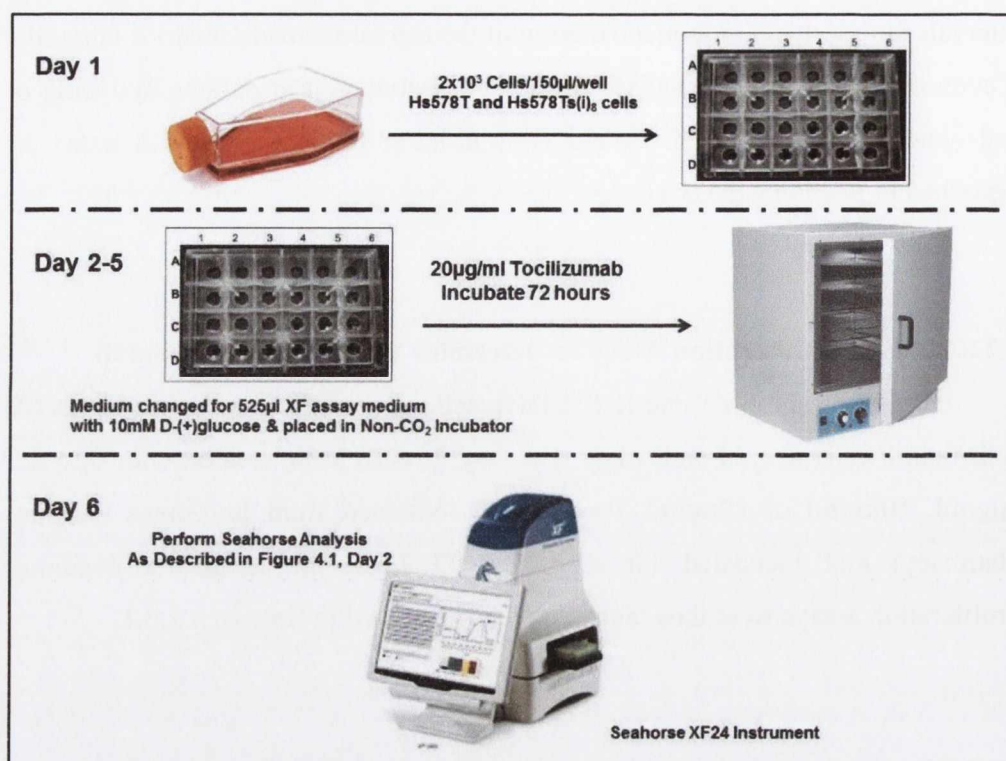


Figure 4.5: Schematic representation of Seahorse Extracellular Flux analysis of Hs578T and Hs578Ts(i)₈ cell line variants including Tocilizumab treatment.

4.2.8.3.1. Data and Statistical Analysis

The OCR and ECAR were plotted as illustrated in Figure 4.2 A and Figure 4.2 B, respectively. Statistical analysis for the basal OCR, Proton leak (FCCP addition- non-mitochondrial respiration), ATP-linked (basal oxidative phosphorylation-after oligomycin A added), maximal respiration and reserve capacity (maximal respiration-basal respiration) was performed using the Student's t-test to compare the basal OCR, maximal OCR and non-mitochondrial acidification of the Hs578Ts(i)₈-UT cells to the Hs578Ts(i)₈-Tocilizumab treated cells. The Student's t-test was also performed to determine the statistical significance of the proton leak of the Hs578Ts(i)₈-UT cells compared to the Hs578Ts(i)₈-UT cells and the Hs578Ts(i)₈-Tocilizumab treated cells compared to the Hs578Ts(i)₈-Tocilizumab treated cells (*i.e.* readings for the Hs578T(i)₈-UT cells after FCCP addition compared to Hs578Ts(i)₈-UT readings for non-mitochondrial respiration) the same was done for the ATP-linked and reserve capacity for each the untreated and treated cells. For graphical representation of significance *p<0.05, **p<0.01, ***p<0.001 for the Hs578Ts(i)₈-UT cells

compared to the Hs578Ts(i)₈-Tocilizumab treated cells and [#]p<0.05, ^{##}p<0.01, ^{###}p<0.001 for the Hs578Ts(i)₈-UT cells compared to Hs578Ts(i)₈-UT cells or Hs578Ts(i)₈-Tocilizumab treated cells compared to Hs578Ts(i)₈-Tocilizumab for proton leak, ATP-linked and reserve capacity.

The same was performed for the ECAR, specifically, the Student's t-test was performed to compare the basal glycolysis, maximal glycolysis and non-glycolytic acidification of the Hs578Ts(i)₈-UT cells compared to the Hs578Ts(i)₈-Tocilizumab treated cells. Student's t-test was also performed to determine the statistical significance of the glycolytic reserve (*i.e.* Maximal glycolytic capacity-basal glycolysis) of:

Hs578Ts(i)₈-UT compared to Hs578Ts(i)₈-UT

Hs578Ts(i)₈-Tocilizumab treated compared to Hs578Ts(i)₈-Tocilizumab treated.

4.2.9. Analysis of NmU and IL-6 in Plasma Specimens

4.2.9.1. Isolation of EVs from Plasma Specimens, Protein Extraction and Characterisation

EVs were isolated from healthy control, obese and MetS participants' plasma specimens as described in Section 3.2.2. Control specimens were collected by Dr. Maria Jose Santos-Martinez, School of Pharmacy and Pharmaceutical Sciences and Trinity Biomedical Sciences Institute. Blood specimens were spun at 1000g for 115mins, plasma aliquoted and stored at -80°C. Isolated EVs were re-suspended in 100µl of PBS. For lysis, 100µl of cell extraction buffer supplemented with protease inhibitor cocktail was added to EVs re-suspended in PBS and incubated on ice for 30mins with vortexing every 10mins for 10secs. EV lysates were subsequently centrifuged at 13,000g at 4°C for 10mins. The resulting supernatants were transferred to fresh Eppendorf tubes. Total protein concentration of EV preparations was determined using the Bio-Rad BCA protein assay dye reagent as described in Section 3.2.3.1. EV characterisation was performed as described in Section 3.2.3.

4.2.9.2. NmU ELISA

NmU was quantified in the plasma from control, obese and MetS participants plasma by ELISA (Peninsula Laboratories, Cat. #: S-1253.0001 and Cloud-clone; Cat. #: CEB025Hu) and in the EVs isolated from the plasma from control, obese and MetS participants by ELISA (Cloud-clone; Cat. #: CEB025Hu) (Table 4.2). ELISAs were performed according to the manufacturers' instructions as described Section 4.2.7.1. EV NmU concentrations were normalised to the EV protein concentrations described in Section 3.3.1.

Table 4.2: Female healthy control, obese participants' and MetS participants' specimens selected for EV isolation.

Healthy Control Specimens		Obese Specimens		MetS Specimens	
Specimen I.D.	Age	Specimen I.D.	Age	Specimen I.D.	Age
005-ST	22	074-AAT	42	040-DHP	46
006-AY	28	009-KL	29	008-DCS	57
007-IR	52	013-JS	57	077-HK	27
008-CE	55	038-EK	61	042-BJ	62
009-MAU	58	030-ABV	60	006-BH	67
010-MA	69	088-SW	62	019-DB	64
011-CA	71	095-EPL	66	098-KH	52
012-OZ	31	093-IH	78	036-KAC	64
013-LI	42	048-SC	20	007-ST	55
014-MAC	51	039-CH	22	079-MBJ	65
015-HE	56	037-SDO	34	045-AM	65
		025-AM	29	082-INA	67
				071-SM	67

4.2.9.3. IL-6 ELISA

[Performed by Dr. Mette Bohl, Department of Endocrinology and Internal Medicine, Aarhus University Hospital, Denmark]

IL-6 was quantified on the plasma of control, obese and MetS participant's (Table 2.4) by ELISA (Cat. HS600B, R&D Systems, Minneapolis, MN, USA).

4.3. Results

4.3.1. Analysis of the Metabolism of Hs578T and Hs578Ts(i)₈ Cell Line Variants by Seahorse Extracellular Flux Assays

A comprehensive investigation of the differences in the metabolism of the Hs578T cell line and its more aggressive subclone Hs578Ts(i)₈ has not been previously completed. Here, we investigated the metabolism of these cell lines by defining the bioenergetic status of the cells via dissecting out oxygen consumption due to mitochondrial function, non-mitochondrial function, ATP turnover and proton leak. This was achieved using the mitochondrial inhibitors oligomycin A and rotenone/antimycin A (Figure 4.6). Maximal mitochondrial capacity was determined by the addition of the mitochondrial uncoupler, FCCP (Figure 4.6). As expected, addition of oligomycin A (an inhibitor of ATP synthase) (Figure 4.6) to both cell line variants decreased the OCR. Subsequent addition of FCCP increased the OCR, as expected, but not beyond the basal rate; which indicates that mitochondria in both cell line variants were originally working at their maximum capacity *i.e.* before any additions were made. Addition of rotenone/antimycin A completely inhibited mitochondrial OCR and thus any OCR under these conditions gave a measure of the oxygen consumption not due to oxidative phosphorylation (non-mitochondrial oxygen consumption) in these Hs578T and Hs578Ts(i)₈ cell line variants.

When analysing the OCR, the data revealed a significantly lower basal cellular activity (2.1-fold, $p=7.6 \times 10^{-6}$) in Hs578Ts(i)₈ cells when compared to the Hs578T cells (Figure 4.7 A and B). ATP-linked respiration, which is the difference in the basal OCR and the OCR after oligomycin A addition (Figure 4.2 A), was significantly decreased after oligomycin A addition in Hs578T cells ($p=0.00019$) and Hs578Ts(i)₈ cells ($p=0.03$) (Figure 4.7 A and B), as expected. After FCCP addition, the rate of oxygen consumption increased, as expected, in both cell line variants and the greater maximal OCR capacity (2.5-fold) in Hs578T cells when compared to their Hs578Ts(i)₈ cell line variant was significantly different ($p=6.8 \times 10^{-6}$). Analysing the reserve capacity, which is the difference in the maximal capacity and the basal OCR (Figure 4.2 A), showed both Hs578T ($p=0.20$) and Hs578Ts(i)₈ ($p=0.56$) cell line variants to be working at their maximum capacity (Figure 4.9 A and B). After rotenone/antimycin A

addition mitochondrial respiration was completely inhibited in both Hs578T and Hs578Ts(i)₈ cell line variants and this levelled off at an OCR below the basal rate. The aforementioned decrease from basal OCR was significant for both Hs578T (3.7-fold, $p=0.000013$) and Hs578Ts(i)₈ (1.9-fold, $p=0.0015$) cell line variants (Figure 4.7 A and B). However, what is evident from these results is that the Hs578Ts(i)₈ cells are devoting very little OCR to mitochondrial oxidative phosphorylation when compared to the Hs578T cells. Quantifying this, we can see that the mitochondrial OCR for the Hs578Ts(i)₈ cells (basal OCR minus non-mitochondrial OCR) is 75 pMoles/min/ μ g protein. This is compared to 292 pMoles/min/ μ g protein for Hs578T cell. So Hs578Ts(i)₈ cells have a 3.9-fold lower mitochondrial activity under basal conditions. This begs the question as to whether the reduced mitochondrial oxygen consumption in Hs578Ts(i)₈ cells is due to the presence of fewer mitochondria in Hs578Ts(i)₈ cells, when compared to Hs578T cells.

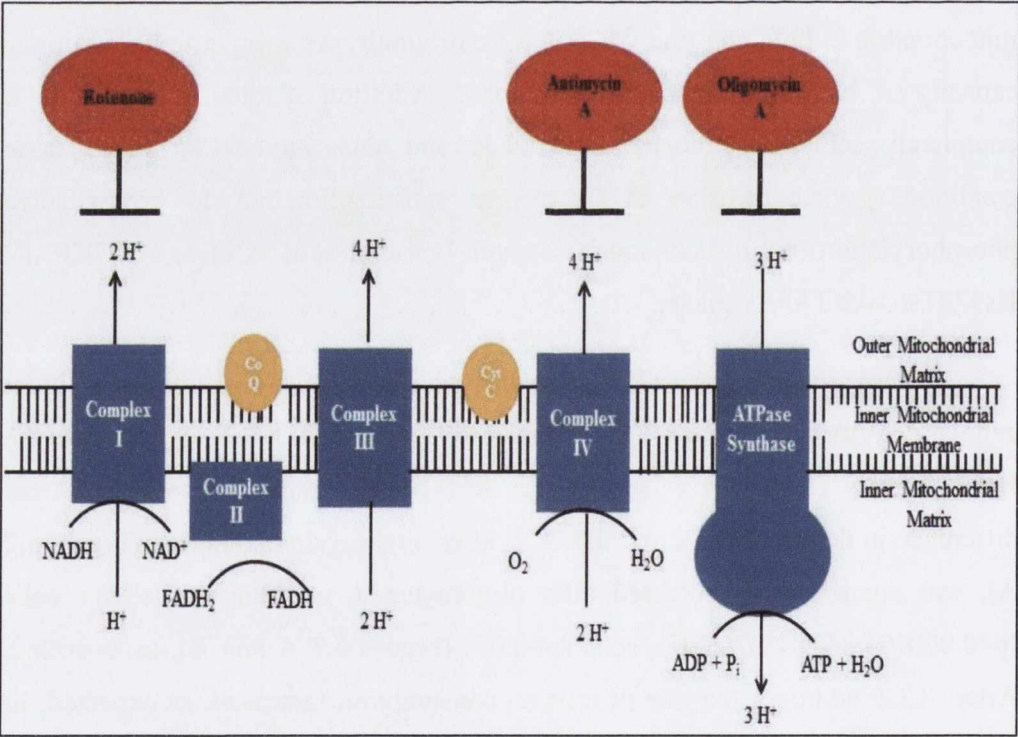


Figure 4.6: Illustration of the targets of drugs used to analyse the metabolism of cells

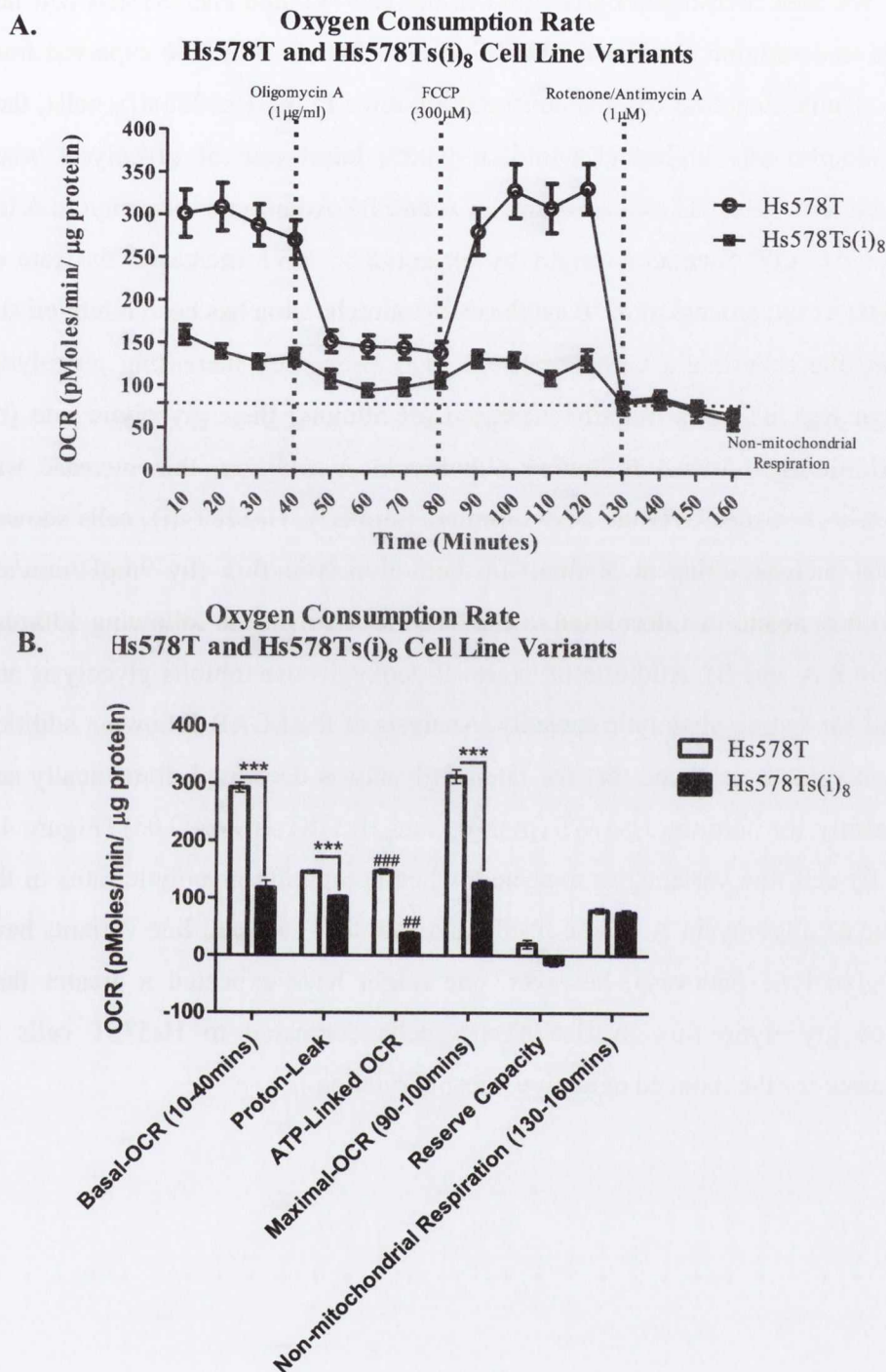
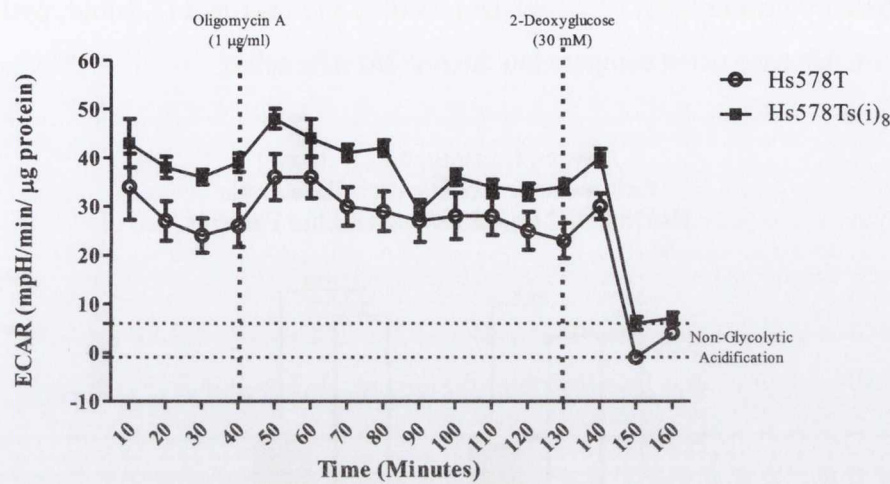


Figure 4.7: Oxygen consumption rate of Hs578T and Hs578Ts(i)₈ cell line variants

A and B The oxygen consumption rate was measured before and after subsequent additions of oligomycin A, FCCP and rotenone/antimycin A over a 2hr 40min period. Proton leak (FCCP addition- non-mitochondrial respiration), ATP-linked (basal oxidative phosphorylation-after oligomycin A added) and reserve capacity (maximal oxidative phosphorylation-basal oxidative phosphorylation) were also determined. Data is expressed as the mean $\pm$ SEM of 3 biological repeats. Statistical analysis was performed using the Student's t-test. ***/### p < 0.001, # p < 0.01, * Hs578T vs Hs578Ts(i)₈, # Hs578T vs Hs578T and Hs578Ts(i)₈ vs Hs578Ts(i)₈.

We then investigated glycolysis in the Hs578T and Hs578Ts(i)₈ cell line variants as determined by the ECAR (Figure 4.2 B). As might be expected from the lower mitochondrial oxygen consumption rates in the Hs578Ts(i)₈ cells, they had a significantly higher (1.5-fold, $p=0.005$) basal rate of glycolysis when compared to the Hs578T cells (Figure 4.8 A and B). Addition of oligomycin A (an inhibitor of ATP Synthase) might be expected to have increased the rate of glycolysis as the process of ATP synthesis by mitochondria has been inhibited and therefore the cells might compensate for that absence by increasing glycolysis. Although Hs578T cells initially increased (at 50mins) their glycolytic rate (by 8.2mpH/min/mg protein) following oligomycin A addition, this increase was completely abolished over the next 130mins. Similarly, Hs578Ts(i)₈ cells showed an initial increase (also at 50mins) in their glycolytic flux (by 9mpH/min/mg protein) but, again, this decreased to the basal levels over the following 130mins (Figure 4.8 A and B). Addition of excess 2-deoxyglucose inhibits glycolysis and is useful for testing glycolytic capacity. Analysis of the ECAR following addition of 2-deoxyglucose showed that the rate of glycolysis decreased dramatically and significantly for both the Hs578T ($p=0.01$) and Hs578Ts(i)₈ ($p=0.03$) (Figure 4.8 A and B) cell line variants, as expected, when comparing glycolytic rates in the presence of oligomycin A. These results indicate that both cell line variants have active glycolytic pathways, however, one might have expected a greater than observed glycolytic flux in Hs578Ts(i)₈ cells compared to Hs578T cells to compensate for the reduced oxidative phosphorylation.

**A. Extracellular Acidification Rate
Hs578T and Hs578Ts(i)₈ Cell Line Variants**



**B. Extracellular Acidification Rate
Hs578T and Hs578Ts(i)₈ Cell Line Variants**

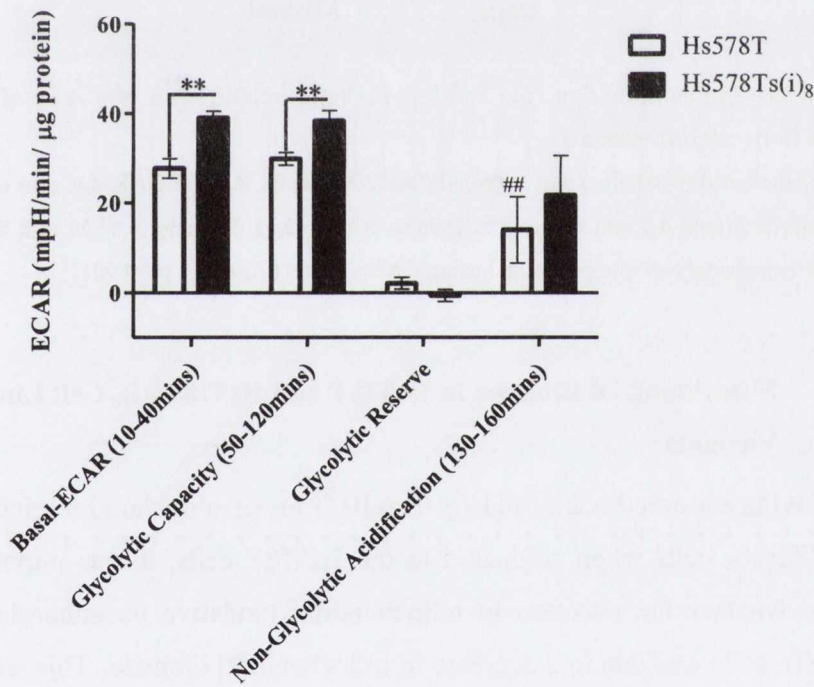


Figure 4.8: Extracellular acidification rate of Hs578T and Hs578Ts(i)₈ cell line variants

A and B. The extracellular acidification rate was measured, before and after sequential additions of oligomycin A and 2-deoxyglucose over a 2hr 40min period. Glycolytic reserve (glycolytic capacity-basal glycolysis) was also determined. Data is expressed as the mean ± SEM of 3 biological repeats. Statistical analysis was performed using the students t-test., **/## p<0.01, *Hs578T vs Hs578Ts(i)₈, # Hs578T vs Hs578T.

The aforementioned concern and discrepancy can be quantified by plotting the OCR/ECAR ratio. It can be seen in Figure 4.9 that the Hs578T cells have a significantly greater basal (2.2-fold, $p=1.5 \times 10^{-5}$) and maximal (2.3-fold, $p=0.001$) OCR/ECAR ratio when compared to the Hs578Ts(i)₈ cells.

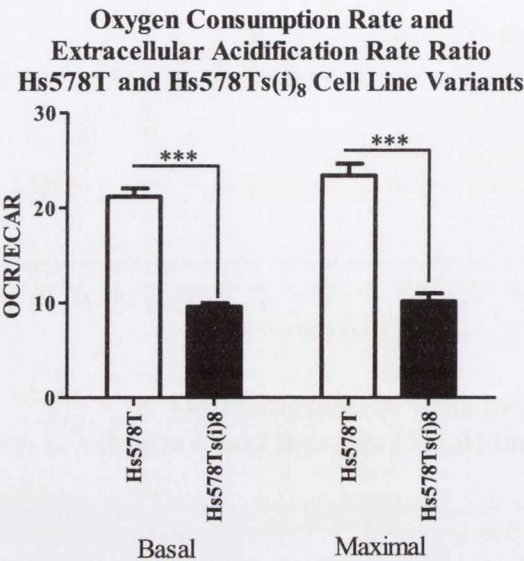


Figure 4.9: Oxygen consumption rate and Extracellular acidification rate ratio of Hs578T and Hs578Ts(i)₈ cell line variants

The ratio of the basal (0-40mins) and maximal (90-120mins) OCR and ECAR was also calculated from the data in Figure 4.7 and Figure 4.8. Data is expressed as the mean $\pm$ SEM of 3 biological repeats. Statistical analysis was performed using the Student's t-test. *** $p < 0.001$.

4.3.2. Mitochondrial Biomass in Hs578T and Hs578Ts(i)₈ Cell Line Variants

Having observed a 2.1-fold ($p=7.6 \times 10^{-6}$) lower mitochondrial activity in the Hs578Ts(i)₈ cells when compared to the Hs578T cells, it was important to determine whether the decrease in mitochondrial oxidative phosphorylation in Hs578Ts(i)₈ cells was due to a decrease in mitochondrial biomass. This was done by two methods (i) a spectrophotometric assay of citrate synthase, and (ii) a measure of immunoblot intensity for a mitochondrial protein, namely the voltage dependent anion channel 1 (VDAC1) protein [217]. β -actin was used as a loading control for this immunoblot and the corresponding densitometry graphs are displayed in Appendix I, Section G, Figure G.1.

Ultimately, we found no significant difference between the mitochondrial biomass of Hs578T and Hs578Ts(i)₈ cell line variants when assessed by the citrate synthase assay (Figure 4.10 A) and immunoblot for VDAC1 (Figure 4.10 B). Therefore, the 2.1-fold decrease in oxidative phosphorylation of Hs578Ts(i)₈ cells cannot be explained by a decrease in the mitochondrial biomass of the cells.

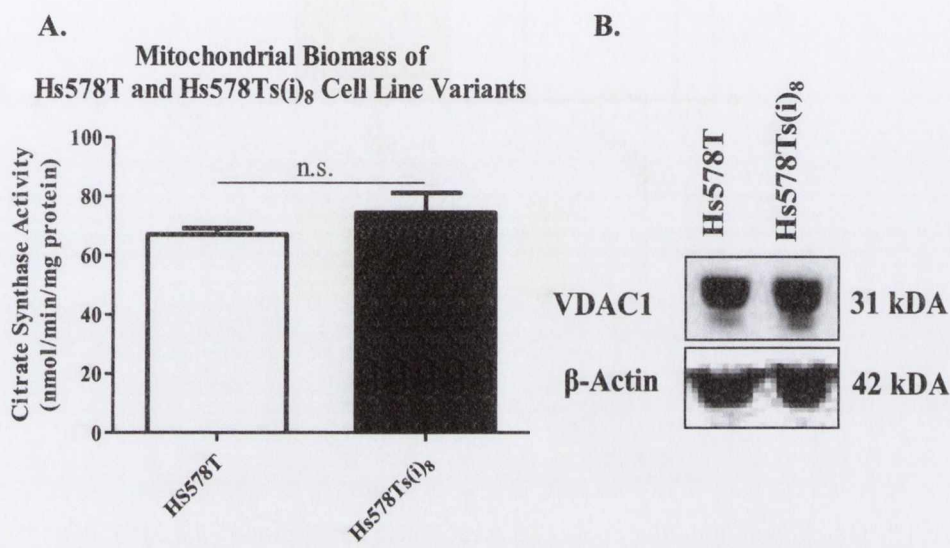
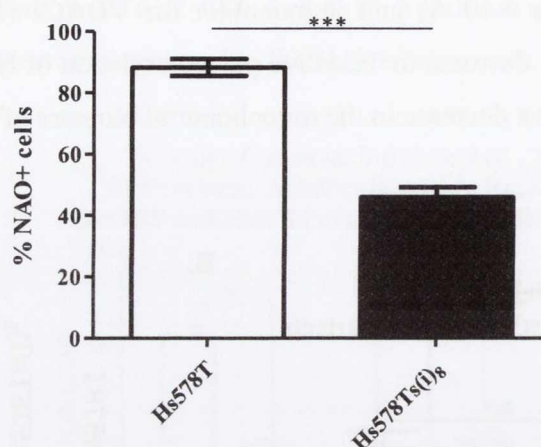


Figure 4.10: Citrate synthase activity and immunoblot for VDAC1.
Analysis of mitochondrial biomass by **A.** the citrate synthase assay and **B.** immunoblot for VDAC1. Data is expressed as the mean $\pm$ SEM of 3 3 biological repeats, where results were not significant.

4.3.3. Analysis of 10-N-Nonyl Acridine Orange (NAO) Staining by Flow Cytometry

In order to determine whether mitochondrial dysfunction or possibly a lower mitochondrial substrate supply might be the explanation for the reduction in the oxidative phosphorylation of the Hs578Ts(i)₈ cells, we assessed NAO staining by flow cytometry. NAO binds to cardiolipin in the mitochondrial membrane and its accumulation in the mitochondria is dependent on the mitochondrial membrane potential [218]. NAO showed a significantly ($p=0.0006$) decreased level of staining in Hs578Ts(i)₈ cells ($46\pm3.2\%$ NAO+ cells) compared to Hs578T cells ($88\pm2.8\%$ NAO+ cells) (Figure 4.11), indicating a lower mitochondrial membrane potential in the more aggressive Hs578Ts(i)₈ cell line variant and, therefore, some mitochondrial dysfunction or a lower mitochondrial substrate supply.

A. **Acridine Orange Staining**
of Hs578T and Hs578Ts(i)₈ Cell Line Variants



B.

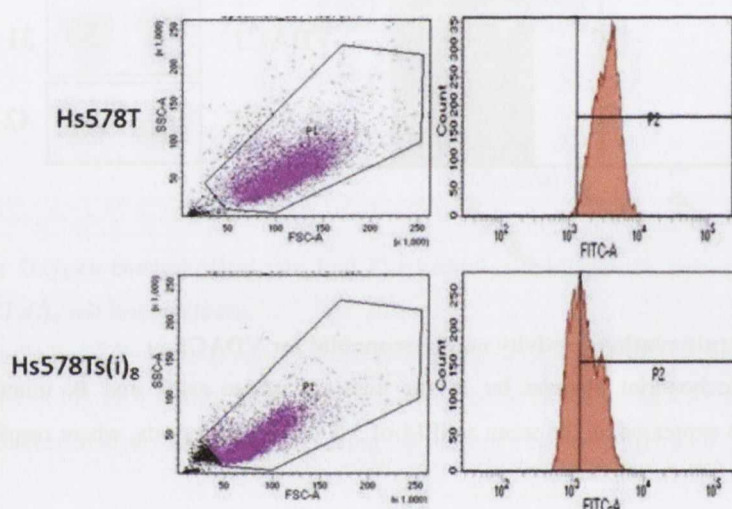


Figure 4.11: Analysis of 10-N-Nonyl Acridine Orange staining of Hs578T and Hs578Ts(i)₈ cells

A. Hs578T and Hs578Ts(i)₈ cell line variants were stained with 10 μ M NAO and the intensity of staining was analysed by flow cytometry for FITC. **B.** FACS Diva analysis of results showing forward and side scatter of NAO staining. Data is expressed as the mean $\pm$ SEM of 3 biological repeats and 3 technical repeats. Statistical analysis was carried out using the Student's t-test. *** $p < 0.001$.

4.3.4. Analysis of the Effects of Dichloroacetate (DCA) on Hs578T and Hs578Ts(i)₈ Cell Line Variants

4.3.4.1. Cytotoxicity Assay for DCA

To help narrow down the mechanism behind the altered metabolic phenotype of Hs578Ts(i)₈ cells, we decided to investigate the possible cause for

decreased mitochondrial substrate supply, that has been observed as the mechanism of lower oxidative capacity in other cancer cells [219]. Dichloroacetate (DCA) is an inhibitor of pyruvate dehydrogenase kinase (PDK). PDK inhibits pyruvate dehydrogenase (PD), which in turn converts pyruvate to acetyl CoA inside the mitochondria. The resulting acetyl CoA enters the Krebs cycle and provides reducing equivalents for oxidative phosphorylation. Therefore, if PD is inhibited by PDK in Hs578Ts(i)₈ cells, treatment with DCA should enhance PD activity and increase substrate supply for oxidative phosphorylation (Figure 4.12).

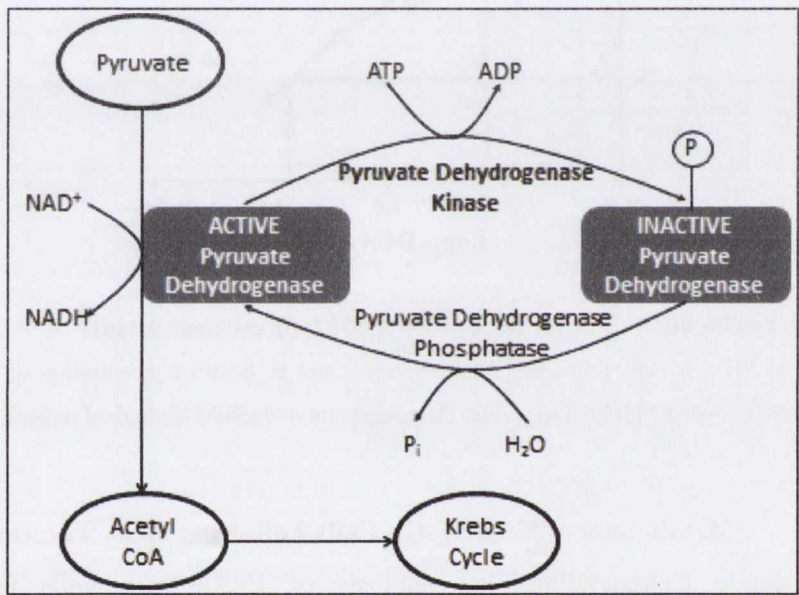


Figure 4.12: Modulation of PD by PDK and the processing of pyruvate to Acetyl CoA.

Pyruvate dehydrogenase converts pyruvate to acetyl CoA which enters the Krebs cycle. However, pyruvate dehydrogenase kinase can inhibit pyruvate dehydrogenase activity. ATP– adenosine triphosphate, ADP– adenosine diphosphate, P- phosphate, NAD– oxidised nicotinamide adenine dinucleotide, NADH– reduced nicotinamide adenine dinucleotide.

In order to determine a suitable concentration of DCA to use while keeping the cells viable, a cytotoxicity assay was performed to determine the IC_{50} of DCA for these cell line variants. The cytotoxicity assay determined the lowest non-toxic concentration of DCA as 5mM (Figure 4.13 B), while the IC_{50} was determined to be 25mM (Figure 4.13). Therefore, we selected 5mM for future assays.

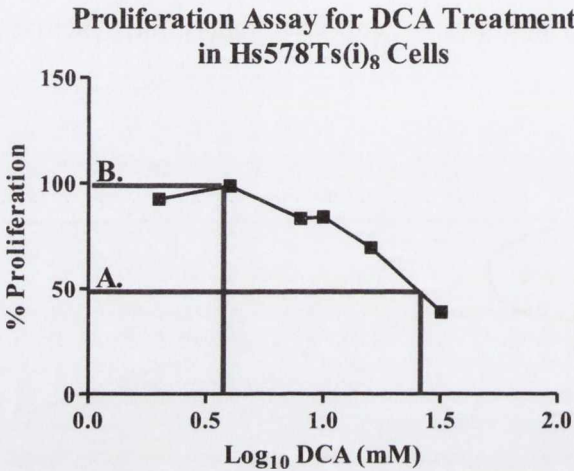
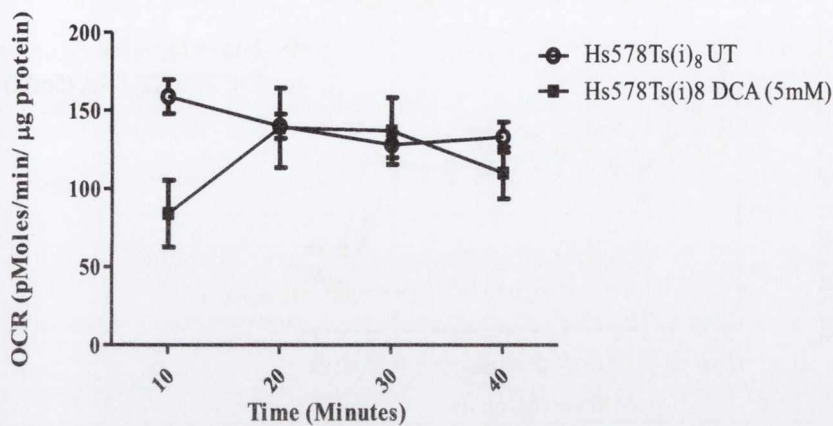


Figure 4.13: Proliferation assay for DCA in the Hs578Ts(i)₈ cell line variant
A. IC_{50} of Hs578Ts(i)₈ cells following DCA treatment and B. lowest concentration of DCA that does not inhibit growth of Hs578Ts(i)₈ cells. Data represent $n=3 \pm SEM$ biological repeats.

4.3.4.2. Metabolism of Hs578Ts(i)₈ Cells Following DCA Treatment

Seahorse Extracellular Flux analysis of Hs578Ts(i)₈ cells following treatment with 5mM DCA showed DCA did not increase the oxidative phosphorylation of these cells (Figure 4.14 A and B). This suggests that PDK is not involved in the switch of the Hs578Ts(i)₈ cells to glycolysis.

A. Basal Oxygen Consumption Rate
Hs578Ts(i)₈ Cells following DCA (5mM) Treatment



B. Basal Oxygen Consumption Rate
Hs578Ts(i)₈ Cells Following DCA (5mM) Treatment

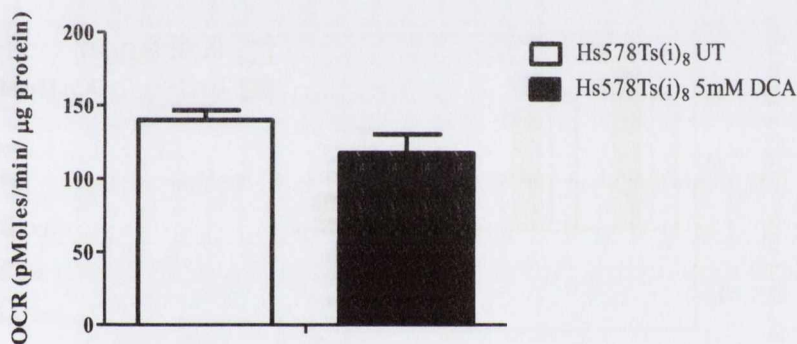
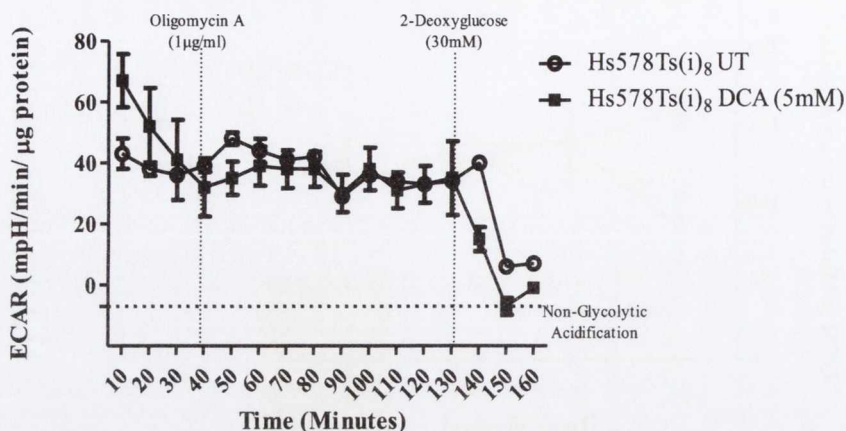


Figure 4.14: Basal oxygen consumption rate Hs578Ts(i)₈ cells after DCA treatment

A and B. The basal oxygen consumption rate was measured over a 40min period following DCA treatment. Data is expressed as the mean $\pm$ SEM of 3 biological repeats, where results were not significant.

Again following DCA treatment there was no significant change in the glycolytic rate of these cells (Figure 4.15). Again, suggesting PDK is not involved in the metabolic switch of the Hs578Ts(i)₈ cells to glycolysis.

A. Extracellular Acidification Rate Hs578Ts(i)₈ Cells Following DCA (5mM) Treatment



B. Extracellular Acidification Rate Hs578Ts(i)₈ Cells Following DCA (5mM) Treatment

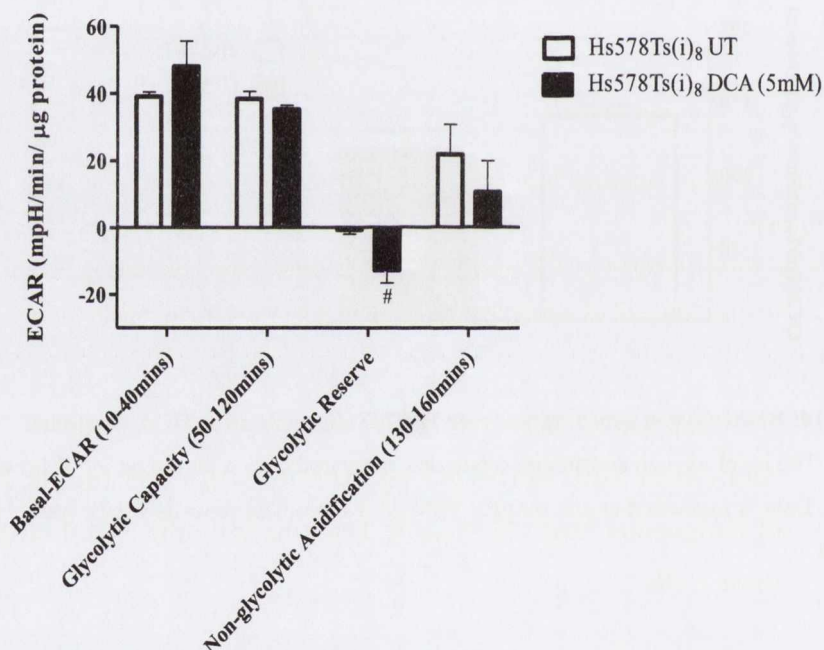


Figure 4.15: Extracellular acidification rate Hs578Ts(i)₈ cells following DCA treatment

A and B. The extracellular acidification rate was measured, before and after subsequent additions of oligomycin A and 2-deoxyglucose over a 2hr 40min period. Glycolytic reserve (glycolytic capacity-basal glycolysis) was also determined. Data is expressed as the mean $\pm$ SEM of 3 biological repeats. Statistical analysis was performed using the Student's t-test., [#] $p < 0.05$, [#] Hs578Ts(i)₈ DCA treated vs Hs578Ts(i)₈ DCA treated.

As is expected based on the preceding OCR and ECAR data, following DCA treatment, the OCR/ECAR ratio shows no difference in the rate of oxidative phosphorylation or glycolysis (Figure 4.16).

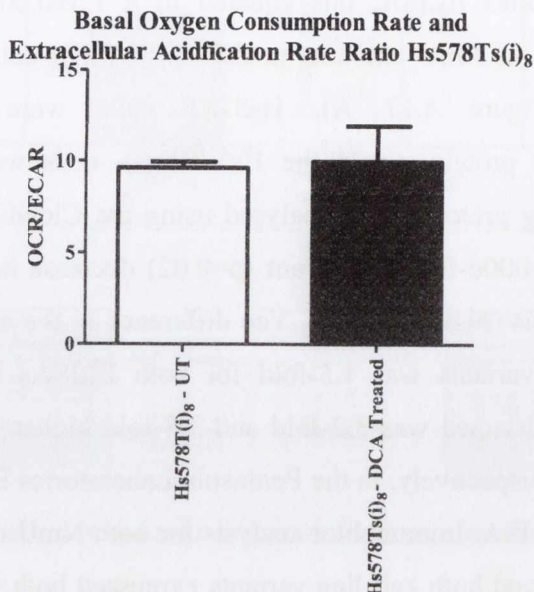


Figure 4.16: Oxygen consumption rate and Extracellular acidification rate ratio of Hs578Ts(i)8 cells

The ratio of the basal (0-40mins) OCR and ECAR was calculated from the data in Figure 4.14 and Figure 4.15. Data is expressed as the mean ± SEM of 3 biological repeats, where results were not significant.

4.3.5. Analysis of NmU in Hs578T and Hs578Ts(i)8 Cells and the Effect of Exogenous NmU-25 Peptide Treatment on the Metabolism of these Cells

4.3.5.1. NmU is Decreased in the more Aggressive Hs578Ts(i)8 Cells Compared to Hs578T Cells

NmU has been reported to have an association with feeding behaviour, metabolism and breast cancer; it was therefore decided to assess the levels of NmU in Hs578T and Hs578Ts(i)8 cells to (i) evaluate the levels of NmU in our TNBC cell line variants; (ii) assess whether the addition of exogenous NmU-25 peptide to Hs578Ts(i)8 cells could increase their oxidative phosphorylation; (iii) evaluate the effect of exogenous NmU peptide on Hs578T(i)8 cells in terms of mitochondrial biomass and membrane potential.

Intracellular NmU concentration in Hs578T and Hs578Ts(i)₈ cells was measured by two ELISAs and amounts were compared between both. NmU levels in the Hs578T cells were found to be $0.029 \pm 0.002 \text{ ng}/\mu\text{g}$ protein and $0.019 \pm 0.0005 \text{ ng}/\mu\text{g}$ protein in the Hs578Ts(i)₈ cells when measured using the Peninsula Laboratories ELISA, this equated to a 1.5 ± 0.0005 -fold significant ($p=0.007$) decrease in NmU amounts in the Hs578Ts(i)₈ cells compared to the Hs578T cells (Figure 4.17 A). Hs578T cells were found to have $0.007 \pm 0.0006 \text{ ng}/\mu\text{g}$ protein, while the Hs578Ts(i)₈ cells were found to have $0.005 \pm 0.0006 \text{ ng}/\mu\text{g}$ protein when analysed using the Cloud-Clone ELISA, this equated to a 1.5 ± 0.0006 -fold significant ($p=0.02$) decrease in NmU amounts in the Hs578Ts(i)₈ cells (Figure 4.17 B). The difference in the amounts of NmU in the two cell line variants was 1.5-fold for both ELISAs used, however the amounts of NmU detected was 3.2-fold and 3.8-fold higher in the Hs578T and Hs578Ts(i)₈ cells, respectively, in the Peninsula Laboratories ELISA compared to the Cloud-clone ELISA. Immunoblot analysis for both NmU receptors (NmU R1 and NmU R2) showed both cell line variants expressed both receptors at similar levels (Figure 4.17 C). β -actin was used as a loading control and densitometry graphs are displayed in Appendix I, Section H, Figure H.1.

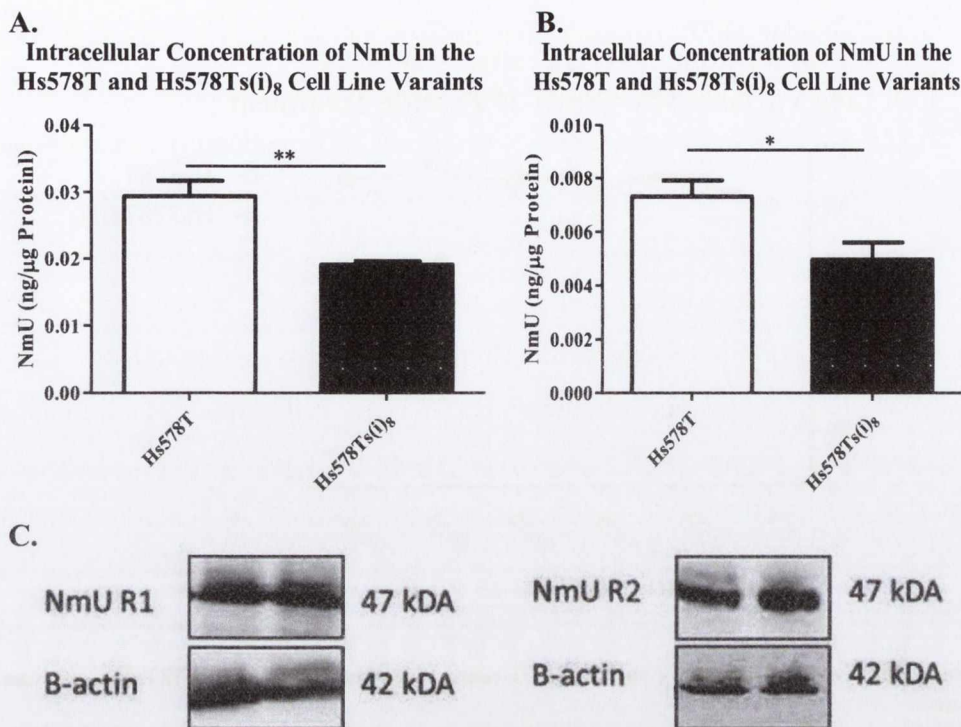


Figure 4.17: Analysis of NmU and NmU receptors 1 and 2 in Hs578T and Hs578Ts(i)₈ cell line variants

A. Concentration of NmU in Hs578T and Hs578Ts(i)₈ cells as determined by Peninsula Laboratories ELISA. **B.** Concentration of NmU in Hs578T and Hs578Ts(i)₈ cells as determined by Cloud-Clone ELISA. **C.** Immunoblot for NmU receptors 1 and 2. Data is expressed as the mean $\pm$ SEM of 3 biological repeats and three technical repeats for the NmU ELISA. Statistical analysis was performed using the Student's t-test. ** $p < 0.01$ and * $p < 0.05$.

4.3.5.2. Proliferation Assay for NmU-25 Peptide

Previous studies in our laboratory used exogenous NmU-25 (Bachem, Cat. #: H5538) for *in vitro* work at a concentration of $1\mu\text{M}$ [112]. It was, therefore, decided to assess the concentration of NmU that may increase the proliferation of the Hs578T and Hs578Ts(i)₈ cells, as increased NmU levels are associated with a poor outcome in HER2-overexpressing breast cancer (See Section 1.2.2). A proliferation assay was performed for a range of NmU-25 peptide concentrations around $1\mu\text{M}$ (0 – $10\mu\text{M}$). There was an increase in the growth of both cell line variants following NmU treatment; however this increase in growth was not significant for any concentration (Figure 4.18).

**Proliferation of Hs578Ts(i)₈ vs Hs578T
Cell Line Variants after NmU-25 Peptide Treatment**

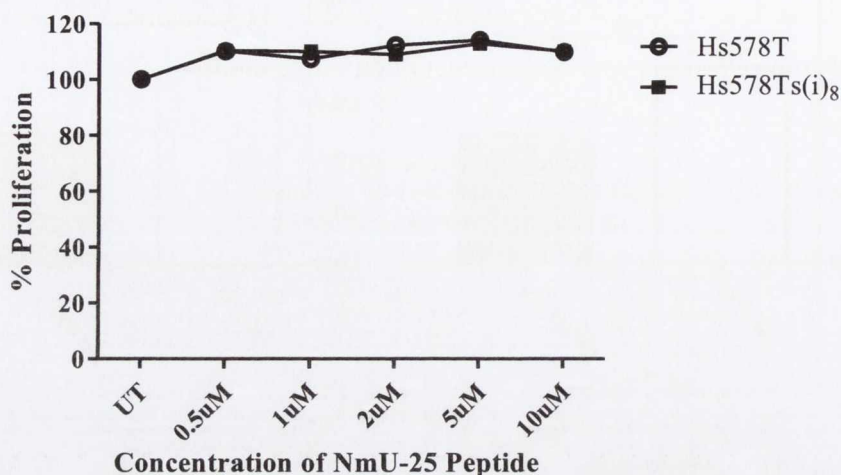


Figure 4.18: Proliferation assay for NmU-25 peptide in Hs578T and Hs578Ts(i)₈ cell line variants

Proliferation assay for Hs578T and Hs578Ts(i)₈ with increasing concentrations of NmU-25. Data is expressed as the mean $\pm$ SEM of 3 3 biological repeats.

4.3.5.3. Metabolism of Hs578T and Hs578Ts(i)₈ Cell Line Variants following NmU-25 Peptide Treatment

Based on our observations in the proliferation assays, 5 μ M NmU-25 peptide was selected for metabolic studies utilising the Seahorse Extracellular Flux Analyser, as NmU stimulated the highest amount of growth in these cell line variants, although not significantly (Figure 4.18). Treatment of Hs578T cells with 5 μ M NmU-25 peptide for 72 hours resulted in significant alterations in their metabolism. The basal OCR of Hs578T cells was significantly ($p=0.0003$) lowered following treatment. Treatment with oligomycin A reduced the OCR in the NmU-25 treated cells and untreated cells to the same level. However, NmU had a dramatic and significant ($p=7.4 \times 10^{-05}$) inhibitory effect on maximal OCR, following FCCP addition (312.8 ± 11.8 pMoles/min/ μ g protein versus 185 ± 6.34 pMoles/min/ μ g protein) in Hs578T cells. This difference was reflected in the reserve capacity ($p=0.02$) *i.e.* when non-mitochondrial OCR was taken into account. Specifically, the drop in OCR following rotenone/antimycin A for the untreated Hs578T cells is significant ($p=0.000013$), indicating that Hs578T cells were consuming oxygen for oxidative phosphorylation by the mitochondria. However, upon NmU-25 treatment the drop in OCR after rotenone/antimycin A is drastically decreased compared to the drop before NmU treatment, however it is

still significant ($p=0.02$). The non-mitochondrial respiration was also significantly ($p=0.004$) increased following NmU-25 treatment (Figure 4.19 A and B). So, in general, NmU-25 appears to dramatically reduce mitochondrial function and, in particular, electron transport chain function in mitochondria.

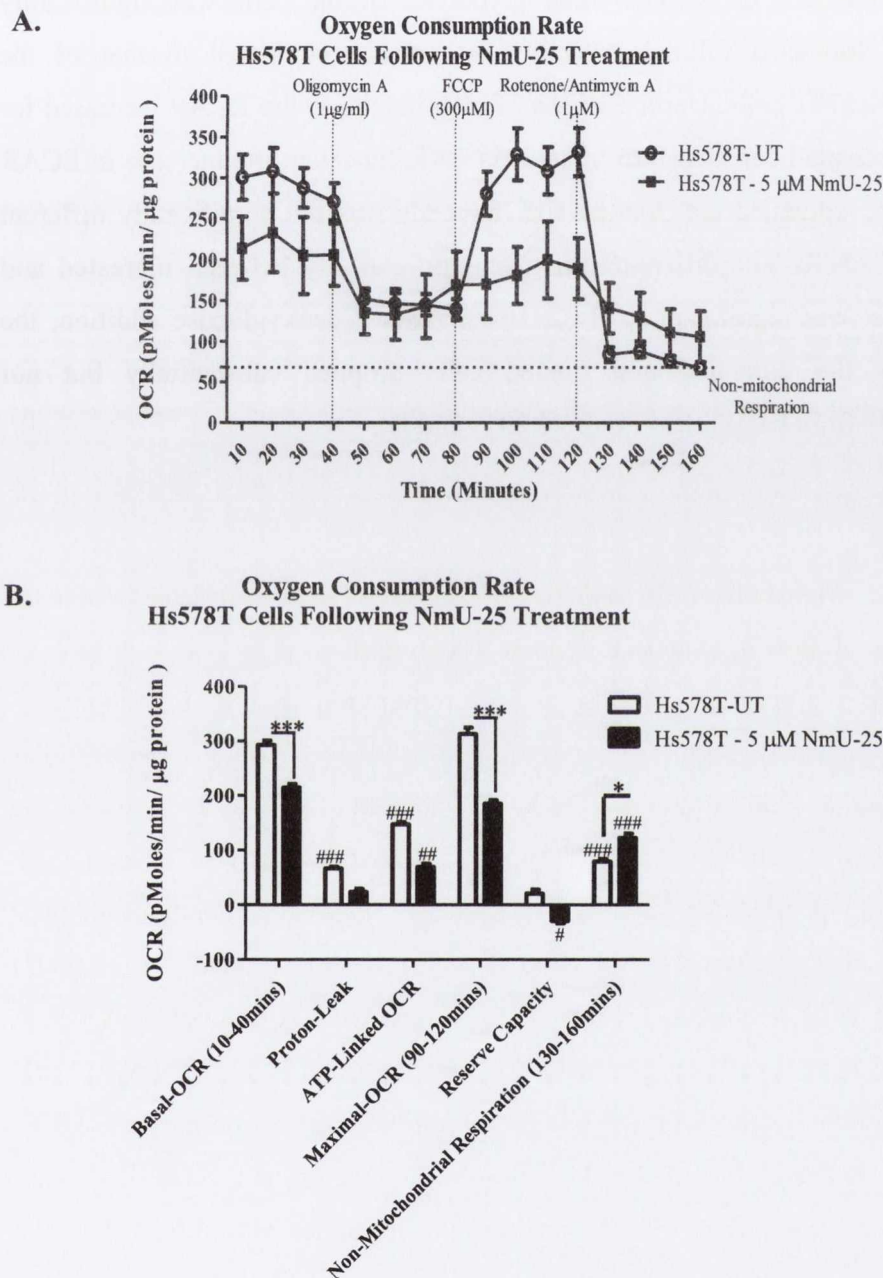


Figure 4.19: Oxygen consumption rate of Hs578T cells following NmU-25 peptide treatment
A and B. The oxygen consumption rate was measured before and after sequential additions of oligomycin A, FCCP and rotenone/antimycin A over a 2hr 40min period. Proton leak (FCCP addition- non-mitochondrial respiration), ATP-linked (basal oxidative phosphorylation-after oligomycin A added) and reserve capacity (maximal oxidative phosphorylation-basal oxidative

phosphorylation) were also determined. Data is expressed as the mean $\pm$ SEM of 3 biological repeats. Statistical analysis was performed using the Student's t-test. ***^{###} $p < 0.001$, ^{##} $p < 0.01$, ^{*/#} $p < 0.05$, * Hs578T-UT vs Hs578T-NmU treated, [#] Hs578T-UT vs Hs578T-UT and Hs578T-NmU treated vs Hs578Ts-NmU treated.

The ECAR for Hs578T cells following NmU-25 treatment showed the same trend as the OCR. The basal glycolysis of the cells was significantly ($p=0.002$) decreased following NmU-25 treatment compared to that of the untreated Hs578T cells. Upon addition of oligomycin A, the ECAR increased for both the untreated and NmU-25 treated Hs578T; however, the increase in ECAR for both the untreated and treated Hs578T cells was not significantly different from basal OCR. The difference in glycolytic capacity between untreated and treated cells was significant ($p=1.22 \times 10^{-5}$). After 2-deoxyglucose addition, the ECAR for the untreated and treated cells dropped substantially but not significantly from basal rates (Figure 4.20 A and B).

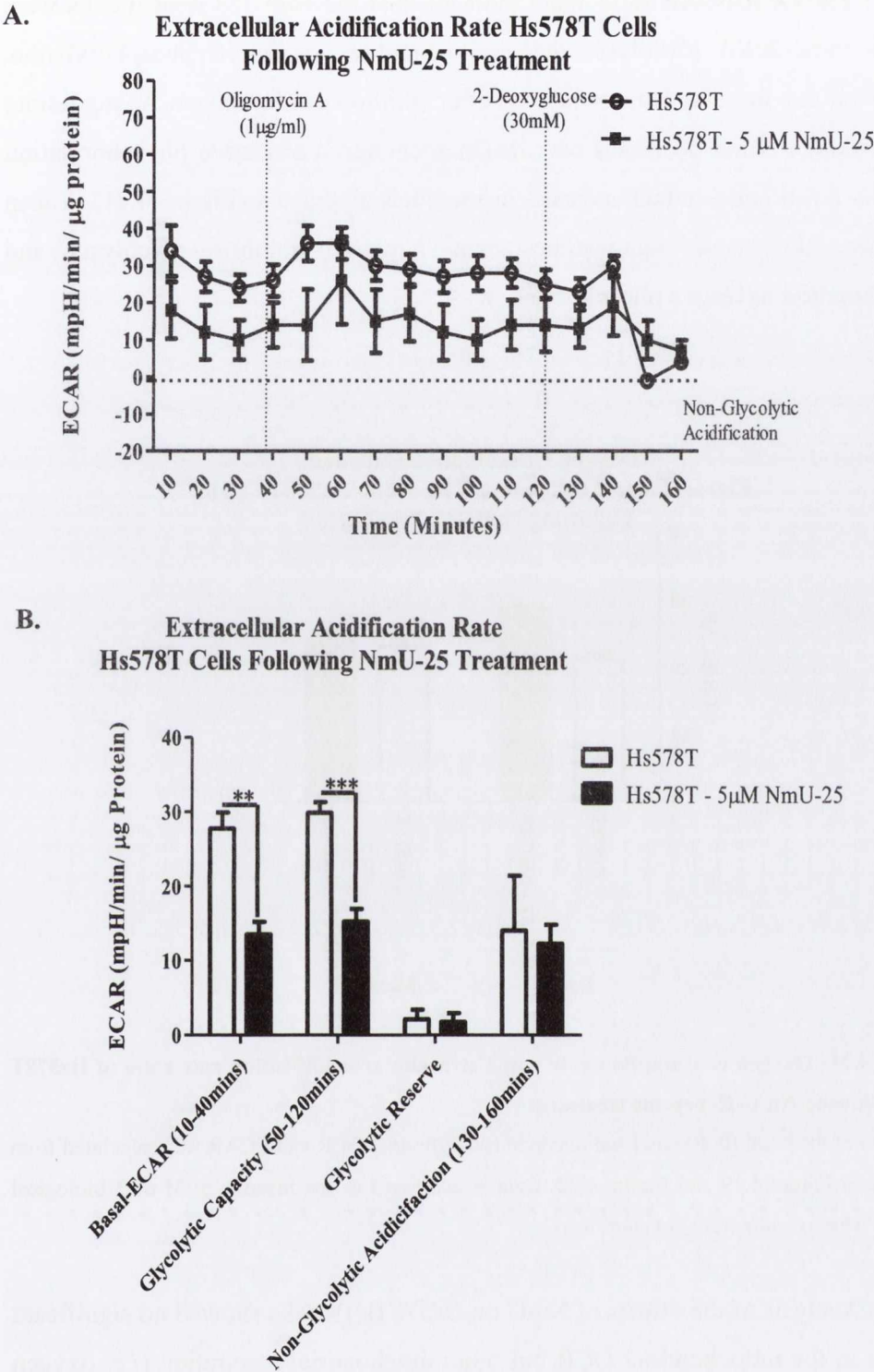


Figure 4.20: Extracellular acidification rate of Hs578T cells after NmU-25 peptide treatment

A and B. The extracellular acidification rate was measured, before and after sequential additions of oligomycin A and 2-deoxyglucose over a 2hr 40min period. Glycolytic reserve (glycolytic capacity-basal glycolysis) was also determined. Data is expressed as the mean $\pm$ SEM of 3 biological repeats. Statistical analysis was performed using the students t-test., *** $p < 0.001$.

The OCR/ECAR ratio again indicates that the NmU-25 treated cells were using more basal glycolysis than mitochondrial oxidative phosphorylation. However, this increase is not observed after addition of oligomycin A, suggesting that although NmU decreases maximal mitochondrial oxidative phosphorylation there is not a concomitant increase in maximal glycolysis (Figure 4.21). Taken together, this data demonstrates that NmU-25 inhibits glycolysis and mitochondrial oxidative phosphorylation.

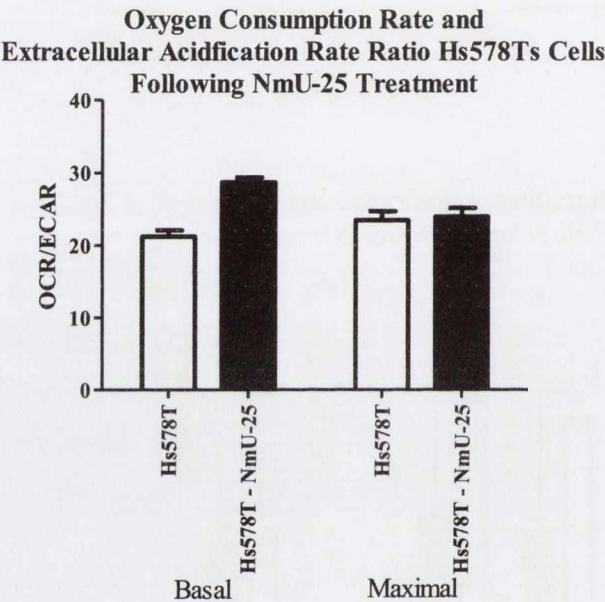
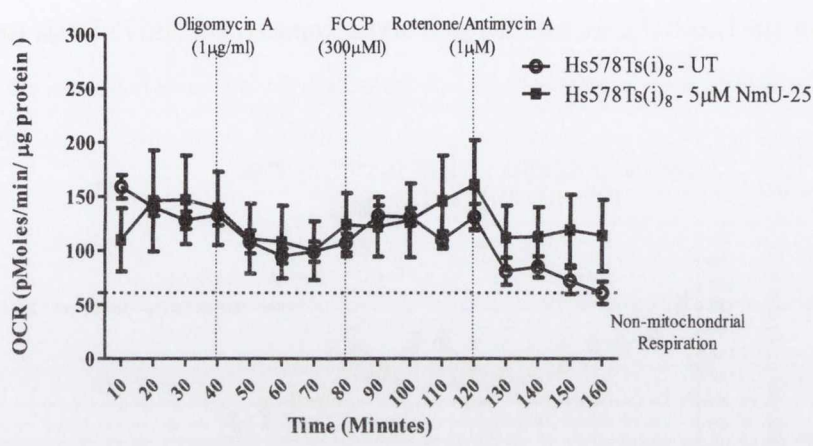


Figure 4.21: Oxygen consumption rate and Extracellular acidification rate ratio of Hs578T cells following NmU-25 peptide treatment

The ratio of the basal (0-40mins) and maximal (90-120mins) OCR and ECAR was calculated from the data in Figure 4.19 and Figure 4.20. Data is expressed as the mean ± SEM of 3 biological repeats, where results were not significant.

Analysis of the effects of NmU on Hs578Ts(i)₈ cells showed no significant change in the mitochondrial OCR but non-mitochondrial respiration (*i.e.* oxygen consumption not due to oxidative phosphorylation) was increased significantly ($p=0.002$) following NmU-25 treatment (Figure 4.22 A and B).

A. Oxygen Consumption Rate Hs578Ts(i)₈ Cells
Following NmU-25 Treatment (5 μ M)



B. Oxygen Consumption Rate
Hs578T Cells Following NmU-25 Treatment

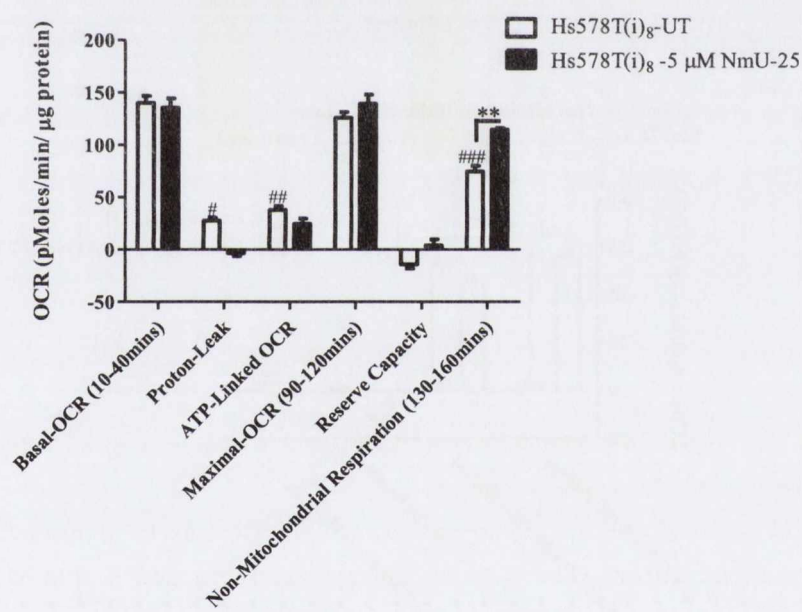


Figure 4.22: Oxygen consumption rate of Hs578Ts(i)₈ cells following NmU-25 peptide treatment

A and B. The oxygen consumption rate was measured before and after sequential additions of oligomycin A, FCCP and rotenone/antimycin A over a 2hr 40min period. Proton leak (FCCP addition- non-mitochondrial respiration), ATP-linked (basal oxidative phosphorylation-after oligomycin A added) and reserve capacity (maximal oxidative phosphorylation-basal oxidative phosphorylation) was also determined. Data is expressed as the mean $\pm$ SEM of 3 3 biological repeats. Statistical analysis was performed using the Student’s t-test. ^{###} $p < 0.001$, ^{**/##} $p < 0.01$, [#] $p < 0.05$, * Hs578Ts(i)₈-UT vs Hs578Ts(i)₈-NmU-25 treated, [#] Hs578Ts(i)₈-UT vs Hs578Ts(i)₈-UT.

Interestingly, the addition of NmU-25 had a dramatic effect on cell glycolysis (Figure 4.23 A and B), with glycolytic flux being attenuated following NmU-25 treatment. The basal ECAR was significantly decreased following NmU treatment ($p=1.3 \times 10^{-6}$), as was the glycolytic capacity of Hs578Ts(i)₈ ($p=2.4 \times 10^{-11}$).

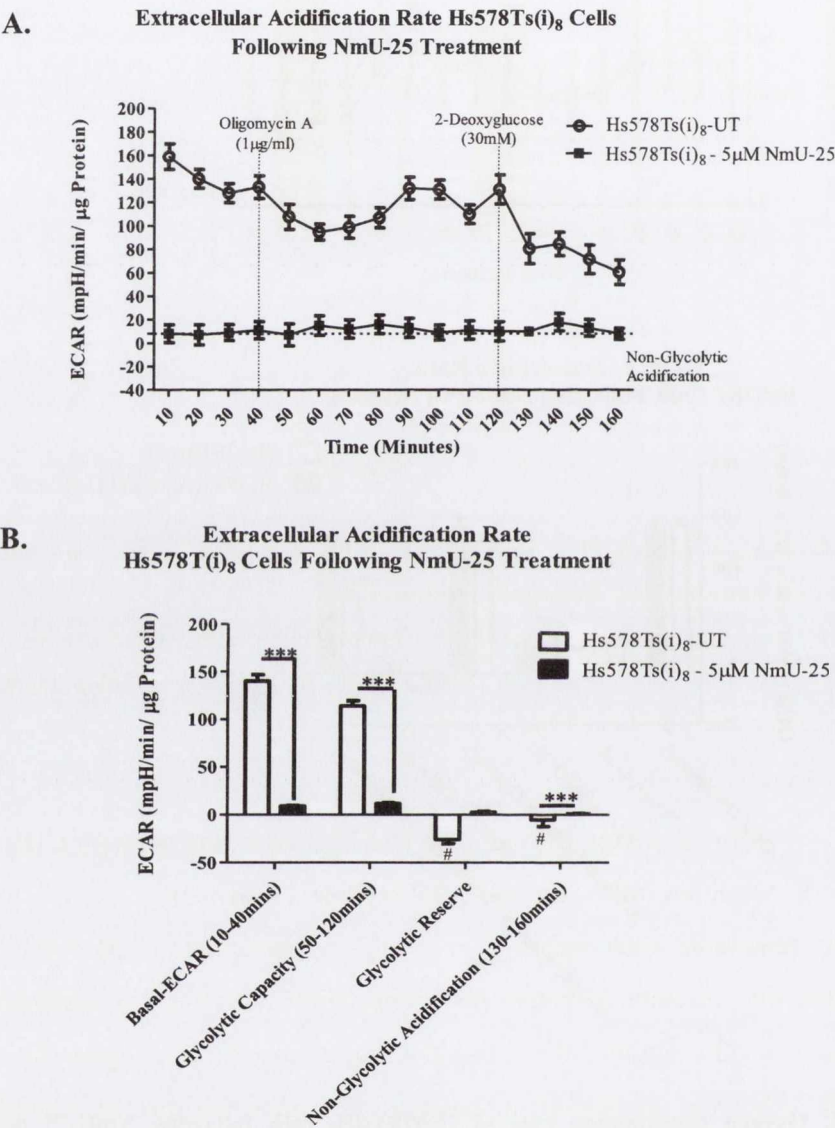


Figure 4.23: Extracellular acidification rate of Hs578Ts(i)₈ cells following NmU-25 peptide treatment

A and B. The extracellular acidification rate was measured, before and after sequential additions of oligomycin A and 2-deoxyglucose over a 2hr 40min period. Glycolytic reserve (glycolytic capacity-basal glycolysis) was also determined. Data is expressed as the mean $\pm$ SEM of 3 biological repeats. Statistical analysis was performed using the students t-test., *** $p<0.001$, # $p<0.05$, * Hs578Ts(i)₈-UT vs Hs578Ts(i)₈-NmU-25 treated, # Hs578Ts(i)₈-UT vs Hs578Ts(i)₈-UT.

The OCR/ECAR ratio again indicated that NmU was attenuating the glycolysis taking place in the cells (Figure 4.24).

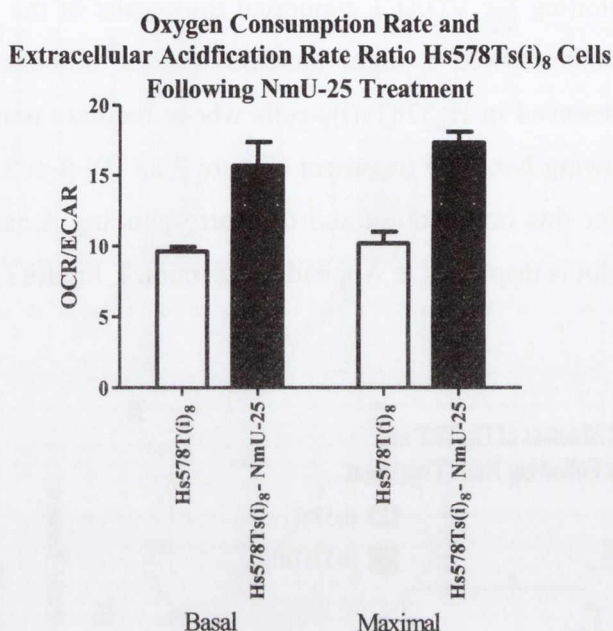


Figure 4.24: Oxygen consumption rate and Extracellular acidification rate ratio of Hs578Ts(i)₈ cells following NmU-25 peptide treatment.

The ratio of the basal (0-40mins) and maximal (90-120mins) OCR and ECAR was also calculated from the data in Figure 4.22 and Figure 4.23. Data is expressed as the mean $\pm$ SEM of 3 biological repeats, where results were not significant.

4.3.5.4. Mitochondrial Biomass of Hs578T and Hs578Ts(i)₈ Cell Line Variants following NmU-25 Peptide Treatment

The functional data relating to NmU-25 treatment of Hs578T cells suggests that flux through oxidative phosphorylation halved when compared to that of untreated cells. On the other hand, the limited oxidative phosphorylation of Hs578Ts(i)₈ cells was unaffected. In order to determine whether the significant decrease in the oxidative phosphorylation of Hs578T cells following NmU-25 treatment was due to a reduction in the mitochondrial biomass, the citrate synthase assay was performed. Interestingly, no significant decrease in mitochondrial abundance was observed; if anything, the biomass of Hs578T cells was increased by NmU-25 treatment and tended towards significance ($p=0.056$) (Figure 4.25 A). Consistent with this lack of significance in mitochondrial

abundance, there was no significant difference in membrane potential (Figure 4.26).

Immunoblotting for VDAC1 supported the results of the citrate synthase assay. Hs578T cells showed a slight increase in their mitochondrial biomass, which was not observed in Hs578Ts(i)₈ cells whose biomass remained the same prior to and following NmU-25 treatment (Figure 4.25 B). β-actin was used as a loading control for this immunoblot and the corresponding densitometry graphs for this immunoblot is displayed in Appendix I, Section I, Figure I.1.

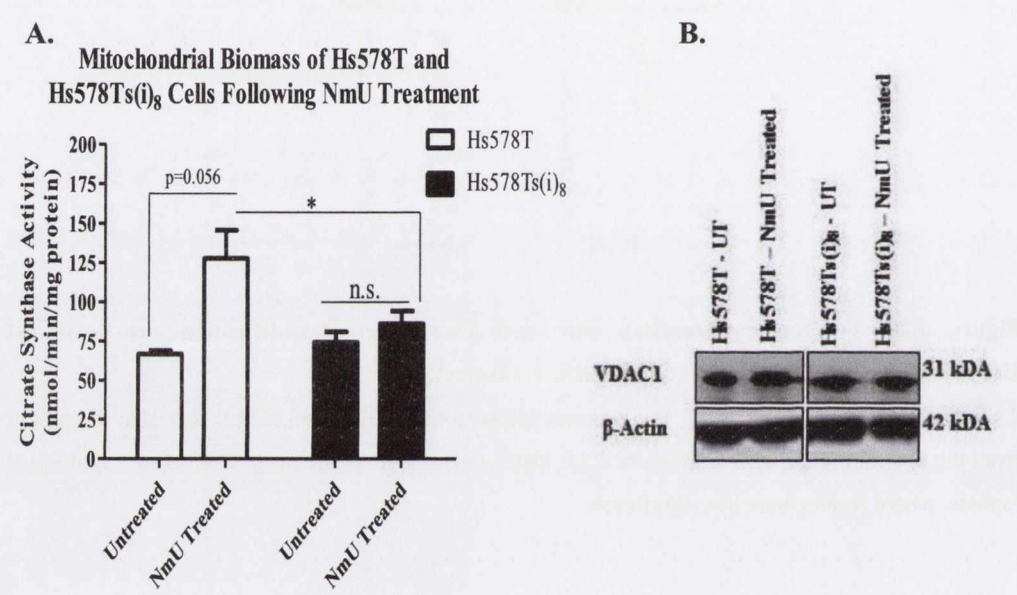
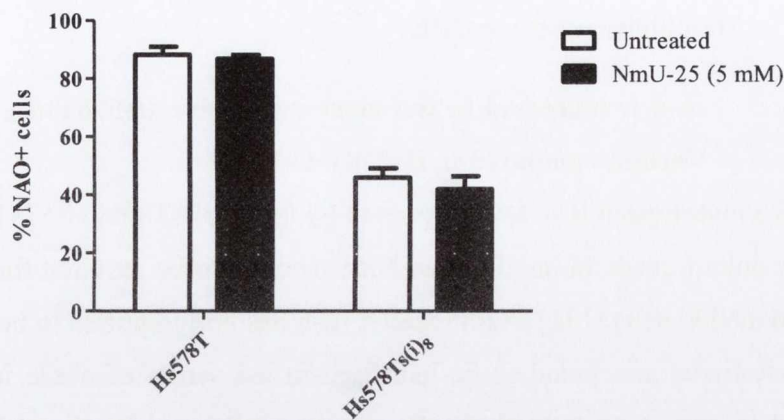


Figure 4.25: Citrate synthase assay and immunoblot for VDAC1 following NmU-25 peptide treatment.
 Analysis of mitochondrial biomass by **A.** the citrate synthase assay and **B.** immunoblot for VDAC1. Data is expressed as the mean ± SEM of 3 3 biological repeats, where *p<0.05.

4.3.5.5. Analysis of NAO Staining by Flow Cytometry following NmU-25 Peptide Treatment

Treatment of Hs578T and Hs578Ts(i)₈ cell line variants with NmU had no effect on the mitochondrial membrane potential (Figure 4.26 A and B).

A. Acridine Orange Staining of Hs578T and Hs578Ts(i)₈ Cells Following NmU-25 Treatment



B.

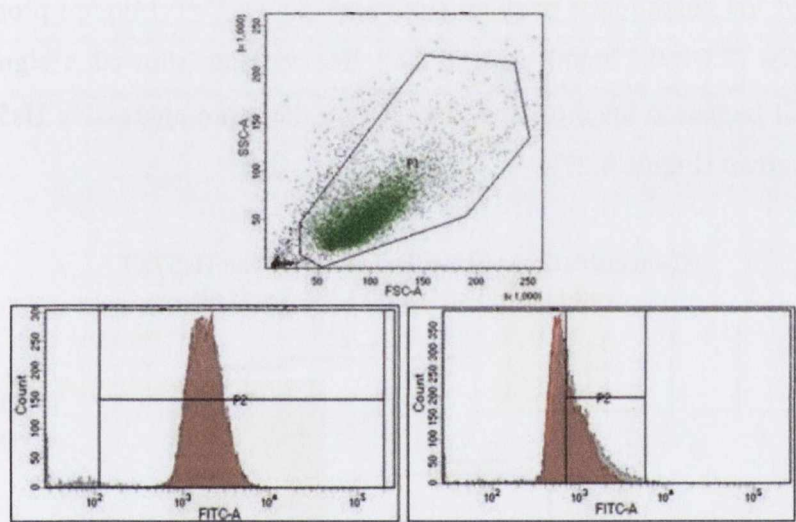


Figure 4.26: Analysis of 10-N-Nonyl Acridine Orange staining of Hs578T and Hs578Ts(i)₈ cell line variants by Flow Cytometry after NmU-25 peptide treatment

A. Hs578T and Hs578Ts(i)₈ cell line variants were stained with 10μM NAO and the intensity of staining was analysed by flow cytometry for FITC. **B.** FACSDiv analysis of results showing forward and side scatter of cells and overlap of Hs578T with NmU-25 treated Hs578T and Hs578Ts(i)₈ with NmU25 treated Hs578Ts(i)₈ cells, respectively. Data is expressed as the mean ± SEM of 3 biological repeats.

4.3.6. Analysis of IL-6 in Hs578T and Hs578Ts(i)₈ Cell Line Variants and the Effect of the IL-6 Inhibitor Tocilizumab on the Metabolism of these Cells

4.3.6.1. IL-6 is increased in the more aggressive Hs578Ts(i)₈ Cell Line Variant compared to Hs578T Cells

We investigated IL-6 levels secreted by the Hs578T and Hs578Ts(i)₈ cells into their culture medium, as IL-6 has been reported to be required for optimum growth of TNBC cells [121]. Additionally, IL-6 has been reported to be produced by adipose tissue and found to be increased in the serum of obese individuals compared to non-obese individuals (See Section 1.2.2 and Section 4.1). ELISA analysis of the conditioned medium from Hs578T (1.75 ± 0.14 ng/ml protein) and Hs578Ts(i)₈ (2.64 ± 0.17 ng/ml protein) cell line variants showed a significantly ($p=0.0002$) increased level of IL-6 released by the more aggressive Hs578Ts(i)₈ cell line variant (Figure 4.27).

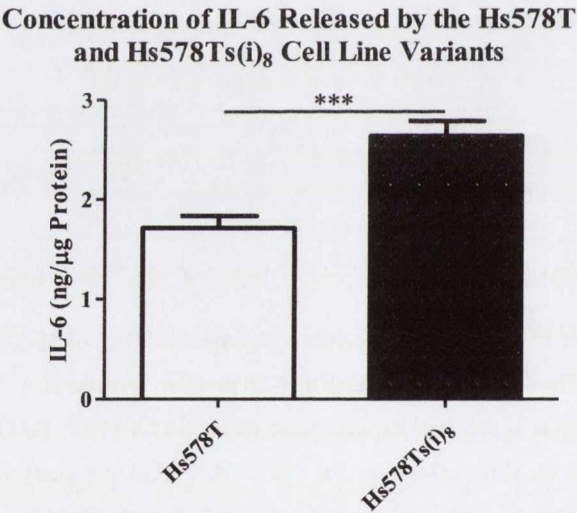


Figure 4.27: Levels of IL-6 in the conditioned media of Hs578T and Hs578Ts(i)₈ cell line variants

Data is expressed as the mean $\pm$ SEM of 3 biological repeats, where *** $p<0.001$.

4.3.6.2. Proliferation Assay for the IL-6 Receptor Inhibitor Tocilizumab

As the levels of IL-6 released by the Hs578Ts(i)₈ cell line variant are higher when compared to Hs578T cells, we investigated potential metabolic alterations in Hs578Ts(i)₈ cells following treatment with a humanised monoclonal

antibody against the interleukin-6 receptor (IL-6R) named Tocilizumab. A proliferation assay was performed for a range of Tocilizumab concentrations (0–20µg/ml) to determine the concentration of Tocilizumab that would not have cytotoxic effects on Hs578Ts(i)₈ cells. The upper cut-off of 20µg/ml was based on previous studied in our laboratory using Tocilizumab for *in vitro* work using HCC1954 cells. There was no cytotoxic effect of Tocilizumab on these cells at the highest concentrations after a 72 hour incubation (Figure 4.28).

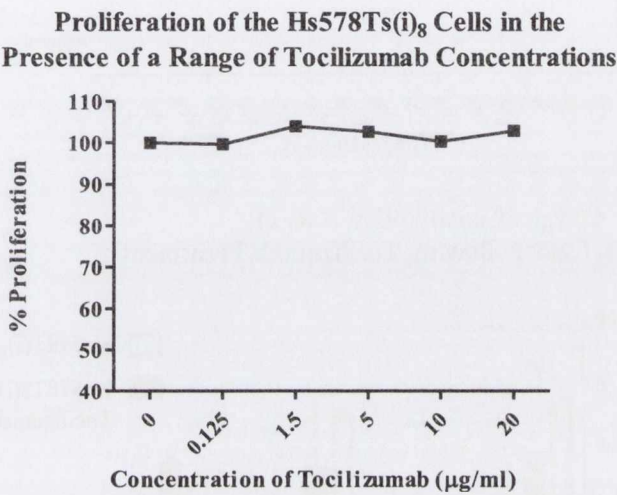


Figure 4.28: Proliferation assay for Tocilizumab in Hs578Ts(i)₈ cells
Data is expressed as the mean ± SEM of 3 biological repeats.

4.3.6.3. Metabolism of Hs578Ts(i)₈ Cells following Tocilizumab Treatment

Following a 72 hour incubation with 20µg/ml of Tocilizumab, the basal OCR of Hs578Ts(i)₈ cells was significantly ($p=0.0007$) decreased by about a third, as was the OCR, due to maximal oxidative phosphorylation ($p=0.0002$) (Figure 4.29 A and B). Remarkably, upon addition of rotenone/antimycin A, the OCR did not fall below the level of oxidative phosphorylation following oligomycin A addition, indicating Tocilizumab was completely inhibiting mitochondrial oxidative phosphorylation (Figure 4.29 A and B).

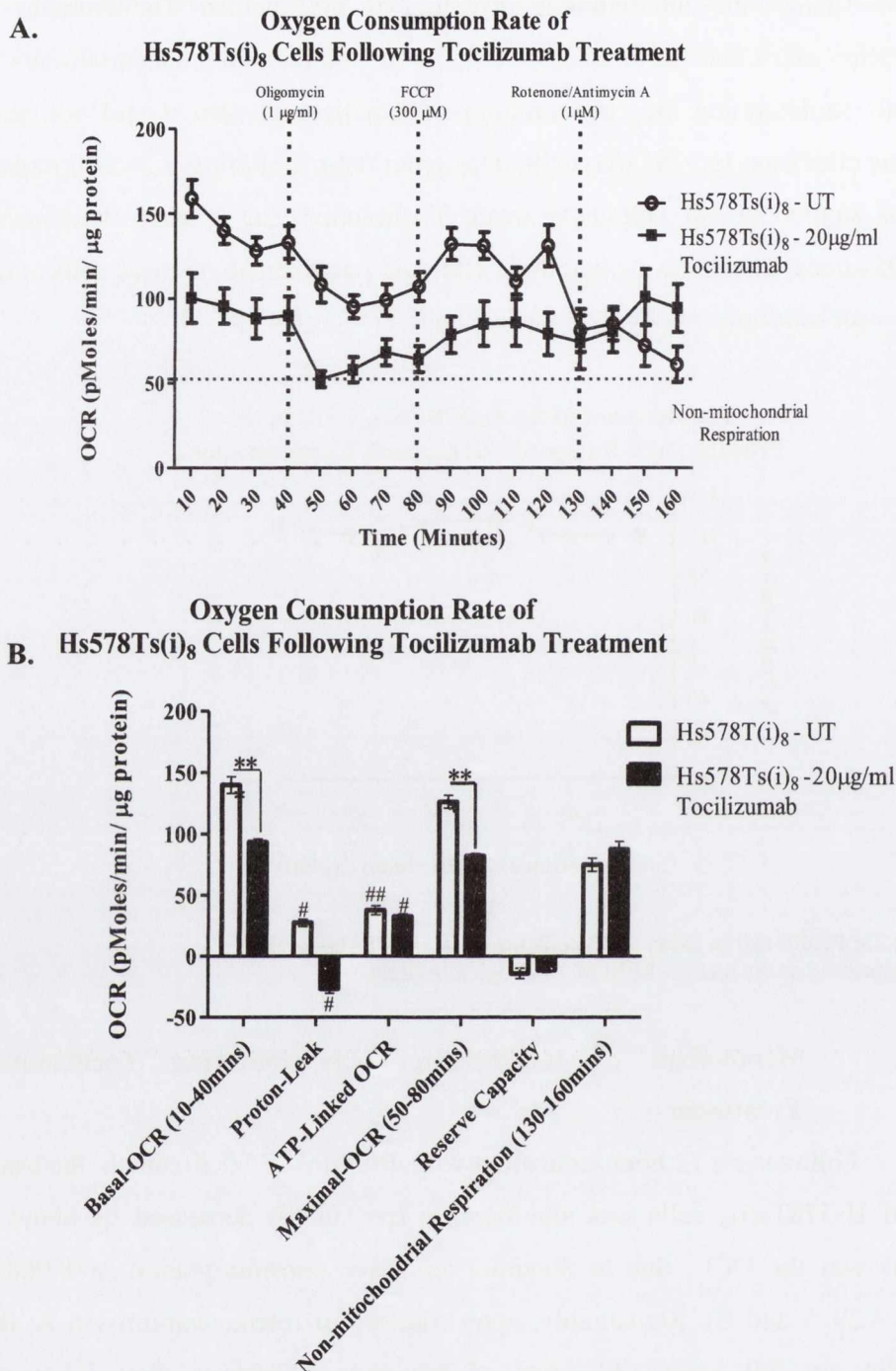


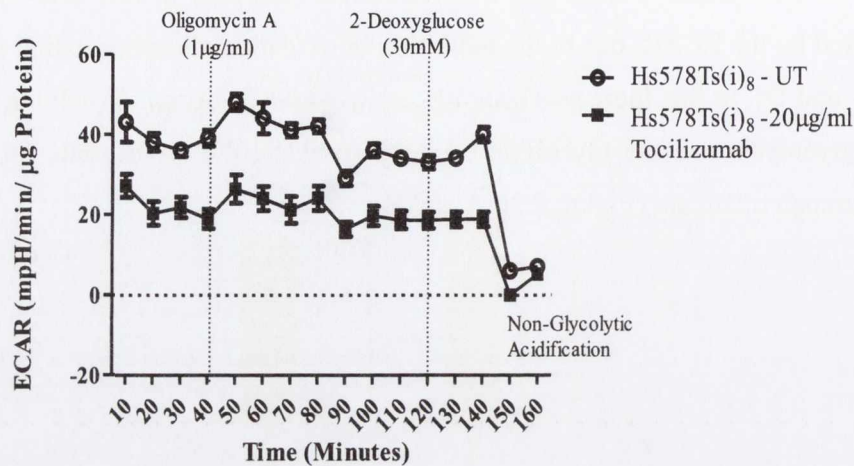
Figure 4.29: Oxygen consumption rate Hs578Ts(i)₈ cells after Tocilizumab treatment

A and B. The oxygen consumption rate was measured before and after sequential additions of oligomycin A, FCCP and rotenone/antimycin A over a 2hr 40min period. Proton leak (FCCP addition- non-mitochondrial respiration), ATP-linked (basal oxidative phosphorylation-after oligomycin A added) and reserve capacity (maximal oxidative phosphorylation-basal oxidative phosphorylation) was also determined. Data is expressed as the mean $\pm$ SEM of 3 biological repeats. Statistical analysis was performed using the Student's t-test. **/### $p < 0.01$, # $p < 0.05$, *

Hs578Ts(i)₈-UT vs Hs578Ts(i)₈-Tocilizumab treated, # Hs578Ts(i)₈-UT vs Hs578Ts(i)₈-UT and Hs578Ts(i)₈- Tocilizumab treated vs Hs578Ts(i)₈-Tocilizumab treated.

Furthermore, there was no concomitant increase in cell glycolysis, as measured by the ECAR, due to the inhibition of oxidative phosphorylation (Figure 4.30 C and D). In fact there was a significant ($p=0.0003$) decrease (halving) in the basal glycolysis and the glycolytic capacity ($p=9.5\times10^{-6}$) of the cells following Tocilizumab treatment (Figure 4.30 A and B).

A. Extracellular Acidification Rate of Hs578Ts(i)₈ Cells Following Tocilizumab Treatment



B. Extracellular Acidification Rate of Hs578Ts(i)₈ Cells Following Tocilizumab Treatment

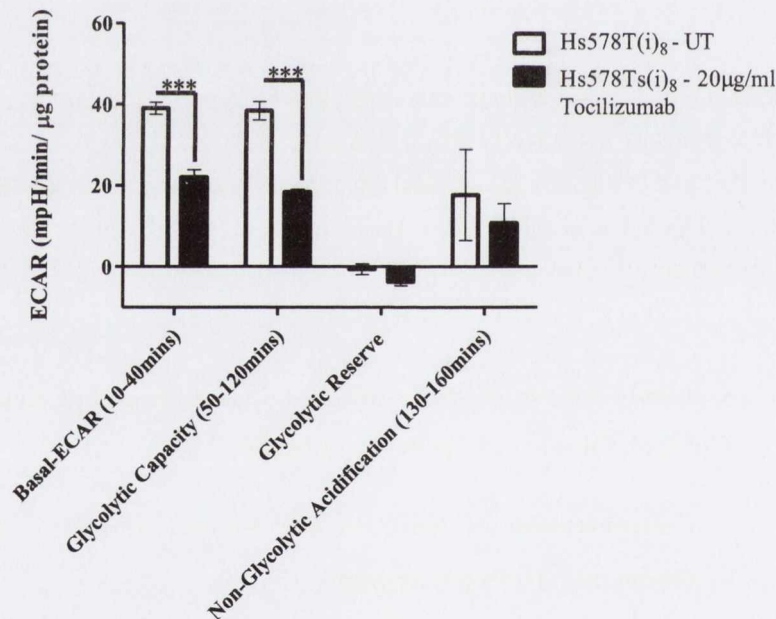


Figure 4.30: Extracellular acidification rate of Hs578Ts(i)₈ cell following Tocilizumab treatment

A and B. The extracellular acidification rate was measured, before and after sequential additions of oligomycin A and 2-deoxyglucose over a 2hr 40min period. Glycolytic reserve (glycolytic capacity-basal glycolysis) was also determined. Data is expressed as the mean $\pm$ SEM of 3 biological repeats. Statistical analysis was performed using the Student’s t-test., ***p<0.001.

The reduced cellular oxygen consumption and reduced glycolysis due to Tocilizumab are also reflected in the lack of difference in OCR/ECAR in Hs578Ts(i)₈ cells with and without Tocilizumab treatment (Figure 4.31)

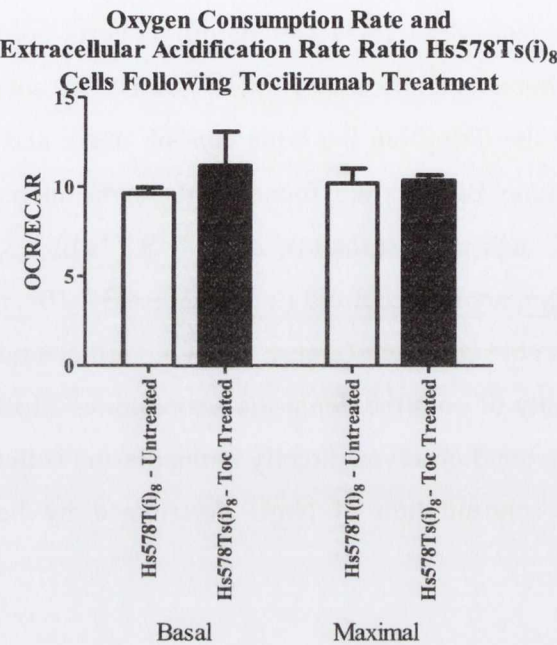


Figure 4.31: Oxygen consumption rate and Extracellular acidification rate ratio of Hs578Ts(i)₈ cells before and after Tocilizumab treatment.

The ratio of the basal (0-40mins) and maximal (90-120mins) OCR and ECAR was also calculated from the data in Figure 4.20 and Figure 4.21. Data is expressed as the mean ± SEM of 3 biological repeats, where results were not significant.

4.3.7. Clinical Analysis of NmU and IL-6 in the Plasma of Healthy Control, Obese and MetS participants

4.3.7.1. Concentration of NmU in the Plasma and EVs of Control, Obese and MetS participants

Analysis of NmU in the plasma of control (n=11), obese (n=12) and MetS (n=13) female participants established NmU to be significantly (p=0.03) altered in the healthy control, obese and MetS cohorts when analysed by one-way *ANOVA*. Post-hoc Tukey analysis indicated NmU was significantly decreased (p=0.025) in the plasma of the obese cohort (0.89±0.05ng/ml) compared to the control cohort (1.16±0.1ng/ml). The MetS cohort (0.93±0.06ng/ml) also showed decreased NmU levels compared to the controls, and this was tending towards significance (p=0.06) (Figure 4.32 A). These NmU levels were determined using ELISAs

purchased from Peninsula Laboratories. Unfortunately, this NmU ELISA could no longer be considered reliable; therefore we purchased NmU ELISAs from Cloud-clone. We again assessed the NmU levels in the plasma of the same control, obese and MetS participants. We found NmU to be slightly increased in the obese (5.44 ± 0.71 ng/ml) and MetS (5.3 ± 0.49 ng/ml) cohorts compared to controls (4.76 ± 0.59 ng/ml) but not significantly (Figure 4.32 B). We subsequently assessed the NmU levels in the EVs from the same control, obese and MetS participants using the Cloud-clone ELISA. We found NmU to be increased in the obese ($2.5 \times 10^{-6} \pm 3.06 \times 10^{-7}$ ng/ μ g EV protein), and MetS ($3 \times 10^{-6} \pm 3.4 \times 10^{-7}$ ng/ μ g EV protein) cohorts compared to controls ($2.31 \times 10^{-6} \pm 4.34 \times 10^{-7}$ ng/ μ g EV protein) but, again, this was not significant (Figure 4.32 C). Further studies are required to determine the validity of both the Peninsula Laboratories ELISA and the Cloud-clone ELISA as the trend observed directly in the plasma is the opposite for both ELISAs, also the concentration of NmU determined by both ELISA varies substantially.

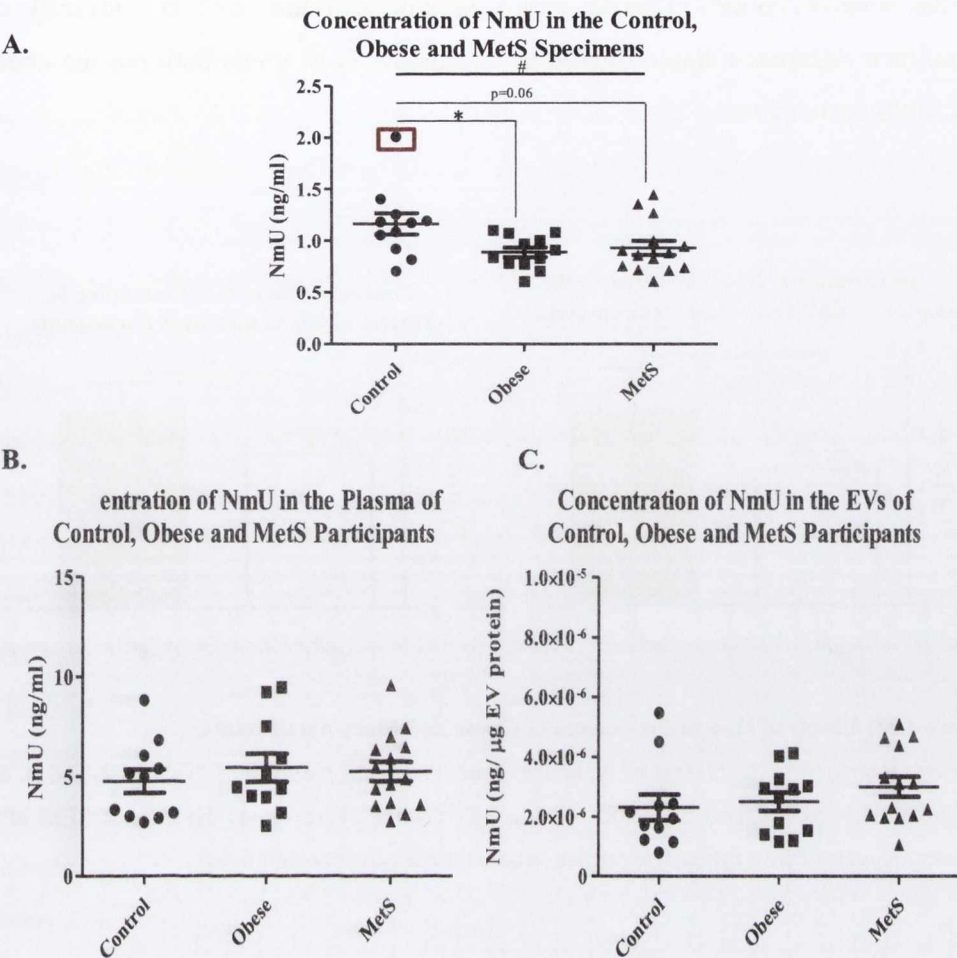


Figure 4.32: NmU detected in the Control, Obese and MetS Specimens

A. Assessed in plasma using the Peninsula Laboratories ELISA **B.** Assessed in plasma using the Cloud-clone ELISA **C.** EVs from Healthy Control, Obese and MetS participants' specimens assessed by the Cloud-clone ELISA. Specimen boxed in red was an outlier as determined by the Grubbs test and was excluded for significance testing. Data is expressed as the mean $\pm$ SEM of 3 technical repeats of NmU for each specimen. Significance testing was performed using one-way ANOVA, $^{\#}p<0.05$ and post-hoc Tukey analysis, $*p<0.05$.

4.3.7.2. Concentration of IL-6 in the Plasma of Control, Obese and MetS Participants

Analysis of the levels of IL-6 in the plasma of the female participants in the obese and MetS cohorts (Table 4.2-obese and MetS) by ELISA determined IL-6 was increased in the MetS cohort ($2.30 \pm 0.5 \text{ pg/ml protein}$) compared to the obese cohort ($1.59 \pm 0.2 \text{ pg/ml protein}$) by 1.44 ± 0.48 -fold (Figure 4.33 A). Similarly, analysis of IL-6 in the plasma of the whole study cohort (Table 2.4) determined IL-6 was higher in the MetS cohort ($1.98 \pm 0.3 \text{ pg/ml protein}$) compared

to the control cohort ($1.56 \pm 0.2 \text{ pg/ml protein}$) (Figure 4.33 B). Overall no significant difference was observed in circulating IL-6 levels between the obese and MetS participants.

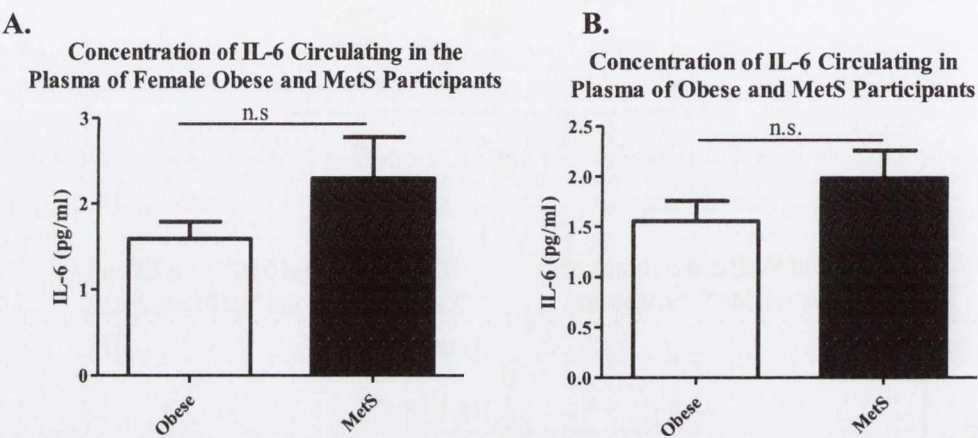


Figure 4.33: Levels of IL-6 in the plasma of Obese and MetS participants. IL-6 was measured in the plasma of **A.** female obese and MetS participants (Table 4.2) and **B.** all obese and MetS participants by ELISA (Table 2.2). Data is expressed as the mean $\pm$ SEM of 3 technical repeats of IL-6 for each specimen, where results were not significant.

4.4. Discussion

Every cell in the body undergoes cell division in order to replace dead or damaged cells and also for cell growth. Cell division is a myriad of strictly regulated processes that involve an intricate balance of signals that both direct a cell to divide but also direct a cell to undergo apoptosis, as appropriate. Numerous factors, such as soluble growth factors, cell membrane receptors, adhesion molecules and extracellular matrix molecules have a role in the control of cell division. Cancer is a result of loss of this intricate balance of factors. As cells mutate, they overcome the control signals in order to proliferate at their own rate, developing their own set of characteristics that enable them to grow independently and uncontrollably, which are known as the 'Hallmarks of Cancer' [220]. Cancer cells overcome the requirement for growth factor signals to grow. They become self-sufficient in growth signals and become insensitive to anti-growth signals. They can evade signals for apoptosis, acquire the ability to sustain angiogenesis and can also acquire the ability to invade surrounding tissues in order to metastasise [220]. Eleven years after the first publication of the 'Hallmarks of Cancer' the authors published a second paper where they highlighted the emerging 'Hallmarks of Cancer'; one of which was deregulation of cellular metabolism [145] (See Section 1.3 and 4.1).

Breast cancer is a heterogeneous group of carcinomas, initiating in the epithelial cells of the mammary glands, which are morphologically and clinically diverse [221]. Through molecular classification this heterogeneous disease was classified into four key categories: (i) Normal-like, (ii) Luminal- estrogen receptor positive (ER+) and progesterone receptor positive (PR+) (iii) Human epidermal growth factor receptor 2+ (HER2+)- overexpression of the HER2 oncogene and (iv) Basal-like- generally lack ER, PR and HER2 receptors, and are mostly classified as TNBC. Luminal and HER2-overexpressing breast cancers have yielded tremendous results from targeted therapies, such as tamoxifen and aromatase inhibitors as therapies for ER+ breast cancer patients [42-44] and trastuzumab (Herceptin) [222] and lapatinib [223] as therapies for HER2-overexpressing tumours. However, TNBC remains without a targeted treatment, due to the lack of ER and PR expression and HER2-overexpression.

TNBC is a highly aggressive, heterogeneous breast cancer subtype which is responsible for 15-20% of breast cancer cases [224]. TNBC is associated with a shorter time to the occurrence of relapse [225], higher mortality rates than receptor positive (42.2% versus 28%, hazard ratio=3.2 for TNBC), a higher risk of developing distant metastasis (hazard ratio=2.6 for TNBC compared to receptor positive tumours) [210] and TNBC diagnoses commonly occur at a later stage, after metastasis. This cancer subtype tends to show relative sensitivity to neoadjuvant anthracycline-based chemotherapeutic treatments [226]. A neoadjuvant chemotherapeutic agent routinely used for TNBC is cisplatin, a DNA cross-linking agent shown to be effective in clinical trials [227]. African American and Hispanic women and younger women usually have a higher incidence of TNBC [210]. Both observational and laboratory studies have suggested that obesity may promote TNBC through IR, secretion of pro-angiogenic adipokines, such as leptin and inflammation, such as IL-6 [228]. Obesity has been shown to account for a 42% increased risk of TNBC in premenopausal women with a BMI equal to or greater than 30kg/m² (See Section 4.1) [210]. As of yet there is no biomarker for TNBC progression or therapy response.

In this chapter, we aimed to investigate the alterations in the metabolic phenotype of two TNBC cell line variants- Hs578T and its more aggressive subclone Hs578Ts(i)₈— as a potential mechanism for aggressive TNBC and also to investigate Neuromedin U (NmU) and Interleukin 6 (IL-6) as potential biomarkers for MetS. We subsequently aimed to determine the role of NmU-peptide and IL-6 modulating therapy (an anti-IL-6R antibody) on the metabolism of these cells.

Firstly, we determined the metabolic phenotype of Hs578T and Hs578Ts(i)₈ cell line variants using the Seahorse Extracellular Flux Analyser. The profile of the OCR following sequential addition of inhibitors of the electron transport chain and uncoupler— oligomycin A, FCCP and rotenone/antimycin A— illustrated that substantial oxidative phosphorylation is occurring in Hs578T cells with significantly less (2.1±1.2-fold) oxidative phosphorylation occurring in the aggressive Hs578Ts(i)₈ cell line variant. Furthermore, quantification of mitochondrial abundance was assessed in both cell line variants using citrate synthase activity and VDAC1 protein detection, with the magnitude of the mitochondrial membrane potential being measured as a further index of

mitochondrial function, as we hypothesised that the lower oxygen consumption rates in Hs578Ts(i)₈ cells when compared to Hs578T cells, might be due to (i) reduced mitochondrial abundance, (ii) reduced supply of substrates to mitochondria or (iii) dysfunctional/aberrant mitochondria, we went on to address some of these possibilities. In doing so, we were able to establish from citrate synthase assays and VDAC1 immunoblotting that the mitochondrial biomass was equivalent in Hs578T and Hs578Ts(i)₈ cell line variants, ruling out a decrease in mitochondria to be the reason behind reduced oxidative phosphorylation. Moving forward then, a reduced supply of substrates to the electron transport chain might not only manifest itself as a reduction in mitochondrial oxygen consumption but also a reduced mitochondrial membrane potential. NAO staining established a significantly reduced (2 ± 0.9 -fold) mitochondrial membrane potential in mitochondria from Hs578Ts(i)₈ cells compared to Hs578T cells, indicating that the mitochondria of Hs578Ts(i)₈ cells were dysfunctional. This suggests either reduced supply of substrates to the mitochondria or that the mitochondrial are, in fact, dysfunctional/aberrant. One of the many possible causes of reduced substrate supply to mitochondria in cancer cells is increased activity of pyruvate dehydrogenase kinase (PDK), which phosphorylates and inhibits pyruvate dehydrogenase (PD) and, thus, chokes supply of Acetyl CoA to the Krebs cycle [219]. We postulated that increased PDK activity might explain the reduced oxygen consumption in Hs578Ts(i)₈ cells. Therefore, we assessed the cytotoxicity of adding Dichloroacetic acid (DCA), a known inhibitor of PDK, to our cells. However, DCA did not increase oxygen consumption in Hs578Ts(i)₈ cells; at least not at the concentration and incubation time we selected to use. This indicates that increased PDK activity is probably not involved in the metabolic switch between Hs578T and Hs578Ts(i)₈ cell line variants. Thus, further research is required to delineate the molecular basis of the reduced mitochondrial function in Hs578Ts(i)₈ cells.

The Seahorse Extracellular Flux analyser not only measures oxygen consumption but simultaneously measures extracellular acidity rate, a surrogate for glycolysis. In light of the differential in the mitochondrial oxygen consumption rates between Hs578T and Hs578Ts(i)₈ cell line variants, we predicted that glycolytic flux might be increased in Hs578Ts(i)₈ cells compared to Hs578T cells. Hs578Ts(i)₈ had a 1.4 ± 0.7 -fold higher glycolytic flux than Hs578T

cells. This value was lower than expected, based on the predicted rate of ATP supply.

Next, we investigated the effects of metabolic-related factors on the metabolism of our TNBC cell line variants. One such factor is the neuropeptide neuromedin U (NmU). NmU has been shown to have a plethora of functions including a role in feeding behaviour, energy homeostasis and resistance of HER2-overexpressing breast tumours to chemotherapeutic drugs, thus having a negative outcome in HER2-overexpressing breast cancer [109, 112] (See Section 1.2.2). We began by investigating the intracellular levels of NmU in Hs578T and Hs578Ts(i)₈ cell line variants. We found NmU to be significantly ($p=0.007-0.02$) decreased in the more aggressive Hs578Ts(i)₈ cell line variant. As NmU was found to be decreased in Hs578Ts(i)₈ cells compared to Hs578Ts cells, we hypothesised that if we added exogenous NmU-25 peptide to Hs578Ts(i)₈ cells we could increase the mitochondrial oxidative phosphorylation of the cells to that of Hs578T cells. Interestingly, NmU-25 peptide treatment of Hs578T cells resulted in a significant decrease in the basal oxidative phosphorylation by 1.4 ± 0.7 -fold and the maximal oxidative phosphorylation by 1.7 ± 0.5 -fold. A concomitant increase in cell glycolysis was not observed; in fact, the basal glycolysis was significantly decreased following NmU-25 peptide treatment. On the other hand, treatment with NmU-25 peptide had no effect on the mitochondrial oxidative phosphorylation of Hs578Ts(i)₈ cells but did significantly inhibit glycolysis. In this sense, adding NmU-25 peptide to Hs578T cells pushes them towards a Hs578Ts(i)₈ phenotype and possibly pushes Hs578Ts(i)₈ cells towards a more aggressive phenotype. These findings also suggest that treatment with NmU-25 peptide induces changes in metabolic pathways other than oxidative phosphorylation and glycolysis, although it cannot be ruled out that NmU induces a decrease in energy generation from various metabolic pathways. In saying that oxidative phosphorylation and glycolysis are the primary ATP generating pathways that other pathways such as fatty acid synthesis pathway feed into.

The second metabolic factor that we investigated was interleukin 6 (IL-6). We have shown that NmU may induce the release of IL-6 from HER2-overexpressing cell lines and HER2-overexpressing cells resistant to chemotherapeutic drugs (Martinez *et al*, Submitted) thus, we investigated IL-6 in our cell line variants. IL-6 is known as the endocrine cytokine as it has a role in

energy, feeding and metabolism [114] and is required by TNBC for growth [121] (See Section 1.2.2). We found IL-6 to be significantly ($p=0.0002$) increased in the more aggressive Hs578Ts(i)₈ cell line, which was expected as TNBC is thought to rely on IL-6 for optimum growth. From this data, we hypothesised that blocking the IL-6 feed-back loop in Hs578Ts(i)₈ cells would push the cells back to Hs578T phenotype. We tested this hypothesis by using a clinically-available monoclonal antibody named Tocilizumab, which works by binding to the IL-6 receptor and inhibiting its signalling. Treatment of Hs578Ts(i)₈ cells with Tocilizumab significantly decreased their mitochondrial oxidative phosphorylation. Similarly, the glycolysis of Hs578Ts(i)₈ cells was also significantly decreased. IL-6 and its receptor appear to play a role in TNBC cell metabolism; although its precise nature remains unclear and needs further investigation.

Having determined NmU levels to be decreased in the Hs578Ts(i)₈ cells compared to the Hs578T cells the next logical step was to determine the amount of NmU in the clinical specimens from healthy control, obese and MetS participants as a biomarker for MetS. Analysis of NmU in the plasma of healthy controls and participants with obesity and MetS determined NmU to be decreased in the obese cohort compared to the control cohort and the MetS cohort was decreased compared to the control cohort but not significantly; however this was observed when using an NmU ELISA from Peninsula Laboratories. This result was not observed when NmU levels were assessed using an ELISA from Cloud-clone. Further analysis of the validity of these NmU ELISAs is required. Having assessed the levels of IL-6 released by the Hs578Ts(i)₈ cells and found it to be significantly increased in these cells compared to the Hs578T cells, again, the next logical step was to evaluate the levels of IL-6 circulating in the plasma of the obese and MetS participants. This had been assessed by Bohl *et al* [229] in Denmark. Analysis of the raw data supplied by Bohl *et al* for the levels of IL-6 in the plasma of our obese and MetS participants showed IL-6 to be increased in the MetS cohort compared to the obese cohort however this difference was not significant.

4.5. Conclusion

This study illustrates that as TNBC progresses to a more aggressive, invasive and migratory phenotype there is a metabolic switch from mitochondrial oxidative phosphorylation towards glycolysis. From our preliminary results, this switch is not due to a decrease in mitochondrial biomass but is potentially the result of diminished supply of substrates to the mitochondria or a decrease in mitochondrial function. Thus, mitochondrial dysfunction could potentially act as biomarker for aggressive TNBC. This switch does not appear to be a result of an increase in the expression of PDK as treatment with its inhibitor DCA did not result in the switch of the more aggressive TNBC cell line to back oxidative phosphorylation. Transcriptomic and proteomic analysis of the Hs578T and Hs578Ts(i)₈ cell line variant may help in the search to pin-point the switch taking place in the Hs578Ts(i)₈ cell which confers greater aggressiveness on these cells. Having assessed the amounts of NmU and IL-6 in the plasma of the control, obese and MetS cohorts we cannot conclude that NmU could not be a biomarker for MetS as further study is required. However, it appears IL-6 is not a biomarker for MetS, at least not in this study cohort.

Chapter Five: Discussion & Conclusion

5.1. Discussion

The primary aim of this PhD project was to investigate potential minimally-invasive biomarkers, either freely-circulating in the plasma or contained within EVs, for metabolic syndrome (MetS) independent of obesity. These biomarkers would function to aid in diagnosing, prognosing and determining response to therapy in MetS individuals. The second aim was to determine whether plasma or EVs are superior sources of biomarkers for MetS or if a combination of both is more beneficial for biomarker discovery. The final aim was to investigate the association of MetS and obesity with the increased risk of TNBC. We began this investigation by analysing the underlying metabolic phenotype of two triple negative breast cancer (TNBC) cell line variants (a parental cell line and its clonal aggressive isogenic variant), to determine the influence of metabolism on TNBC, which has been epidemiologically linked to MetS and obesity [206]

As is discussed in Chapter 2, the current methods for diagnosing and prognosing MetS are insufficient to identify all MetS individuals due to the numerous definitions available, leading to clinician bias. Also, the definitions require numerous tests, *i.e.* oral glucose tolerance testing, measurement of body mass index/waist circumference, measurement of triglycerides and finally blood-pressure [155]. To provide a simple, minimally-invasive method for diagnosing MetS, blood-based biomarkers were investigated, as blood procurement is the least invasive procedure available. Plasma was isolated from blood as it is superior for RNA research compared to serum. Exiqon have shown that plasma has lower miRNA expression levels (cycle threshold values) compared to serum indicating higher miRNA content. However, this is only true for plasma isolated from EDTA tubes as heparin inhibits the enzymes of the reverse transcription (RT) and PCR reactions [230]. For these reason, we collected blood in EDTA tubes. miRNA research is difficult in bio-fluids due to low levels of RNA, high levels of inhibitors of the RT reaction, susceptibility to pre-analytical variables and difficulty normalising the expression data. To overcome these problems we first assessed the RNA by NanoDrop for 260/280 values, performed small RNA Bioanalyzer analysis, determined an input volume of RNA for the RT reaction in order to avoid inhibitors of the RT reaction and in order to avoid unreliable RNA concentrations due to phenol contamination and preformed a pre-screen to

identify endogenous control RNAs for normalising the data. The NanoDrop analysis determined the quality of the RNA to be lower than expected from a cellular sample, however, previous work performed in our laboratory had found similar 260/280 values for RNA isolated from the conditioned medium of cultured breast cancer cell lines. From the small RNA Bioanalyser analysis, we determined a relatively high presence of miRNAs in the total RNA sample isolated. Finally, from the pre-screen, five potential endogenous control RNAs for normalising the miRNA profiling data were identified and placed on custom miRNA profiling panels. Following pre-analysis of the RNA for subsequent miRNA profiling the miRNA profiling was performed and miR-150-5p and miR-486-5p were selected as endogenous control RNAs for this study cohort. Subsequently, miR-758-3p was identified in the discovery and validation stages of this research as a potential biomarker for MetS as it was detected in the plasma from the obese participants but undetected in the plasma from the MetS participants. Numerous potential mRNA targets of miR-758-3p were identified using the predictive tool/database miR-walk. Two targets of interest which were further investigated were the cholesterol efflux regulatory protein ABCA1/CERP and the breast cancer resistance protein ABCG2/BCRP. ABCA1 was of interest as MetS is associated with increased LDL-cholesterol and decreased HDL-cholesterol. ABCG2 was of interest as MetS and obesity are associated with an increased risk of TNBC and may have an underlying role in drug resistance [231]. We investigated the expression of these two proteins in two TNBC cell line variants by over-expression of miR-758-3p and inhibition of miR-758-3p in relevant cell line variants to confirm miR-758-3p targeted these proteins. This however could not be shown in our TNBC cell line variants. However, in a recent study by Ramirez *et al* [164] they demonstrated that miR-758-3p targets ABCA1 in liver and macrophage cell lines. Our attempt to modulate miR-758-3p was unsuccessful; this may be due to miR-758-3p action being primarily in the macrophages and liver.

The miRNA profiling in Chapter 2 confirmed plasma contains miRNAs and at a substantial percentage of the total RNA content (60-65%) and so plasma is a suitable source of miRNA biomarkers. Studies in our laboratory have initially focused on plasma or serum as potential sources of biomarkers [167, 168, 194]. In recent years, however, a new area of biomarker research has begun which focuses

on nano- and micron-sized extracellular vesicles (EVs) detected in biological fluids wherein the EVs themselves and their substantial cargo have been shown to be biomarkers for disease (See Section 1.2) [188, 191]. Thus, research in this PhD aimed to investigate EVs as biomarkers for MetS from the biological plasma specimens and their miRNA cargo also as potential biomarkers for MetS. This research then evolved, to compare the miRNA content freely circulating in the plasma compared to the EV-miRNA content. In Chapter 3 miRNA profiling was performed in a similar manner to Chapter 2, but this time focused on miRNAs isolated from EVs which had been isolated from the plasma of healthy control, obese and MetS participants. Here we found the expression of fourteen miRNAs to be of interest as they were significantly altered in the obese cohorts and MetS cohorts compared to controls. Upon validation, miR-100-5p showed the same trend in the profiling and validation stages. Comparison of the miRNAs freely circulating in the plasma and contained within EVs showed miR-758-3p as a potential plasma-miRNA biomarker for MetS, however this was not detected in the EVs of the healthy control, obese or MetS cohorts, therefore the process of EV isolation is not required to identify this miRNA for diagnosis of MetS. Eleven miRNAs were detected both freely-circulating in the plasma and enclosed within EVs. Analysis of the C_T values showed the levels of the miRNAs were lower freely-circulating in the plasma compared to those of the miRNAs enclosed within the EVs, and six of these miRNAs were significantly decreased in the EVs compared to the plasma of the obese and MetS participants, however, the six miRNAs were different in each cohort. Overall, we concluded from this analysis that it is not simply a case of freely-circulating miRNA or EV-miRNA biomarkers for MetS, or one being superior over the other; plasma-miRNAs and EV-miRNAs are of equal importance for biomarker discovery for MetS. It is a combination of both that may hold the most potential for diagnosing, prognosing and determining response to therapy in individuals with MetS.

Fifty-two individuals enrolled in this study and completed the 12-week dietary intervention. miRNA profiling of the RNA isolated from the plasma of these participants following the 12-week diets showed miR-758-3p was only detected in the obese cohort, as was seen at visit 2, baseline. Comparison of the miRNA levels in the MetS cohort compared to the obese cohort following the 12-week diets showed 16 of the 21 miRNAs detected were significantly decreased.

Again, miR-758-3p was undetected in the MetS cohort following the 12-week diets and detected in the obese cohort. Analysis of the four diets individually indicated miR-758-3p was undetected in the MetS cohort for 3 diet groups *i.e.* high MC-SFA-whey, high MC-SFA-casein and low MC-SFA-casein; miR-758-3p was however detected in the low MC-SFA-whey diet group. Interestingly, the clinical outcome for participants on the whey diet irrespective of MC-SFA content had a decreased post-prandial chylomicron load. Indicating whey supplementation as a potential dietary treatment for MetS. Furthermore, as miR-758-3p was detected in the MetS cohort following the low MC-SFA-whey diet miR-758-3p may be used as a tool for monitoring response to whey supplementation in MetS individuals.

The final aim of this project was to investigate the metabolic phenotype of two TNBC cell line variants, Hs578T and its more aggressive subclone Hs578Ts(i)₈. We decided to investigate the metabolic phenotype of these two cell line variants for a number of reasons:

- (i) We were interested in the role of metabolism in the progression of TNBC to aggressive TNBC. This is because epidemiological studies have shown MetS and obesity both increase of the risk of TNBC.
- (ii) If a difference in the metabolic phenotype was detected the switch taking place during the selection process of the Hs578Ts(i)₈ cells may be responsible for the altered metabolic phenotype.
- (iii) The effect of Neuromedin U (NmU) on the metabolism of these cell line variants should be investigated to determine if NmU has a role in the progression of TNBC, as NmU is associated with obesity since knockout mice (NmU^{-/-}) are obese and is associated with poor outcome in HER2-overexpressing breast cancer.
- (iv) Finally, to investigate the levels of IL-6 (which have been shown to be increased in TNBC) in both cell line variants as we have recently shown NmU may drive IL-6 secretion in HER2-overexpressing breast cancer cells (Martinez *et al*, submitted) and determine if the anti-IL-6 antibody Tocilizumab had an effect on the metabolism of these cells.

Investigation of the metabolic phenotype of a parental TNBC and an aggressive TNBC cell line variant demonstrated that during the progression to

aggressive TNBC the metabolic phenotype of these cells changes dramatically. Specifically, the parental TNBC cell line variant used mitochondrial oxidative phosphorylation to generate adenosine triphosphate (ATP) for their energy demands. Conversely, the more aggressive TNBC cells had a significantly reduced level of mitochondrial oxidative phosphorylation compared to the parental TNBC cells. The aggressive TNBC cells had switched to using glycolysis to produce their energy, as glycolysis produces ATP energy faster than mitochondrial oxidative phosphorylation but at a less efficient rate. It is hypothesised that cancer cells switch to using glycolysis in order to increase the uptake and incorporation of nutrients to increase growth rates. In an attempt to determine the switch taking place in the parental TNBC as it progressed to a more aggressive TNBC we investigated pyruvate dehydrogenase kinase (PDK). PDK acts to inhibit pyruvate dehydrogenase (PD) which functions to convert pyruvate to Acetyl CoA, this is subsequently shuttled into the Krebs cycle for mitochondrial oxidative phosphorylation. It has been shown that breast cancer can have an increased level of PDK, thus inhibiting PD and the conversion of pyruvate to acetyl CoA (See Section 4.1). Thus, we treated the aggressive TNBC cells with Dichloroacetate in order to inhibit PDK. However, PDK was not implicated in the switch seen in the TNBC cell line variants at the concentration and incubation time used for this study.

We investigated the potential of two metabolic factors- NmU and IL-6- as biomarkers for obesity and MetS and their role in the metabolism of the TNBC cell line variants. NmU has been associated with feeding behaviour and energy expenditure and interestingly we have shown NmU is associated with a poor outcome in HER2-overexpressing breast cancer. Therefore we assessed the amounts of NmU in our TNBC cell line variants. We hypothesised NmU would be increased in the more aggressive cell line variant. However upon evaluation NmU was decreased in the more aggressive cell line variant compared to the parental cell line variant. We next hypothesised that as NmU is decreased in the aggressive TNBC cell line variant compared to the parental TNBC cell line variant, treatment of the aggressive TNBC cell line with exogenous NmU-25 peptide would revert the aggressive cell line back to the phenotype of the parental TNBC cell line variant. Interestingly, this was not observed, NmU-25 treatment did not improve the metabolic phenotype of these cells, in fact it inhibited the

glycolysis of the aggressive TNBC cell line. Remarkably, treatment of the parental TNBC cells with NmU-25 resulted in decreased oxidative phosphorylation. Of note, the glycolysis of the parental TNBC cells was not increased following the decrease in oxidative phosphorylation with NmU-25 treatment. These results illustrate that NmU acted to inhibit the cells primary source of ATP generation, *i.e.* mitochondrial oxidative phosphorylation for the parental TNBC cells and glycolysis for the aggressive TNBC cells.

Additionally, NmU has been associated with obesity as NmU^{-/-} mice are obese and peripheral administration of NmU to these mice decreases their weight and increases their energy expenditure. Thus, we hypothesised NmU would be decreased in the plasma of these obese and MetS participants. Upon analysis, the levels of NmU in the plasma of obese and MetS participants were significantly decreased in the plasma of obese participants compared to controls and tending towards significantly decreased in the plasma of MetS participants compared to controls. We also investigated NmU levels in the EVs isolated from the same specimens, however, the ELISA used to determine plasma NmU levels could no longer be considered reliable, thus we measured the level of NmU in EVs and re-measured NmU levels in the same plasma specimens using an ELISA from another supplier. This ELISA showed no difference in NmU levels in the plasma or EVs. Therefore, further analysis of the validity of both ELISAs is required in order to evaluate if circulating NmU has potential as a biomarker for MetS.

We have recently shown that NmU may drive IL-6 production in HER2-overexpressing cells and IL-6 has been reported to be required for optimum growth of TNBC. Consequently, we investigated IL-6 levels secreted by our TNBC cell line variants and found IL-6 secretion to be significantly increased in the aggressive TNBC cells compared to the parental TNBC cells. Therefore, we investigated if inhibition of the IL-6 receptor in the aggressive TNBC cells, using a monoclonal antibody named Tocilizumab, could revert these cells to the phenotype of the parental TNBC cells. However, Tocilizumab significantly decreased the basal and maximal mitochondrial oxidative phosphorylation of the aggressive TNBC cells. The same trend was seen for the glycolysis of these cells, which was significantly decreased following Tocilizumab treatment. Analysis of IL-6 in the plasma of the obese and MetS participants in this study found IL-6 to

be increased in MetS participants compared to the obese participants but not significantly.

Our findings show for the first time the metabolic switch in the progression of TNBC to aggressive TNBC and this has potential as a mechanism for progression to aggressive TNBC. Also, the discovery that the mitochondria of the aggressive TNBC cell line variant are dysfunctional or lacking a substrate supply may offer potential for a biomarker for TNBC.

5.2. Conclusion

This PhD project has investigated minimally-invasive biomarkers for MetS either isolated directly from plasma of obese and MetS individuals who enrolled in a 12-week dietary intervention or enclosed in EVs isolated from the same plasma specimens. From this investigation we have found two potential biomarkers for MetS, *i.e.* miR-758-3p freely-circulating in the plasma and miR-100-5p enclosed within EVs. We have also demonstrated that EV quantities have potential as minimally-invasive biomarkers for MetS and they can distinguish MetS from obesity. Furthermore, we have shown that miR-758-3p holds promise as a biomarker for dietary intervention of low MC-SFAs and whey protein. The metabolic alterations triggered by obesity and MetS can influence tumorigenesis and obesity has been epidemiologically linked to TNBC. Our investigation of the metabolic phenotype of two TNBC cell line variants found there to be a significant alteration in the metabolic phenotype of the these cell line variants which holds potential a possible mechanistic link for obesity, MetS and TNBC, however this requires further investigation.

5.3. Future Directions

- *Assessment of ABCA1 in the macrophages of obese and MetS participants compared to controls.*

In chapter 2 we identified miR-758-3p as a potential blood-based biomarker for MetS. We attempted to immunoblot for ABCA1 in the plasma of obese and MetS participants, however there was no signal detected as ABCA1 is found in macrophages. Macrophages can be isolated from peripheral blood; however they must be isolated from fresh blood. This was not possible during this study but investigation of ABCA1 in the macrophages of obese, MetS and healthy controls would be the next step in this investigation. This would however require the initiation of a secondary MetS study.

- *Does the 12-week dietary intervention have any effect in EV quantity?*

In chapter 2, we demonstrated that the plasma from obese and MetS participants contained EVs and that there was a significant increase in EV quantities in the MetS cohort compared to the control cohort. Similarly there was a significant increase in the MetS cohort compared to the obese cohort. For this reason the next step in this study would be to isolate EVs from the plasma of the obese and MetS participants following the 12-week dietary intervention and compare the quantity of EVs in each cohort.

- *Does Dichloroacetate inhibit pyruvate dehydrogenase at a lower concentration and shorter incubation time?*

In Chapter 4, we demonstrated that Dichloroacetate (DCA) was non-toxic to the Hs578Ts(i)₈ cell line variant at 5mM, so we performed a Seahorse Extracellular Flux assay at this concentration. However, investigation of DCA at lower concentrations and possibly lower incubation times is warranted. Therefore, a Seahorse Extracellular Flux assay titrating DCA should be performed.

- *Does NmU inhibit oxidative phosphorylation and glycolysis at lower concentrations?*

In Chapter 4, we demonstrated that NmU was non-toxic to the Hs578T and Hs578Ts(i)₈ cell line variants at 5μM, so we performed a Seahorse

Extracellular Flux assay at this concentration. However, investigation of NmU at lower concentrations and possibly reduced incubation times is warranted. Thus, a Seahorse Extracellular Flux assay titrating NmU should be performed.

- *Assessment of the mitochondrial oxidative phosphorylation and glycolysis in a broad range of TNBC cell lines*

In Chapter 4 we found a significant decrease in the basal and maximal oxygen consumption rates of a parental and more aggressive TNBC cell line pair. To investigate if these findings are specific to this Hs578T and Hs578Ts(i)₈ cell line pair or seen in all TNBC cell lines a broad range of TNBC cell lines should be assessed.

- *Is the mitochondrial function of obese and MetS individuals altered?*

In chapter 4 we showed that the mitochondrial function of the Hs578Ts(i)₈ cells is somewhat dysfunctional. An interesting study would be to investigate the mitochondrial function of adipocytes from obese and MetS individuals with TNBC as Yin *et al* [232] have previously shown the mitochondrial function of adipocytes isolated from the omental and subcutaneous areas of obese individuals compared to non-obese individuals was significantly decreased as measured by the OCR.

- *Assessment of NmU in the plasma of obese and MetS Individuals with TNBC*

In chapter 4 we found NmU to be significantly decreased in the aggressive TNBC cell line and they may be decreased in the obese and MetS participants plasma specimens. Assessment of the levels of NmU in the plasma of participants with TNBC who are also obese compared to obese/MetS participants and lean participants with TNBC would be interesting to perform.

- *Is NmU locally acting or freely- circulating in the plasma of obese participants?*

We hypothesised that the decrease in NmU levels in the obese and MetS participants to be significant as NmU^{-/-} mice exhibit obesity while NmU

wild-type mice are lean. We therefore hypothesise that NmU is acting locally in the brain where it can target NmU R2 in lean participants; therefore NmU circulating in the plasma may not give an accurate account of NmU in obesity and MetS. Determination of NmU action in obese and MetS participants is now required to determine its potential as a blood-based biomarker for MetS effectively.

- *Effect of Hs578T cell EVs on the Hs578Ts(i)₈ cells metabolic phenotype*
EVs have been shown to confer properties of their cells of origin to recipient cells with which they are co-cultured. Assessing if EVs isolated from the Hs578T cells and co-cultured with the Hs578Ts(i)₈ cells could confer their properties which allow oxidative phosphorylation should be performed.

Chapter Six

6.1. References

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Appendix I

A. Study Dietary Intervention and Meal Tests

WP1 (Section 1.4) endeavoured to produce two types of milk that differed in small and medium chain saturated fatty acid (MC-SFA) chain length (C_4 - C_{14}), while C_{16} remained constant. To achieve this, production of high levels (C_4 - C_{14}) and low levels (C_4 - C_{12}) of MC-SFAs in 50 Holstein cattle was induced.

Low *de novo* synthesis of MC-SFAs was induced in these cattle by replacing their barley feed with fractionated palm fat; and high *de novo* synthesis of MC-SFAs was induced by lowering the fat content in their feed. Their feed was, therefore, composed of grass silage, maize silage, beet pulp, extracted rape seed and barley. After three weeks on these feeds, milk was collected and used for the production of butter, rolls and muffins. This process resulted in the synthesis of MC-SFAs differing in C_4 - C_{14} content. The difference being 4.8g/ 100g milk.

Following the successful synthesis of these differing MC-SFAs, WP2 performed a 12-week randomised, controlled, parallel and isocaloric dietary intervention. At visit 2, after all blood specimens were procured, MetS and obese subjects were divided into 4 groups (Table 2.4) and subsequently commenced one of four 12-week diets, based on different combinations of MC-SFAs and proteins (whey or casein). Their daily intake of fat was: C_4 - C_{12} = 0.9g/day or C_4 - C_{14} = 2.2g/ day, resulting in a daily energy intake from study diet of 4.500 kJ (~35-60% total daily energy intake).

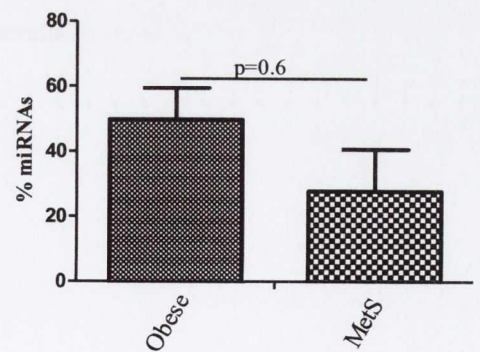
B. miRNAs Analysed in the Pre-screen

Table B.1: Table of miRNAs analysed in the pre-screen [See Section 2.2.4.2]

miR-652-3p	miR-145-5p	miR-139-5p	miR-16-5p	miR-148b-3p	miR-301a-3p	miR-331-3p	miR-18b-5p
miR-502-3p	miR-1	miR-194-5p	miR-136-5p	miR-339-5p	miR-140-5p	miR-144-5p	miR-28-3p
miR-181a-5p	miR-185-5p	miR-375	let-7b-5p	miR-20a-5p	miR-151a-5p	miR-142-3p	miR-335-5p
miR-339-3p	miR-382-5p	miR-660-5p	let-7i-3p	Blank (H2O)	miR-130b-3p	miR-210	miR-324-3p
miR-221-3p	miR-486-5p	miR-451a	miR-152	miR-17-5p	miR-122-5p	let-7d-3p	miR-204-5p
miR-409-3p	miR-32-5p	miR-574-3p	miR-106b-5p	miR-500a-5p	miR-20a-3p	miR-199a-5p	miR-182-5p
let-7f-5p	miR-26a-5p	let-7a-5p	miR-30c-5p	miR-30d-5p	miR-423-5p	miR-605	let-7g-5p
miR-154-5p	miR-133b	miR-551b-3p	miR-15b-3p	miR-378a-3p	miR-629-5p	miR-766-3p	miR-15b-5p
miR-27b-3p	miR-143-3p	miR-128	miR-197-3p	miR-186-5p	miR-101-3p	miR-19a-3p	miR-22-3p
miR-155-5p	let-7d-5p	miR-130a-3p	miR-142-5p	miR-425-5p	miR-200c-3p	miR-584-5p	miR-106b-3p
miR-374b-5p	miR-30a-5p	miR-125a-5p	miR-99b-5p	let-7i-5p	miR-365a-3p	miR-144-3p	miR-199a-3p
miR-140-3p	miR-133a	miR-28-5p	miR-296-5p	miR-346	miR-501-3p	miR-92a-3p	miR-29c-3p
miR-93-5p	miR-222-3p	miR-485-3p	miR-30e-5p	miR-26b-5p	miR-23a-3p	miR-126-3p	miR-19b-3p
miR-92b-3p	miR-20b-5p	miR-497-5p	miR-326	miR-193b-3p	miR-423-3p	miR-363-3p	miR-95
miR-200a-3p	miR-342-3p	let-7b-3p	miR-146a-5p	miR-34a-5p	miR-215	miR-148a-3p	miR-29a-3p
miR-505-3p	UniSp3 IPC	miR-425-3p	cel-miR-39-3p	miR-320b	miR-376a-3p	miR-374a-5p	miR-21-5p
UniSp2	UniSp4	UniSp5	miR-421	miR-885-5p	miR-320a	miR-10b-5p	miR-150-5p
miR-23b-3p	miR-328	miR-132-3p	miR-424-5p	miR-590-5p	miR-22-5p	miR-190a	miR-30e-3p
miR-484	miR-324-5p	miR-25-3p	miR-223-5p	miR-127-3p	miR-338-3p	miR-195-5p	miR-30b-5p
miR-141-3p	miR-532-3p	let-7c	UniSp6	miR-191-5p	miR-2110	miR-29a-5p	miR-543
miR-10a-5p	miR-15a-5p	miR-18a-5p	miR-146b-5p	miR-208a	miR-223-3p	miR-125b-5p	miR-24-3p
miR-106a-5p	let-7e-5p	miR-33a-5p	miR-107	miR-99a-5p	miR-361-3p	miR-18a-3p	miR-29b-2-5p
miR-27a-3p	miR-532-5p	miR-29b-3p	miR-205-5p	miR-16-2-3p	miR-103a-3p	miR-192-5p	miR-495-3p
miR-93-3p	miR-151a-3p						

C. Agilent Small RNA Kit Analysis of miRNA Content in Plasma RNA

A. Percentage of miRNAs Detected in RNA Isolated from Plasma Specimens (Pre-Diet)



B. Percentage of miRNAs Detected in RNA Isolated from Plasma Specimens (Post-Diet)

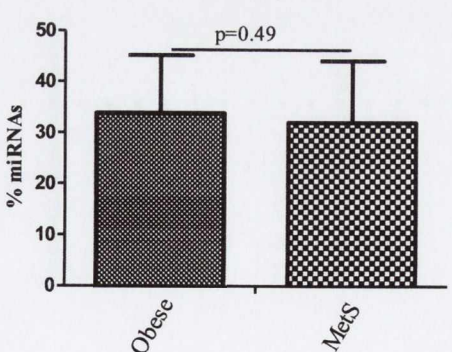


Figure C.1: Percentage of miRNAs detected in the total RNA isolated from the plasma of obese and MetS participants. Percentage miRNAs from total RNA at A. T₀ Visit 2, B. T₀ Visit 7, miRNA profiling at baseline (T₀) [See Section 2.2.3.2]

Table C.1: Percentage of miRNAs detected in the total RNA isolated from the plasma of obese and MetS participants following high fat meal test.

T₃₀	Obese (%)	MetS (%)	P
Visit 2	89.5±0.5	79.3±3.5	0.13
Visit 7	66.3±9.1	77.8±4.8	0.23
T₁₂₀	Obese (%)	MetS (%)	P
Visit 2	74.7±2.0	59.5±16.1	0.46
Visit 7	70.7±10.9	59.7±6.4	0.43
T₂₄₀	Obese (%)	MetS(%)	P
Visit 2	63.7±4.7	45.0±2.0	0.06
Visit 7	54.3±7.1	54.0±1.0	0.97

Percentage of miRNAs present in the total RNA sample isolated from the plasma of obese and MetS participants. Results shown are the mean for the cohort ±the SEM of the cohort, where results were not significant [See Section 2.2.3.2].

D. Custom miRNA Profiling of Plasma from Obese and MetS Participants at Baseline Pre- and Post- 12-week Dietary Intervention

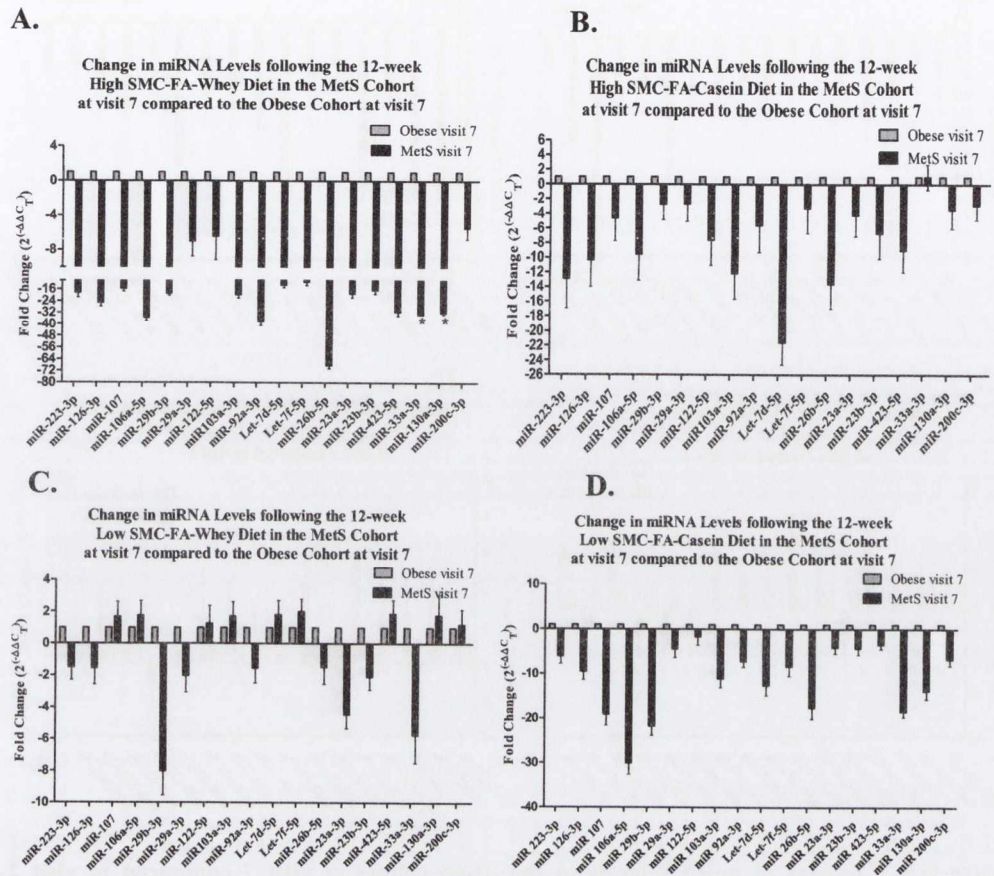


Figure D.1: Changes in miRNA levels in the MetS cohort compared to the Obese cohort at baseline– For each diet

The fold change of the miRNAs analysed in the MetS cohort compared to the obese cohort for **A.** the high-MC-SFA-whey diet, **B.** the high-MC-SFA-casein diet, **C.** the low-MC-SFA-whey diet and **D.** the low-MC-SFA-casein diet. Data represent the mean ± SEM for each cohort, where results were not significant [See Section 2.3.5.4.1].

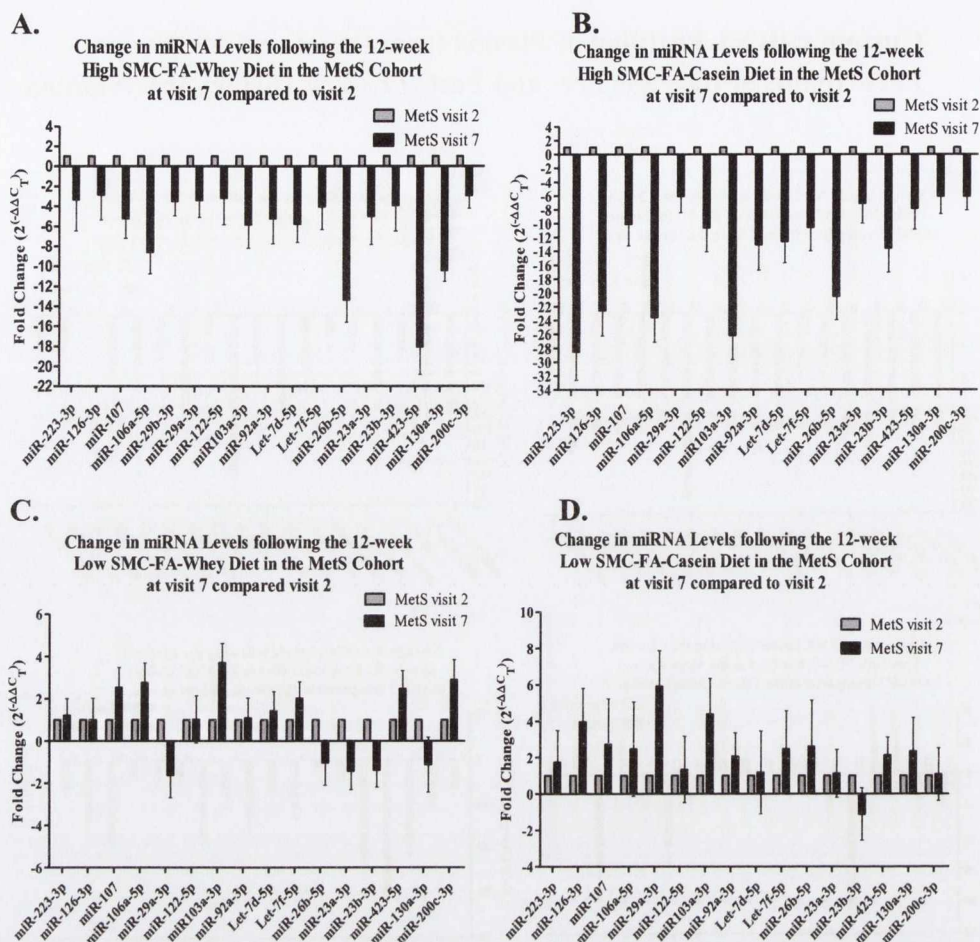


Figure D.2: Changes in miRNA levels in the MetS cohort at visit 7 compared to visit 2 baseline– for each diet

The fold change of the miRNAs analysed in the MetS cohort at visit 7 compared to visit 2 for **A.** the high-MC-SFA-whey diet, **B.** the high-MC-SFA-casein diet, **C.** the low-MC-SFA-whey diet and **D.** the low-MC-SFA-casein diet. Data represent the mean $\pm$ SEM for each cohort, where results were not significant [See Section 2.3.5.4.2].

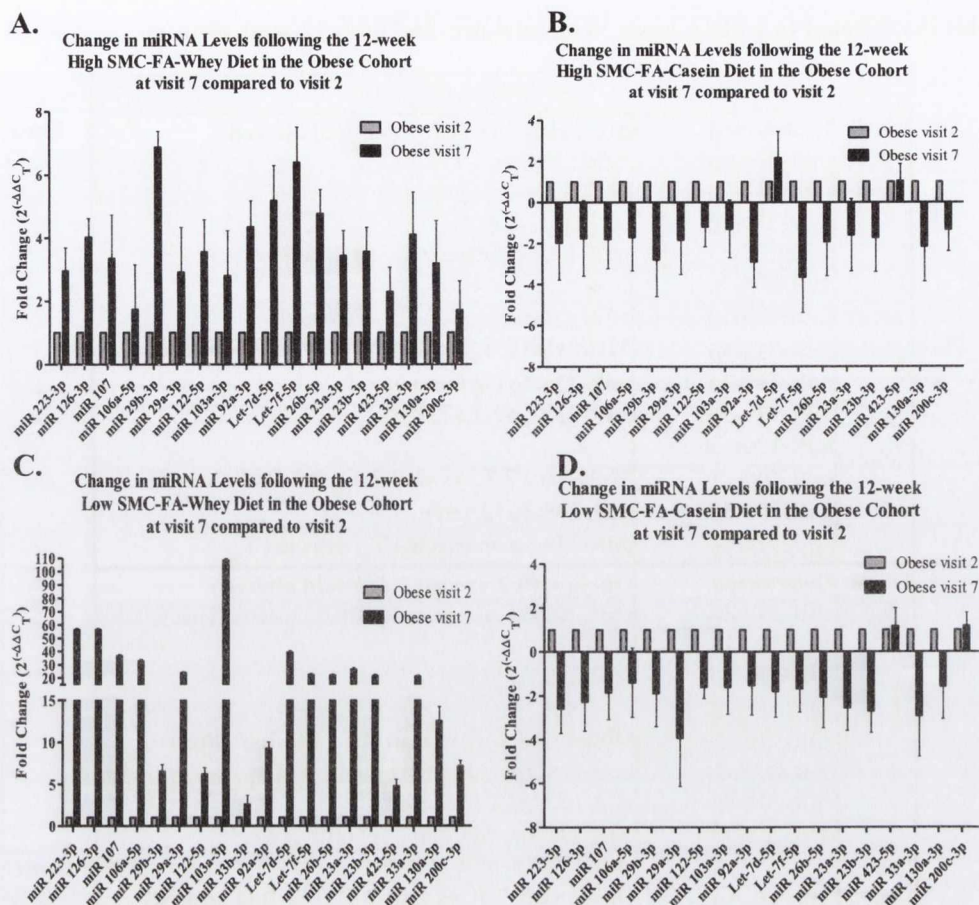


Figure D.3: Changes in miRNA levels in the Obese cohort at visit 7 compared to visit 2 baseline –for each diet

The fold change of the miRNAs analysed in the Obese cohort at visit 7 compared to visit 2 for **A.** the high-MC-SFA-whey diet, **B.** the high-MC-SFA-casein diet, **C.** the low-MC-SFA-whey diet and **D.** the low-MC-SFA-casein diet. Data represent the mean $\pm$ SEM for each cohort, where results were not significant [See Section 2.3.5.4.3].

Table D.1: Change in miRNA levels 30 minutes pre- and post- 12-week diets

Time 30mins (T ₃₀)		
MetS	Undetected	Mets visit 2 vs visit 7 (>2-fold altered)
Visit 2	miR-33a-3p	miR-126-3p (-2.1±1.3)
	miR-33b-3p	let-7d-5p (-2.2±1)
	miR-33b-5p	let-7f-5p (-2.0±1)
	miR-200a-3p	miR-130a-3p detected visit 2, undetected visit 7
	miR-204-5p	
	miR-758-3p	
Visit 7	miR-33a-3p	MetS visit 2 T₀ vs visit 2 T₃₀ (>2-fold altered)
	miR-33b-3p	miR-23a-3p (-2.86±0.9)
	miR-33b-5p	miR-23b-3p (-2.87±0.9)
	miR-130a-3p	
	miR-200a-3p	MetS visit 7 T₀ vs visit 7 T₃₀ (>2-fold altered)
	miR-204-5p	miR-29b-3p (2.1±1)
	miR-758-3p	miR-33a-3p undetected T ₀ , detected T ₃₀
Obese	Undetected	Obese visit 2 vs visit 7 (>2-fold altered)
Visit 2	miR-29b-3p	miR-29b-3p undetected visit 2, detected visit 7
	miR-33a-3p	miR-23a-3p (2.2±1.1)
	miR-33b-3p	miR-23b-3p (2.6±1)
	miR-33b-5p	
	miR-130a-3p	Obese visit 2 T₀ vs visit 2 T₃₀ (>2-fold altered)
	miR-200a-3p	miR 223-3p, miR 126-3p, miR 23a-3p, miR 23b-3p
	miR-204-5p	miR 107, miR 106a-5p
	miR-758-3p	miR 29a-3p, miR 200c-3p, miR-106b-5p
		miR 122-5p, miR 26b-5p
Visit 7	miR-33a-3p	Obese visit 7 T₀ vs visit 7 T₃₀ (>2-fold altered)
	miR-33b-3p	miR 223-3p, miR 107, miR 23a-3p, miR 23b-3p
	miR-33b-5p	miR 126-3p, miR 106a-5p
	miR-130a-3p	miR 29a-3p, miR 423-5p, miR 200c-3p
	miR-200a-3p	miR 122-5p, miR 103a-3p
	miR-204-5p	miR 92a-3p, let-7d-5p, let-7f-5p, miR 26b-5p
	miR-758-3p	
Each Diet Individually		MetS miR-758-3p undetected all diet groups
		Obese miR-758-3p undetected all diet groups

The levels of analysed miRNAs in the plasma of Obese and MetS participants 30 minutes before and after the 12-week dietary intervention that were >2-fold altered. [See Section 2.3.5.5].

Table D.2: Change in miRNA levels 120 minutes pre- and post- 12-week diets

Time 120mins (T ₁₂₀)		
MetS	Undetected	Mets visit 2 vs visit 7 (>2-fold altered)
Visit 2	miR-29b-3p	miR-107 (2.1±1.1), miR-423 (4.2±0.5)
	miR-33a-3p	miR-103a-3p (2.7±1.1), let-7d-5p (2.7±1)
	miR-33b-3p	miR-92a-3p (3.0±0.7), let-7f (2.8±1)
	miR-33b-5p	miR-23a-3p (2.6±0.9)
	miR-130a-3p	miR-23b-3p (2.7±0.8)
	miR-200a-3p	miR-29b-3p was detected visit 2, undetected visit 7
	miR-200c-3p	
	miR-204-5p	
	miR-758-3p	
Visit 7	miR-33a-3p	MetS visit 2 T₀ vs visit 2 T₃₀ (>2-fold altered)
	miR-33b-3p	miR-223-3p (-4.6±0.9), miR-107 (2.8±1.1)
	miR-33b-5p	miR-29a-3p (11±0.9), let-7d-5p (2.3±1.1)
	miR-130a-3p	miR-26b-3p (3.7±1.1), miR-23a-3p (-5.2±1.2)
	miR-200a-3p	miR-15a-5p (4.1±1.1), miR-106b-5p (8.1±0.9)
	miR-200c-3p	miR-29b-3p, miR-130a-3p and miR-200c-3p were detected T ₀ , undetected T ₃₀
	miR-204-5p	MetS visit 7 T₀ vs visit 7 T₃₀ (>2-fold altered)
	miR-758-3p	let-7f-5p (-3.4±1.1), miR-23a-3p (-2.11±1.0)
		miR-107 (-2.2±1.1), let-7d-5p (-2.3±1.1)
		miR-200c-3p was detected visit 2, undetected visit 7
Obese	Undetected	Obese visit 2 vs visit 7 (>2-fold altered)
Visit 2	miR-33a-3p	miR-126 (2.3±0.6), miR-107 (2.5±0.6)
	miR-33b-3p	miR-106a-5p (2±0.7), miR-122-5p (2.7±0.4)
	miR-33b-5p	miR-103a-3p (2.1±0.7), let-7f-5p (3.3±0.6)
	miR-130a-3p	miR-200c-3p were detected visit 2, undetected visit 7
	miR-200a-3p	Obese visit 2 T₀ vs visit 2 T₃₀ (>2-fold altered)
	miR-200c-3p	miR-223-3p, miR-107, miR-29a-3p
	miR-758-3p	let-7d-5p, miR-26b-5p, miR-23a-3p
		miR-106b-5p, miR-200c-3p, miR-92a-3p
		miR-29b-3p undetected T ₀ , detected T ₃₀
Visit 7	miR-33a-3p	Obese visit 7 T₀ vs visit 7 T₃₀ (>2-fold altered)
	miR-33b-3p	miR 223-3p, miR 200c-3p, miR 423-5p
	miR-33b-5p	miR 126-3p, miR 23b-3p, miR 23a-3p
	miR-130a-3p	miR 107, miR 26b-5p, let-7f-5p, miR 29a-3p
	miR-200a-3p	miR 106a-5p, let-7d-5p, , miR 122-5p
	miR-204-5p	miR 29b-3p, miR 92a-3p, , miR 103a-3p
	miR-758-3p	
Each Diet Individually		MetS miR-758-3p undetected all diet groups
		Obese miR-758-3p undetected all diet groups

The levels of analysed miRNAs in the plasma of Obese and MetS participants 120 minutes before and after the 12-week dietary intervention that were >2-fold altered [See Section 2.3.5.5].

Table D3: Change in miRNA levels 240 minutes pre- and post- 12-week diets

Time 240mins (T ₂₄₀)		
MetS	Undetected	Mets visit 2 vs visit 7 (>2-fold altered)
<u>Visit 2</u>	miR-29b-3p	miR-26b-5p (2.4±1.2)
	miR-33a-3p	miR-15a-5p (2.2±1.2)
	miR-33b-3p	miR-106b-5p (2.0±1.2)
	miR-33b-5p	
	miR-130a-3p	MetS visit 2 T₀ vs visit 2 T₃₀ (>2-fold altered)
	miR-200a-3p	miR 223-3p, miR 126-3p, miR 107
	miR-200c-3p	miR 106a-5p, miR 29a-3p, miR 122-5p
	miR-204-5p	miR 103a-5p, miR 92a-3p, let-7d-5p, let-7f-5p
		miR 26b-5p, miR 23a-3p, miR 23b-3p, miR 423-5p,
		miR 130a-3p, miR-15a-5p, miR-106b-5p
		miR-29b-3p, miR-130a-3p and miR-200c-3p
	miR-758-3p	were detected T ₀ , undetected T ₃₀
<u>Visit 7</u>	miR-33a-3p	MetS visit 7 T₀ vs visit 7 T₃₀ (>2-fold altered)
	miR-33b-3p	miR-29a-3p (2.4±0.9), let-7d-5p (2.1±0.8)
	miR-33b-5p	miR-26b-5p (2.8±0.9), miR-423-5p (3.1±0.4)
	miR-130a-3p	miR-106b-5p (2.4±0.8)
	miR-200a-3p	miR-200c-3p detected T ₀ , undetected T ₃₀
	miR-200c-3p	
	miR-204-5p	
	miR-758-3p	
Obese	Undetected	Obese visit 2 vs visit 7 (>2-fold altered)
<u>Visit 2</u>	miR-29a-3p	miR-26b-3p (-2.11±1.16)
	miR-33a-3p	miR-29a-3p was detected at visit 2, detected at visit 7
	miR-33b-3p	
	miR-33b-5p	Obese visit 2 T₀ vs visit 2 T₃₀ (>2-fold altered)
	miR-130a-3p	miR 223-3p, miR 126-3p, miR 107,
	miR-200a-3p	miR 106a-5p, miR 29a-3p, miR 122-5p,
	miR-200c-3p	miR 103a-5p, miR 92a-3p, let-7d-5p,
	miR-204-5p	let-7f-5p, miR 26b-5p, miR 23a-3p, miR 23b-3p
	miR-758-3p	miR 423-5p, miR-15a-5p, miR-106b-5p
		miR-29a-3p and miR-200c-3p detected T ₀ , undetected T ₃₀
<u>Visit 7</u>	miR-29a-3p	Obese visit 7 T₀ vs visit 7 T₃₀ (>2-fold altered)
	miR-29b-3p	miR-223-3p (-2.85±0.6)
	miR-33a-3p	miR-126 (-2.2±0.6)
	miR-33b-3p	miR-23a-3p (-2.39±0.7)
	miR-33b-5p	
	miR-130a-3p	
	miR-200a-3p	
	miR-200c-3p	
	miR-204-5p	
	miR-758-3p	
Each Diet Individually		MetS miR-758-3p undetected all diet groups
		Obese miR-758-3p undetected all diet groups

The levels of analysed miRNAs in the plasma of Obese and MetS participants 240 minutes before and after the 12-week dietary intervention that were >2-fold altered [See Section 2.3.5.5].

E. Table of miRNAs Detected in the Control, Obese and MetS Specimens

Table E.1: miRNAs detected in the control, obese and MetS cohorts [See Section 3.3.3.2]

miRNA	Control	Obese	MetS	miRNA	Control	Obese	MetS	miRNA	Control	Obese	MetS
Let-7g-5p	X		X	miR-106a-5p	X		X	Let-7c	X	X	
miR-329	X	X	X	miR-370	X			miR-19b-3p			X
miR-378a-3p	X	X	X	miR-100-5p		X	X	miR-30b-5p	X		X
miR-103a-3p			X	miR-146a-5p	X			miR-184	X	X	X
miR-221-3p	X	X	x	miR-139-5p			X	miR-21-5p	X	X	X
miR-16-5p	X	X	X	miR-373-5p	X	X		miR-214-3p	X		
miR-25-3p	X			miR-31-5p	X	X		miR-324-3p	X	X	X
miR-20a-5p	X		X	miR-451a	X	X	X	miR-205-5p	X		
miR-24-3p	X	X	X	miR-27b-3p	X	X	X	miR-19a-3p	X		
miR-30c-5p	X	X		miR-491-5p	X	X	X	miR-15a-5p			X
miR-133b	X	X	X	miR-665	X	X	X	Let-7d-3p		X	X
miR-23a-3p	X	X	X	miR-363-3p	X			miR-10b-5p	X	X	
miR-342-3p	X		X	miR-27a-3p		X		Let-7i-5p	X		
miR-934	X	X	X	miR-376c-3p		X	X	miR-652-3p	X	X	
miR-101-3p	X		X	miR-940	X	X	X	miR-126-5p	X	X	X
miR-188-5p	X	X	X	miR-224-5p	X	X		miR-9-3p	X		
miR-26a-5p	X	X	X	miR-320a	X	X		miR-93-5p			X
miR-23b-3p	X	X	X	miR-302c-5p	X			miR-133a	X	X	X
miR-92a-3p	X	X	X	miR-107		X		miR-124-3p	X	X	X
miR-126-3p	X	X	X	miR-662	X			miR-223-3p	X	X	X
miR-125a-5p		X		miR-485-3p	X	X	X	miR-512-5p	X		
miR-346	X	X	X	miR-1-3p	X	X	X	Let-7d-5p	X	X	X
miR-202-3p	X			Let-7f-5p	X	X		miR-495-3p	X	X	X
miR-125b-5p	X	X	X	miR-30e-5p		X	X	miR-596	X		
miR-362-5p	X	X	X	miR-663a	X	X	X	miR-145-5p		X	X
miR-140-3p	X			miR-572	X	X		Let-7a-5p	X	X	X
miR-193a-5p	X			miR-490-3p	X	X	X	miR-128	X	X	X
Let-7b-5p	X	X	X	miR-1			X	miR-206	X	X	X
miR-134	X	X	X	miR-142-3p	X	X	X				

F. miRNA Profiling in the Control, Obese and MetS Cohorts

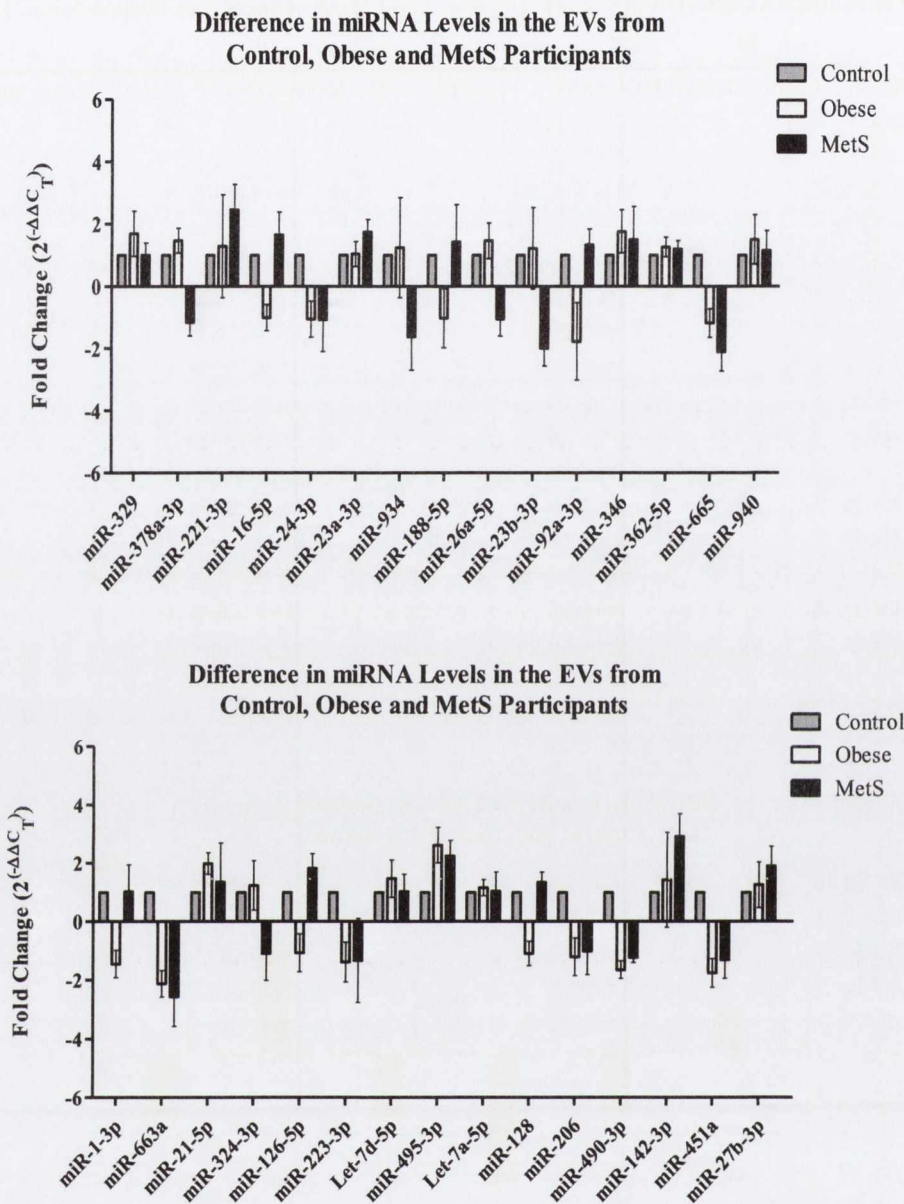


Figure F.1: Change in EV- miRNA levels in the Obese and MetS cohorts compared to healthy controls

Fold change of miRNAs in the Obese and MetS cohorts compared to healthy controls, when detected in all three cohorts. Data represent the mean $\pm$ SEM for each cohort, where results were not significant [See Section 3.3.3.2.1].

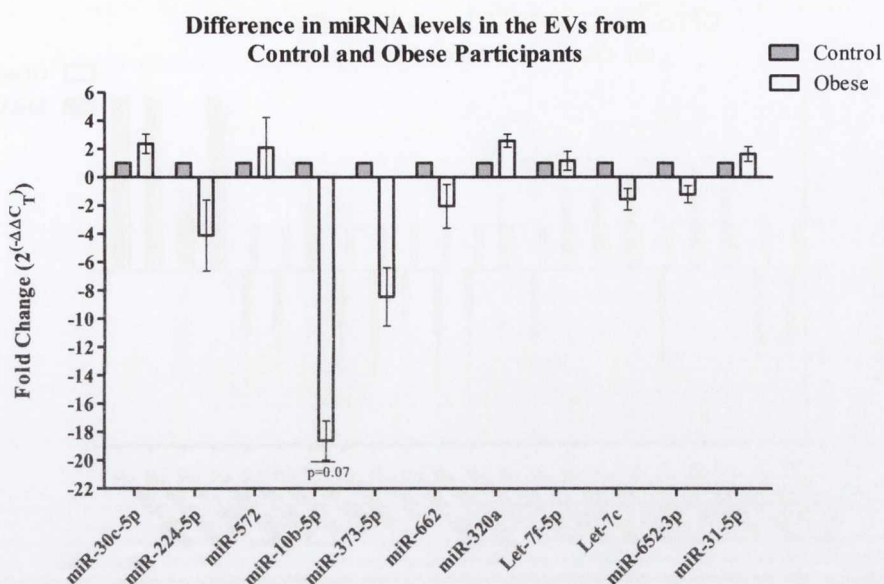


Figure F.2: Change in EV-miRNA levels in the Obese cohort compared to healthy controls. Fold change of miRNAs in the Obese cohort compared to controls when detected in the control and Obese cohorts but undetected in the Mets cohort. Data represent the mean $\pm$ SEM for each cohort, where results were not significant [See Section 3.3.3.2.1].

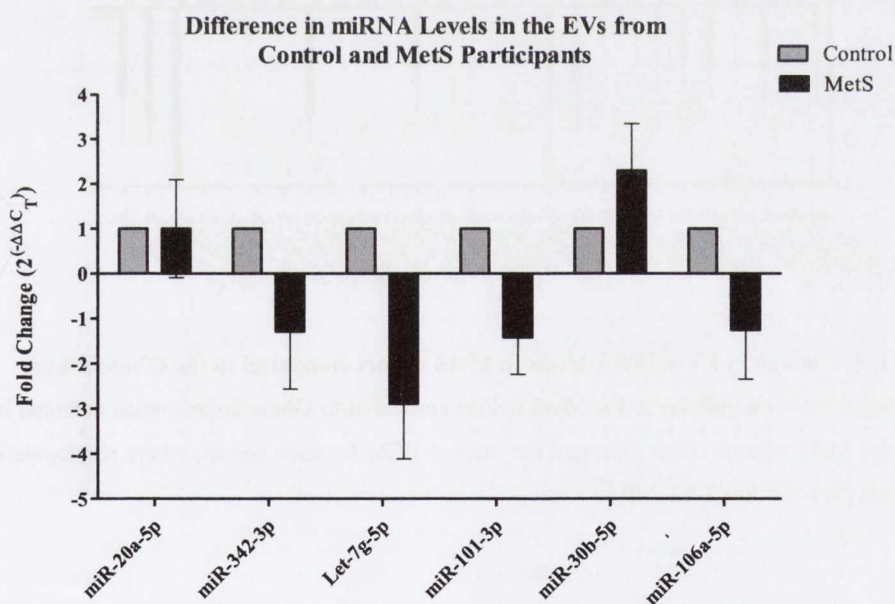


Figure F.3: Changes in EV-miRNA Levels in the MetS cohort compared to the Control Cohort

Fold change for each miRNA in the MetS cohort compared to controls when detected in control and MetS cohorts but undetected in the obese cohort. Data represent the mean $\pm$ SEM for each cohort, where results were not significant [See Section 3.3.3.2.1].

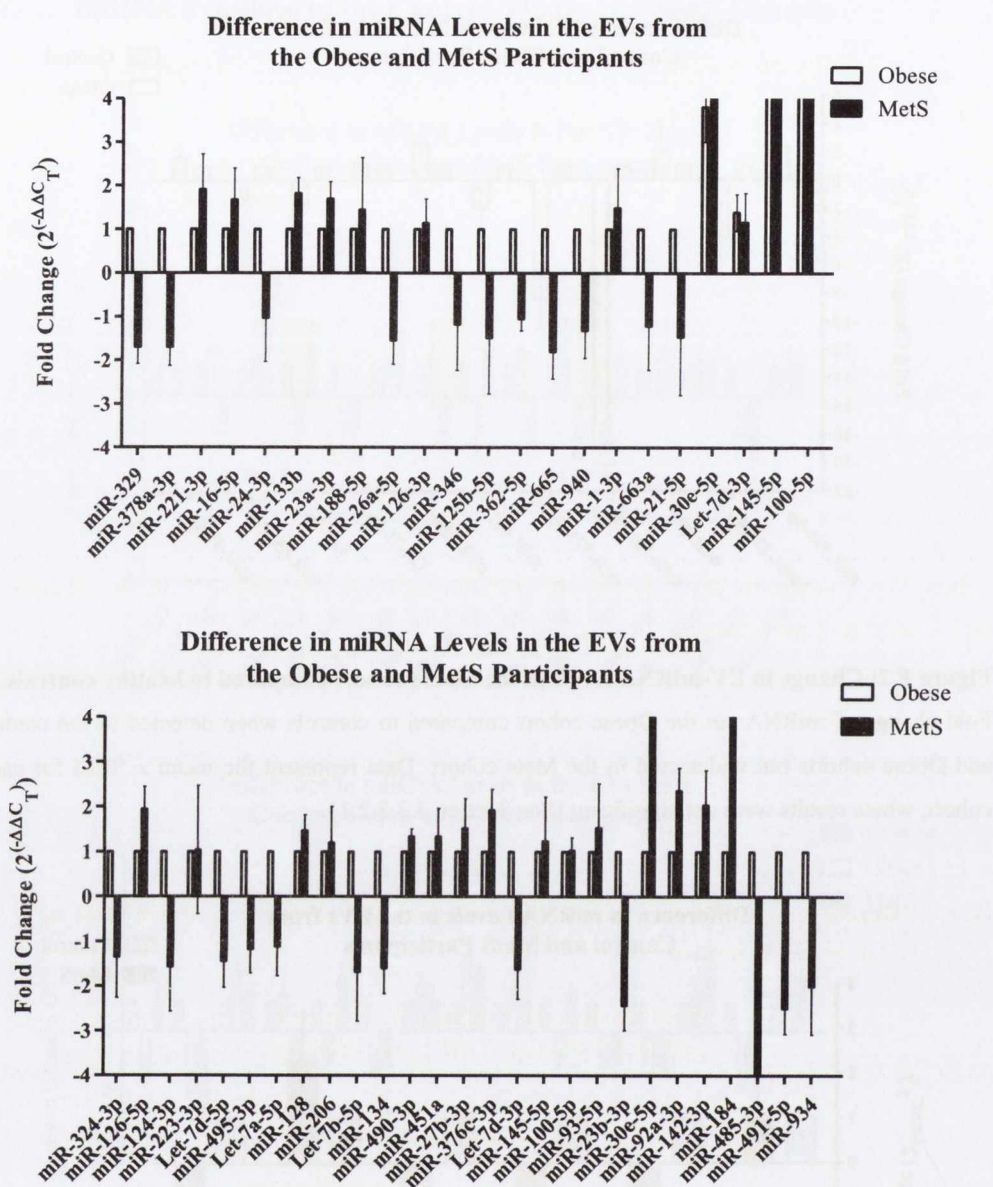


Figure F.4: Change in EV-miRNA levels in MetS cohort compared to the Obese cohort
 Fold change for each miRNA in the MetS cohort compared to Obese cohort when detected in the Obese and MetS cohorts. Data represent the mean $\pm$ SEM for each cohort, where results were not significant [See Section 3.3.3.2.2].

**Levels of miR-100-5p, miR-134 and miR-370
Freely-circulating in the plasma of Obese and MetS Participants**

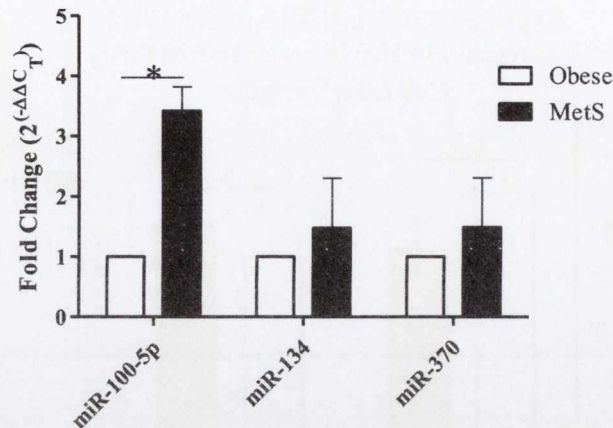


Figure F.5: Change in the levels of miR-100-5p, miR-134 and miR-370 in the plasma of Obese and MetS participants

Change in the levels of 3 miRNAs identified as potential EV-miRNA biomarkers in the Obese and MetS cohorts compared to healthy controls in the plasma of obese and MetS participants. Data represent the mean ± SEM for each cohort, where * p<0.05 [See Section 3.4].

G. Densitometry of VDAC1 Expression in the Hs578T and Hs578Ts(i)₈ Cell Line Variants

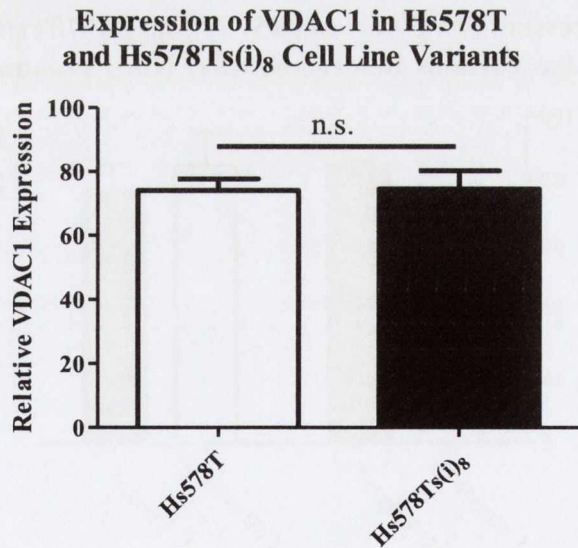


Figure G.1: Densitometry analysis of VDAC1 in the Hs578T and Hs578Ts(i)₈ cell line variants

Densitometry of VDAC1 in the Hs578T and Hs578Ts(i)₈ cell line variants. Data represent n=3±SEM biological repeats, where results were not significant [See Section 4.3.2, Figure 4.10].

H. Densitometry of NmU R1 and NmU R2 Expression in Hs578T and Hs578Ts(i)₈ Cell Line Variants

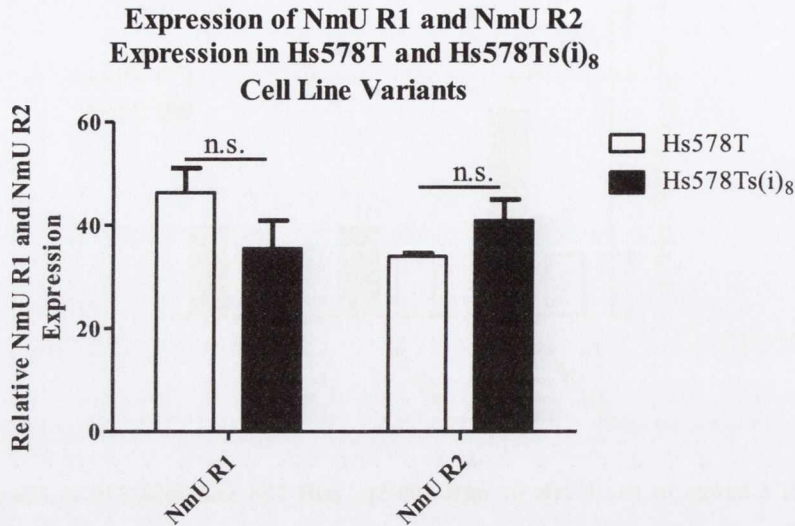


Figure H.1: Densitometry of NmU R1 and NmU R2 expression in Hs578T and Hs578Ts(i)₈ cell line variants

Data represent $n=3\pm\text{SEM}$ biological repeats, where results were not significant [See Section 4.3.5.1, Figure 4.17]

I. Densitometry of VDAC1 Expression in the Hs578T and Hs578Ts(i)₈ Cell Line Variants following NmU Treatment

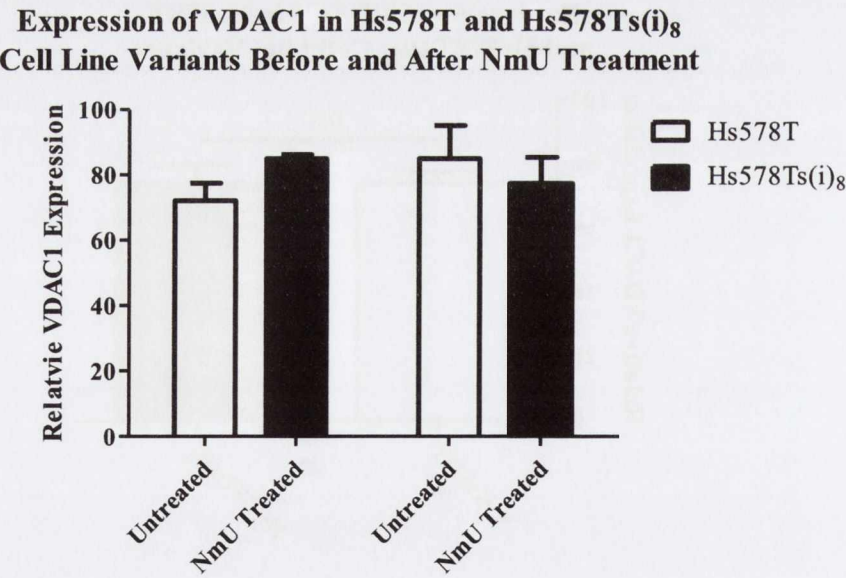


Figure I.1: Densitometry analysis of VDAC1 in the Hs578T and Hs578Ts(i)₈ cell line variants following NmU treatment

Densitometry of VDAC1 in the Hs578T and Hs578Ts(i)₈ cells following NmU treatment. Data represent $n=3\pm\text{SEM}$ biological repeats, where results were not significant [See Section 4.3.5.4, Figure 4.25].

Appendix II

A. Publications

Reviews

O'Neill S and O'Driscoll L. Metabolic syndrome: a closer look at the growing epidemic and its associated pathologies. *Obesity Reviews* 2015 Jan; 16(1): 1-12

O'Neill S, Bohl M, Gregersen S, Hermansen K, and O'Driscoll, L. Blood-based biomarkers for metabolic syndrome. *Trends in Endocrinology and Metabolism* 2016 Jun; 27(6): 363-74.

Book Chapter

S. O'Neill and L. O'Driscoll. Profiling circulating miRNAs from the plasma of individuals with metabolic syndrome. *Methods Mol Biol* (Expected publication May 2016).

B. Qualifications

2013 – Diploma in extracellular vesicle isolation and preparation, Cost-ME-HAD, Universidad Internacional Menéndez Pelayo and Universitat de Valencia.

2015 – Certificate in Statistics, Trinity College Dublin

C. International and National Conference Presentations

Oral Presentation

O'Neill S, Bohl M, Gregersen S, Hermansen K, and O'Driscoll, L. Extracellular vesicles, extracellular vesicles-contained miRNAs and total miRNAs as plasma-based biomarkers for metabolic syndrome. International Society for Extracellular Vesicles (ISEV), Rotterdam, The Netherlands, 5 May 2016.

O'Neill S, Bohl M, Gregersen S, Hermansen K, and O'Driscoll, L. Circulating miRNA biomarkers for metabolic syndrome and obesity and their link with triple negative breast cancer. All Ireland Schools of Pharmacy Conference Queens University, Belfast, 30 March 2015

O'Neill S, Bohl M, Hokamp K, Gregersen S, Hermansen K, and O'Driscoll, L. Potential miRNA biomarkers for metabolic syndrome. Shortlist for Donegan Medal, RAMI Biomed Science section meeting. University College Dublin, 19 June 2014.

O'Neill S, Bohl M, Hokamp K, Gregersen S, Hermansen K, and O'Driscoll, L. Circulating miRNAs: potential biomarkers for metabolic syndrome. All Ireland Schools of Pharmacy Conference. TCD. 14 April 2014.

Poster Presentation

O'Neill S, Bohl M, Hokamp K, Gregersen S, Hermansen K, and O'Driscoll, L. Circulating miRNAs in metabolic syndrome and the effect of dietary intervention on these miRNAs. Young Life Sciences Ireland Conference, TBSI, TCD, 01 March 2014.